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Effect of Nicotinamide on Proliferation, Differentiation, and Energy Metabolism in Bovine Preadipocytes*

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ABSTRACT : This study examined the effects of nicotinamide on proliferation, differentiation, and energy metabolism in a primary culture of bovine adipocytes. After treatment of cells with 100-500 μM nicotinamide, cell growth was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and cellular lipid content was assessed by Oil Red O staining and a triglyceride (TG) assay. Several factors related to energy metabolism, namely adenosine triphosphatase (ATPase) activity, nitric oxide (NO) content, nitric oxide synthase (NOS) activity, the number of mitochondria and the relative expression of glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), peroxisome proliferator-activated receptor-γ (*PPARγ*) and inducible NOS (*iNOS*), were also investigated. Results showed that nicotinamide induced both proliferation and differentiation in bovine preadipocytes. Nicotinamide decreased NO production by inhibiting NOS activity and iNOS mRNA expression, and controlled lipolytic activity by increasing ATPase activity and the number of mitochondria. The present study provides further evidence of the effects of nicotinamide on lipid and energy metabolism, and suggests that nicotinamide may play an important role in the development of bovine adipose tissue *in vivo*. This emphasizes the importance of investigating bovine adipose tissue to improve our understanding of dairy cow physiology. (**Key Words :** Nicotinamide, Bovine Preadipocytes, Nitric Oxide, Triglyceride, Lipolysis, Energy Balance)

INTRODUCTION

Nicotinamide, the amide derivative of vitamin B_3, is the precursor for the coenzyme nicotinamide adenine dinucleotide (NAD^+). Nicotinamide is involved in a wide range of biological processes that include energy production; fatty acid, cholesterol, and steroid biosynthesis; signal transduction; and maintenance of genomic integrity. Nicotinamide has been shown to improve energy status in ischemic tissues by raising intracellular NAD^+ levels available for energy metabolism (Yang et al., 2002). Originally, synthesis of B vitamins by ruminal microbes was thought to be adequate to meet the animal's needs (Hungate, 1966). However, more recent studies suggested that supplementing nicotinic acid in the diet of high producing dairy cows may be beneficial (Jaster and Ward, 1990; Pires and Grummer, 2007). Nicotinamide may be even more effective as an animal nutrition supplement than nicotinic acid (Collins and Chaykin, 1972). In limited studies, nicotinamide has been shown to reduce blood ketones (Kronfeld and Raggi, 1964), increase blood glucose (Talkt et al., 1973), and increase milk production.

High yielding dairy cows in early lactation are in negative energy balance, necessitating the use of body fat as a source of energy (Goff and Horst, 1997). Severe negative energy balance can lead to substantial loss of body condition, subclinical ketosis, susceptibility to disease, production decline, or poor reproductive performance later in lactation (Heuer et al., 1999). Therefore, an understanding of mechanisms regulating fat deposition and metabolism in cattle is important, as the adipocyte plays a central role in overall metabolic regulation, serving both as a storage depot for fatty acids (Morrison and Farmer, 2000) and as an endocrine cell to regulate energy utilization and feeding behavior. As in humans (Hauner, 2005) and mice (Stanley et al., 2005), ruminant adipocytes play an important role as a reserve of energy. Dysregulation of adipocyte proliferation and differentiation causes obesity, lipoatrophy, cardiovascular disease, noninsulin-dependent

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diabetes mellitus (Morrison and Farmer, 2000), and decrease in animal meat quality.

The nuclear receptor peroxisome proliferator-activated receptor-γ (*PPARγ*) is critically required for adipogenesis and insulin sensitivity (Barak et al., 1999; Rosen et al., 1999). In addition to its effects on preadipocyte differentiation and thus on adipocyte number, activation of *PPARγ* stimulates storage of fatty acids in mature adipocytes (Medina-Gomez et al., 2007; Beloor et al., 2009). Niacin dose-dependently inhibited leptin secretion and increased *PPARγ* expression in cultured adipocytes of rabbits (Yang et al., 2008), also could up-regulate *PPARγ* mRNA expression in 3T3-L1 cells (Yu et al., 2007). Glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) is a key enzyme in adipocytes, in which glycolysis is a metabolic pathway for providing substrates for lipogenesis (Catalan et al., 2007). However, *GAPDH* has been reported to participate in several metabolic pathways, including membrane transport and fusion, microtubule assembly, nuclear RNA export, protein phosphotransferase/kinase reactions, translational control of gene expression, DNA replication and repair (Sirover, 1999). Nicotinamide inhibits the enzyme poly-ADP-ribose polymerase (PARP) by blocking ADP-ribosylation, thereby increasing cellular energy and preventing the induction of nitric oxide synthase (NOS) (Pellat-Deceunynck et al., 1994). It has been previously reported that nicotinamide inhibited inducible NOS (iNOS) activity or *NOS* mRNA induction in several cell lines: L929 fibroblasts, a rat insulinoma, and RAW 264.7 macrophages (Hauschildt et al., 1992; Cetkovic-Cvrlje et al., 1993; Pellat-Deceunynck et al., 1994).

