Effect of heat-shock protein B7 on oxidative stress in adipocytes from preruminant calves

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ABSTRACT

Dairy cows with ketosis display excessive lipolysis in adipose tissue. Heat-shock protein B7 (HSPB7), a small heat-shock protein, plays important roles in mediating cytoprotective responses to oxidative stress in rodent adipose tissue. Accordingly, it is assumed that HSPB7 may also play important roles in the antioxidant response in adipose tissue of ketotic cows. Therefore, the aim of this study is to investigate (1) the redox state of adipose tissue in ketotic cows and (2) the role and mechanism of HSPB7 on the regulation of oxidative stress in adipocytes from preruminant calves. An in vivo study consisting of 15 healthy and 15 clinically ketotic cows was performed to harvest subcutaneous adipose tissue and blood samples. In addition, adipocytes isolated from calves were treated with different concentrations of H₂O₂ (0, 12.5, 25, 50, 100, or 200 μ M) for 2 h, transfected with adenovirus-mediated overexpression of HSPB7 for 48 h, or transfected with small interfering RNA of HSPB7 for 48 h followed by exposure to H₂O₂ (200 μ M) for 2 h. Serum concentrations of nonesterified fatty acids and β -hydroxybutyrate were greater in cows with clinical ketosis, whereas serum concentration of glucose was lower. Compared with healthy cows, the malondialdehyde content was greater but the activity of glutathione peroxidase and superoxide dismutase was lower in adipose tissue of clinically ketotic cows. The abundance of HSPB7 and nuclear factor, erythroid 2 like 2 (NFE2L2) was greater in adipose tissue of clinically ketotic cows. In vitro, H₂O₂ treatment induced the overproduction of reactive oxygen species and malondialdehyde, and inhibited the activity of antioxidant enzymes glutathione peroxidase

and superoxide dismutase in adipocytes from preruminant calves. The low concentration of H₂O₂ (12.5, 25, and 50 μ M) increased the abundance of HSPB7 and NFE2L2, but high concentrations of H₂O₂ (100 or 200 μ M) reduced the abundance of HSPB7 and NFE2L2. The overexpression of HSPB7 improved the H₂O₂-induced oxidative stress in adipocytes via increasing the abundance of NFE2L2 and its downstream target genes heme oxygenase-1 (HMOX1) and NADH quinone oxidoreductase 1 (NQO1). Knockdown of HSPB7 markedly inhibited the expression of NFE2L2, HMOX1, and NQO1 and further exacerbated H₂O₂-induced oxidative stress. Overall, these results indicate that activation of the HSPB7-NFE2L2 pathway increases cellular antioxidant capacity, thereby alleviating oxidative stress in bovine adipocytes.

Key words: adipocytes, HSPB7, oxidative stress, NFE2L2

INTRODUCTION

Ketosis is a major metabolic disorder of dairy cows in early lactation, a time during which cows often display a state of negative energy balance (NEB) induced by a low intake of DM and an increase in demands for energy (Roche et al., 2013). Severe NEB initiates fat mobilization of adipose tissue and increases the blood concentration of nonesterified fatty acids (NEFA) and BHB (Loo et al., 2007). It is clear that the intense adipose mobilization play promoting roles in the onset and progression of the ketosis (Grummer, 1993; White, 2015). Metabolic adaptations to NEB trigger catabolic pathways of adipose tissue and increase the systemic reactive oxygen species (ROS) production (Bernabucci et al., 2005; Pedernera et al., 2010), but it is currently unclear that oxidative stress exists in the adipose tissue of ketotic cows. When the antioxidant defenses are unable to neutralize the excessive ROS, oxidative stress

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can be induced. Increasing evidence demonstrates that dairy cows with ketosis display systemic (Sahoo et al., 2009; Li et al., 2016; Youssef and El-Ashker, 2017) and hepatic (Du et al., 2017) oxidative stress. However, the redox state of adipose tissue in ketotic cows remains unclear.

Small heat-shock proteins are a family of molecular chaperones (Yang et al., 2011), exerting housekeeping functions in normal cell metabolism as well as protective functions under stress conditions (Verschuure et al., 2003). Heat-shock protein B7 (**HSPB7**), a member of the small heat-shock proteins family, is preferentially expressed in cardiovascular, skeletal muscle and adipose tissue (Yang et al., 2011). The expression of HSPB7 mRNA was increased in the white adipose tissue of obese rats (Krief et al., 1999). Previous studies also demonstrated that high expression of heat shock protein 70 represents an autoprotective mechanism for biological stress, especially for oxidative stress, in mouse RIF-1 cells and multidrug-resistant human breast carcinoma (MCF-7/ADR) cells and in skeletal muscle, heart, and liver (Salo et al., 1991; Gomer et al., 1996; Morimoto and Santoro, 1998; Lee and Corry, 1998). Collectively, these findings indicate that high expression of HSPB7 may also be an adaptation mechanism to oxidative stress in adipose tissue of obese rodents and humans.

To our knowledge, the abundance and role of HSPB7 in the adipose tissue of ketotic cows has not been characterized. In addition to heat shock protein, the nuclear factor, erythroid 2 like 2 (**NFE2L2**) also has a crucial role in the antioxidant response in humans and rodents (Karttunen et al., 2018; Sherif, 2018). Activation of NFE2L2 initiates transcription of the various antioxidant genes including heme oxygenase-1 (**HMOX1**), NADH quinone oxidoreductase 1 (**NQO1**), glutathione peroxidase (**GSH-Px**), and superoxide dismutase (**SOD**), which together enhance cellular antioxidant capacity (Jung and Kwak, 2010). However, it is unclear whether HSPB7 is associated with stress-induced

NFE2L2 activation or the regulation of oxidative stress (or both) in the adipose tissue of ketotic cows.

Because of the antioxidant role of the HSPB7 and NFE2L2 via regulating of antioxidant genes transcription in adipose tissue of rodents and humans, we hypothesized that HSPB7 and NFE2L2 may also play important roles in mediating cytoprotective responses to oxidative stress in adipose tissue of ketotic cows. Therefore, the aim of this study is to investigate (1) the redox state of adipose tissue in ketotic cows and (2) the role and mechanism of HSPB7 on the regulation of oxidative stress in adipocytes from preruminant calves.

MATERIALS AND METHODS

Animals

The study protocol was approved by the Ethics Committee on the Use and Care of Animals at Jilin University (Changchun, China). Dairy cows were selected