Though one study analyzed the effects of nicotinamide on cell proliferation and the expression of Sirtuim 1 (*SIRT1*) during pig preadipocytes differentiation (Bai et al., 2008), the underlying mechanism on energy metabolism in bovine preadipocytes is not yet fully understood. Therefore, the aim of this study was to investigate proliferation, differentiation, and energy metabolism in bovine adipocytes in the presence of nicotinamide.

MATERIALS AND METHODS

Cell culture and preadipocyte differentiation

Subcutaneous adipose tissue samples from neonatal dairy calves were collected at slaughter. All animals received humane care in compliance with the Guide for the Care and Use of Experimental Animals (Animal Care Committee, 2002). Adipose tissue samples were dissected into small pieces in Hank's balanced salt solution containing 2 mg/ml collagenase I and 0.1% bovine serum albumin (BSA, Sigma) in a sterile 50 ml plastic tube. Following digestion at 37°C for 50 min with gentle shaking in a water bath, the suspension was filtered through sterile nylon mesh with 100 μm pores to remove undigested tissue and mature adipocytes. Cells from the filtrate were seeded at a density of 2×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium/Ham's F12 nutrient medium (DMEM/F12, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and Antibiotic-Antimycotic (containing 100 IU/ml penicillin and 100 μg/ml streptomycin, GIBCO) at 37°C under a humidified 5% CO_2 atmosphere.

After the preadipocytes had reached confluence, differentiation was induced by treatment of the cells with a differentiation-induction medium with DMEM/F12 containing 10 μg/ml insulin, 0.25 μM dexamethasone(DEX, Sigma), and 0.5 mM 1-methyl-3-isobutylmethylxanthine (IBMX, Sigma). After 2 days the differentiation medium was replaced with DMEM/F12 containing 1 mM octanoate (Sigma), 10 mM acetate (Sigma), 10 μg/ml transferrin (Sigma), 3 μg/ml cholesterol (Sigma), 17 mM biotin (Sigma), 100 mM calcium pantothenate (Sigma), and 0.5% BSA (Sigma). The cells were fed with fresh medium every 2 days.

MTT method

An MTT-based tetrazolium dye reduction assay was used to determine cell survival and proliferation rate. Preadipocytes were seeded in 96-well culture plates at a density of 10^4 cells/cm^2. Cells were cultured in 200 μl/well of DMEM/F12 medium containing 10% FBS in the presence of nicotinamide at 0, 100, 200, 300, 400, or 500 μM. After 2, 4, 6, or 8 days, 20 μl of MTT reagent was added to each well of one cell culture plate, and the cells were cultured for a further 4 h. The medium was removed, 150 μl of DMSO was added to each well, and the plate was agitated for 10 min on a shaker to dissolve formazan. Absorbance at 490 nm was measured on a Rayto RT-2100C microplate reader (Shenzhen, China) and corrected for the absorbance of blank wells containing DMSO but no cells.

Oil Red O staining

To examine lipid accumulation, cells were seeded in 6-well culture plates at a density of 5×10^4/cm^2. Nicotinamide at 0, 100, 200, 300, 400, or 500 μM was added to the medium once cells were confluent and had begun to differentiate. After 8 days, the medium was removed, and cells were washed three times with phosphate-buffered saline (PBS) and fixed with 10% formaldehyde for 30 min at room temperature. After washing three times with PBS, cells were stained for at least 1 h with 1% filtered Oil Red O (6:4 Oil Red O stock solution-H_2O, where Oil Red O stock solution comprises 0.5% Oil Red O in isopropyl alcohol, Sigma). The stain was removed, and the cells were washed twice with water and photographed.

Several factors related to energy metabolism analysis

Cells were cultured in differentiation medium in the presence of various concentrations of nicotinamide in 6-well plates for 72 h. Triglyceride (TG) in the cell lysate were detected using a Triglyceride G Test Kit(Beyotime Institute of Biotechnology, Haimeny, China). The NO content in the cell culture medium was determined by the nitric acid deoxidized enzyme method using an NO detection kit, and NOS activity in cells was measured using an NOS detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), Na^+/K^+-ATPase activity and Ca^{2+}/Mg^{2+}-ATPase activity in cells were measured using Na^+/K^+-ATPase activity and Ca^{2+}/Mg^{2+}-ATPase activity detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Each according to the manufacturer's instructions. When counting of mitochondria, cells were incubated with 10 μg/ml rhodamine-123 (R123, Sigma) in PBS according to the manufacturer's instructions, then imaged using an Olympus BX51 fluorescence microscope (Tokyo, Japan). Three fields were examined for each sample and the percentage of cells with a normal, reduced, or increased number of mitochondria was calculated from 100 cells in each field.

Real-time PCR analysis

Cells were cultured for 72 h as described in Section Several factors related to energy metabolism analysis above. Total cellular RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized using PrimeScript™ First Strand cDNA Synthesis Kit (TaKaRa, Japan) from 1 μg of RNA. Real-time PCR were performed using the ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 μl containing SYBR®Premix Ex TaqTM (Perfect Real Time) (TaKaRa). Data was analyzed and values form ΔΔCt were obtained from the ABI 7500 System SDS Software. Relative levels of gene expression were determined by using the '$2^{-\Delta\Delta C(T)}$' Method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Specific primer sequences were synthesized in BIOSUNE Biological Technology Corp (Shanghai, China). And the sequences of the primers were as follows: *PPARγ* (forward: 5'-ATTTACACGATGCTGGCCTC-3'; reverse:5'-GAGGC CAGCATCGTGTAAAT-3'); *iNOS* (forward: 5'-ACCTA CCAGCTGACGGGAGAT-3'; reverse:5'-TGGCAGGGTC CCCTGTGATG-3') (Ulbrich et al., 2006), *GAPDH* (forward: 5'-AGGAGTGGAAGTGGTTCCG-3'; reverse: 5'-GCCCCGGTACTACTCTGTCA-3')(Komatsu et al., 2003) and 18S ribosomal RNA (*18S rRNA*, forward: 5'-CGGTCGGCGTCCCCCAACTT-3'; reverse: 5'- GCGTGC AGCCCCGGACATCTAA-3')(Lehnert et al., 2007).

Statistical analysis

All data were obtained from one independent experiment carried out in triplicate. Main and interactive effects were analyzed by One-way analysis of variance (ANOVA) using SPSS15.0 software. When justified by One-way ANOVA, differences between individual group means were analyzed by the least squares difference (LSD) test. Differences were considered statistically significant at $p<0.05$ and $p<0.01$.

RESULTS

Effect of nicotinamide on proliferation of bovine preadipocytes

Bovine preadipocytes were treated with 0, 100, 200, 300, 400, or 500 μM nicotinamide. After 2, 4, 6, or 8 days, the cells were harvested and analyzed with MTT to test the effect of nicotinamide on proliferation of the preadipocytes (Table 1). Absorbance values after 4-8 days treatment with 200-400 μM nicotinamide were significantly higher than control values ($p<0.05$). Interestingly, treatment with 500 μM nicotinamide caused a decrease in mitochondrial activity, but did not increase cell number ($p<0.05$). These results indicated that proliferation in bovine preadipocytes was enhanced by 100-400 μM nicotinamide, but not by the highest concentration of 500 μM.

Effect of nicotinamide on differentiation of bovine preadipocytes

Differentiation was confirmed by Oil Red O staining of cells and measurement of TG accumulation (Jeong et al., 2008). An increase in the amount of TG accumulation was observed after culture of preadipocytes for 72 h in differentiation-induction medium in the presence of 200 or

Table 1. Effect of nicotinamide on the proliferation of bovine preadipicytes[1]

Day	0 μM	100 μM	200 μM	300 μM	400 μM	500 μM
2	0.071±0.007[a]	0.063±0.003[a]	0.062±0.006[a]	0.055±0.009[a]	0.060±0.015[a]	0.072±0.017[a]
4	0.344±0.011[b]	0.371±0.015[ab]	0.412±0.025[a]	0.413±0.019[a]	0.409±0.010[a]	0.277±0.012[c]
6	0.492±0.032[b]	0.727±0.038[a]	0.795±0.046[a]	0.810±0.006[a]	0.752±0.022[a]	0.380±0.048[c]
8	0.522±0.043[c]	0.870±0.017[ab]	0.895±0.029[a]	0.871±0.062[ab]	0.781±0.015[b]	0.475±0.019[c]

[1] OD value, Mean±SE. Experiments were repeated 3 times.

[a, b, c] Values in the same row with different superscripts differ significantly ($p<0.05$).

Table 2. Effect of nicotinamide on TG content of bovine preadipicytes[1]

Nicotinamide content (μM)	0	100	200	300	400	500
TG content (nM)	1.946±0.063[c]	2.386±0.227[c]	3.453±0.166[b]	6.215±0.761[a]	2.323±0.274[c]	1.695±0.326[d]

[1] Mean±SE. Experiments were repeated 3 times.
[a, b, c, d] Values in the same row with different superscripts differ significantly ($p<0.05$).

Table 3. Effects of nicotinamide on NO, tNOS activity and iNOS activity of bovine preadipocytes[1]

Nicotinamide content (μM)	0	300	500
NO (OD value)	0.115±0.003[a]	0.089±0.001[b]	0.101±0.006[a]
tNOS activity (U/ml)	5.512±0.069[a]	2.529±0.273[b]	2.968±0.265[b]
iNOS activity (U/ml)	1.414±0.047[a]	0.697±0.149[b]	1.196±0.030[a]

[1] Mean±SE. Experiments were repeated 3 times.
[a, b] Values in the same row with different superscripts differ significantly ($p<0.05$).

300 μM nicotinamide. Nicotinamide at 500 μM exerted an inhibitory effect on TG content, which declined by 12.9% relative to control values ($p<0.05$, Table 2). And *GAPDH* mRNA expression greatly increased in the presence of 300 μM nicotinamide and decreased in 500 μM ($p<0.05$, Table 6). These results were confirmed by the Oil Red O staining, which revealed noticeable differences in the appearance of the lipid droplets in the adipocytes exposed to different concentrations of nicotinamide (Figure 1). But *PPARγ* mRNA expression greatly increased in the presence of 500 μM nicotinamide ($p<0.05$, Table 6). These results showed that certain concentrations of nicotinamide enhanced the TG content of the bovine adipocytes and stimulated differentiation into mature cells.

Effect of nicotinamide on NO content, NOS activity and iNOS mRNA expression in bovine preadipocytes

Assessment of NO levels and NOS activity using a detection kit revealed that total NOS (tNOS) activity in the soluble extracts decreased with increased concentration of nicotinamide in the culture medium ($p<0.05$, Table 3). iNOS activity in the presence of 300 μM nicotinamide was significantly lower than the other groups ($p<0.05$), and the decreased of *iNOS* mRNA expression was significantly different from control values ($p>0.05$, Table 6). These result are consistent with the significant inhibition of NO release that was observed in the presence of 300 μM nicotinamide but not at the other groups ($p<0.05$).

Effect of nicotinamide on ATPase activity of bovine preadipocytes

We examined the effects of nicotinamide on the ATPase activity of bovine preadipocytes. Na^+/K^+-ATPase activity and Ca^{2+}/Mg^{2+}-ATPase activity in the presence of 300 μM nicotinamide were higher than the other groups ($p<0.05$, Table 4). ATPase activity in 500 μM nicotinamide did not

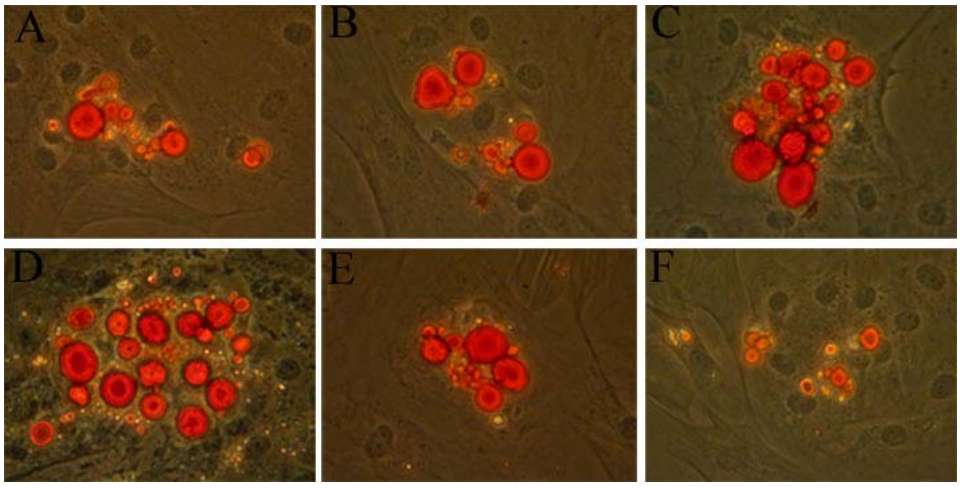

Figure 1. Preadipocytes were treated with 0, 100, 200, 300, 400, or 500 μM nicotinamide for 8 days, at which time the cells were confluent and had begun to differentiate, and then visualized by Oil Red O staining (×400). Lipid droplets were stained bright red. Cells shown in photos A-F were treated with the following concentrations of nicotinamide: (A) 0 μM, (B) 100 μM, (C) 200 μM, (D) 300 μM, (E) 400 μM, and (F) 500 μM nicotinamide.

Table 4. Effects of nicotinamide on Na^+-K^+-ATPase activity and Ca^{2+}-Mg^{2+}-ATPase activity of bovine preadipocytes[1]

Nicotinamide content (μM)	0	300	500
Na^+-K^+-ATPase activity (U/ml)	0.452±0.006[b]	0.483±0.001[a]	0.443±0.003[b]
Ca^{2+}-Mg^{2+}-ATPase activity (U/ml)	0.423±0.004[b]	0.451±0.005[a]	0.420±0.002[b]

[1] Mean±SE. Experiments were repeated 3 times.
[a, b] Values in the same row with different superscripts differ significantly ($p<0.05$).

Table 5. Effects of nicotinamide on number of mitochondria of bovine preadipocytes[1]

Nicotinamide content (μM)	0	300	500
Normal (% cells with different number of mitochondria)	75.33±1.76[A]	5.33±2.90[C]	31.00±4.16[B]
reduced (%cells with different number of mitochondria)	14.66±2.33[B]	1.33±1.33[C]	65.00±3.78[A]
Increased (% cells with different number of mitochondria)	10.00±0.57[B]	93.33±3.33[A]	4.00±0.58[C]

[1] Mean±SE. Experiments were repeated 3 times.
[A, B, C] Values in the same row with different superscripts differ significantly ($p<0.01$).

differ significantly from control values ($p>0.05$). The results showed that certain concentrations of nicotinamide promoted ATPase activity.

Effect of nicotinamide on number of mitochondria in bovine preadipocytes

Mitochondria were visualized by R123 fluorescence staining in preadipocytes. Variation in the intensity of the bright green fluorescence resulting from staining of the mitochondria corresponded to different numbers of mitochondria per cell (Figure 2). Treatment with 300 μM nicotinamide significantly increased the mitochondrial DNA copy number in the preadipocytes ($p<0.05$), whereas 500 μM nicotinamide had an inhibitory effect, as evidenced by a significant decrease in mitochondrial DNA copy number ($p<0.05$) (Table 5).

DISCUSSION

It has been previously reported that nicotinamide can induce both proliferation and differentiation of embryonic stem cells into insulin-producing cells (Vaca et al., 2003), and induce human fetal islet cell differentiation and maturation (Otonkoski et al., 1993). In this study, we found that nicotinamide significantly promoted proliferation and differentiation of bovine preadipocytes. The optimal concentration of nicotinamide was 300 μM, similar to that previously shown to be effective in pig adipocytes (Bai et al., 2008). While nicotinamide inhibits adipocyte differentiation in 3T3-L1 cells (Lewis et al., 1982). These apparent contradictions can be explained by the fact that we used a small doses of nicotinamide treatment (100, 200, 300, 400 and 500 μM), but Lewis et al. (1982) used much higher concentration of nicotinamide (5, 10, 15 and 20 mM). And we already found that higher concentration of nicotinamide (500 μM) didn't enhance proliferation and differentiation in bovine while lower concentration could (100-400 μM).

Mammalian adipocytes are the major site for TG storage, and as such they constitute a long-term energy reservoir. These cells play a critical role in maintaining energy balance and homeostasis of circulating fatty acids by promoting TG break down and fatty acid release. *PPARγ* is

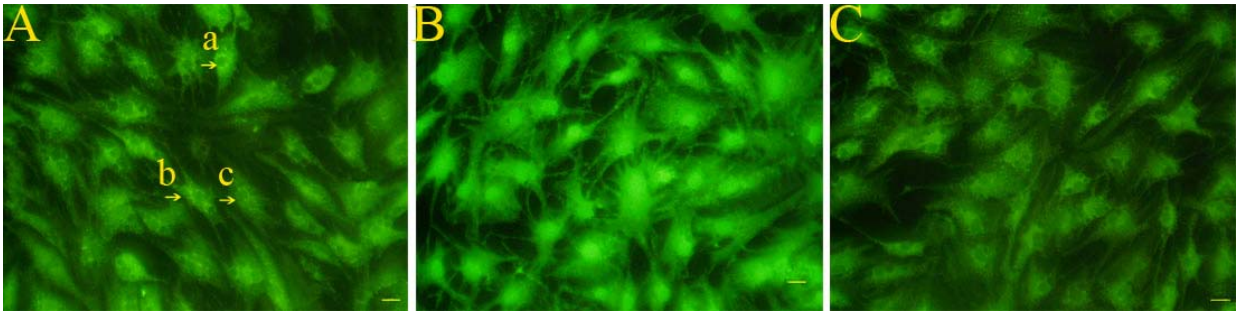

Figure 2. Representative image of bovine preadipocytes stained with mitochondria-specific R123 dye (×400). Cells were treated with 0 (A), 300 (B), or 500 (C) μM nicotinamide and cultured for 72 h until confluence and the onset of differentiation before staining and microscopic analysis. Variation in the intensity of the bright green fluorescence resulting from staining of the mitochondria corresponds to different numbers of mitochondria per cell. Fields were examined for each sample and percentages of the cells with (a) increased, (b) normal, and (c) reduced number of mitochondria were calculated from 100 cells in each field. The scale bar represents 20 μm.

Table 6. Effects of nicotinamide on relative mRNA expression of bovine preadipocytes[1]

Nicotinamide content (μM)	0	300	500
PPARγ	1.000±0.158[b]	0.941±0.256[b]	2.425±0.253[a]
GAPDH	1.000±0.253[b]	1.424±0.249[a]	0.401±0.131[c]
iNOS	1.000±0.127[b]	0.795±0.116[c]	6.046±0.070[a]

[1] Mean±SE. Experiments were repeated 3 times.
[a, b, c] Values in the same row with different superscripts differ significantly ($p<0.05$).

an important transcriptional regulator during adipocyte differentiation (Choi et al., 2003). In this study, 500 μM nicotinamide increased *PPARγ* expression in bovine preadipocytes, which is consistent with that niacin could up-regulate *PPARγ* expression in cultured adipocytes of rabbits (Yang et al., 2008) and in 3T3-L1 cells (Yu et al., 2007). A retrospective analysis of gene expression data has revealed that *GAPDH* expression is sometimes affected by endocrine factors, such as dexamethasone (Oikarinen et al., 1991) and insulin (Rolland et al., 1995a). *GAPDH* showed variability in the expression levels observed in our study. This finding is consistent with previous results in adipose tissue (Rolland et al., 1995a; Rolland et al., 1995b; Barroso et al., 1999; Liu et al., 2003). The relative change of *GAPDH* expression to energy balance supports the possibility of an increased expression in obesity (Catalan et al., 2007).

NO is involved in adipose tissue biology by influencing adipogenesis, insulin-stimulated glucose uptake and lipolysis (Engeli et al., 2004). Lipid accumulation and lipogenic enzymes are also induced by NO in rat white preadipocytes (Yan et al., 2002). However, nicotinamide inhibited NO production in macrophage RAW 264.7 cells by preventing *NOS* mRNA induction, without inhibiting NOS activity (Pellat-Deceunynck et al., 1994). The present work showed that nicotinamide inhibited iNOS activity and *iNOS* mRNA expression, as previously observed in the fibroblast cell line L929 (Hauschildt et al., 1992) and the insulinoma cell line RINm5F (Cetkovic-Cvrlje et al., 1993; Andrade et al., 1996). These apparent contradictions can be explained by the fact that we used a different nicotinamide treatment method and different cells to other researchers.

White adipocytes contain large numbers of mitochondria in their cytosolic compartment to provide a large capacity for adenosine triphosphate (ATP) production. The maintenance of cellular energy reserves and cellular function by nicotinamide are closely tied to the function of mitochondria (Li et al., 2006). Nicotinamide participates in energy metabolism through the tricarboxylic acid cycle by utilizing NAD^+ in the mitochondrial respiratory electron transport chain for the production of ATP, DNA synthesis, and DNA repair (Lin and Guarente, 2003; Magni et al., 2004). It has also been previously shown that NO acts as a key messenger to activate the mitochondrial biogenesis program (Nisoli et al., 2003). Low levels of NO may stimulate mitochondrial proliferation (Bossy-Wetzel and Lipton, 2003). Nicotinamide may use other mechanisms to preserve cellular energy metabolism that could depend upon glycolytic metabolism mediated by glyceraldehyde-3-phosphate dehydrogenase (Kaanders et al., 2002). Furthermore, there exist additional pathways that nicotinamide may use to maintain cellular metabolic homeostasis through the maintenance of mitochondrial membrane potential. We propose that bovine preadipocyte mitochondria may have greater demand for ATP in high yielding dairy cows in early lactation when these animals are in negative energy balance, as the cows must then use body fat as a source of energy. Thus, a nicotinamide additive could regulate energy metabolism in these cows to ameliorate the negative energy balance. In this study, both the number of mitochondria and ATPase activity increased in the presence of 300 μM nicotinamide. This concentration of nicotinamide also resulted in lower NO production and higher *GAPDH* mRNA, suggesting that decreased NO and increased *GAPDH* mRNA may enhance ATP production by giving rise to an increase in the number of mitochondria and in ATPase activity. The results presented here support this hypothesis, but further confirmation is needed through longitudinal observations.

Interestingly, treatment with 500 μM nicotinamide gave a significant decrease in TG levels, ATPase activity, mitochondrial DNA copy number, *GAPDH* mRNA, and increase in NO content, *PPARγ* mRNA and *iNOS* mRNA. Based on previous reports (Lewis et al., 1982), we could anticipate that high concentrations of nicotinamide (500 μM) inhibit adipocyte differentiantion. These results suggest that nicotinamide is a double edged sword (Williams and Ramsden, 2005), and point out that exist the most effective nicotinamide concentration for dairy cow nutrition supplement. We propose that nicotinamide also could induce to activate NOS activity through another pathway, producing excessive NO to inhibit energy metabolisom. However, due to lack of data this hypothesis needs further testing.

In vivo, postpartum serum nicotinic acid concentrations were increased in dairy cows receiving 12 g/d supplemental niacin or nicotinamide (Jaster et al., 1983; Campbell et al., 1994). Nicotinamide concentration of plasma was

unaffected by treatment and ranged from 0.85 to 1.26 μg/ml for cows supplemented with nicotinamide and nicotinic acid respectively (Campbell et al., 1994). Our present study provides further support for the effects of nicotinamide on lipid and energy metabolism, emphasizing the importance of investigating bovine adipose tissue to improve our understanding of dairy cow physiology. Additional work with nicotinamide is needed to substantiate these preliminary results, and to establish the amount to be supplemented to cows to control negative energy balance and reduced ketosis in early lactation.

In conclusion, nicotinamide exerts control at a crucial point in the signaling pathways that control lipolysis and energy balance in bovine preadipocytes, and it can induce both proliferation and differentiation of these cells. Lipolytic activity is controlled by inhibition of NOS activity that gives rise to a decrease in NO production, which in turn increases ATPase activity and the number of mitochondria. Future studies should elucidate how adipocytes respond to nicotinamide treatment with respect to changes in their intracellular energy charge and the molecular mechanism of their metabolic adaptations.

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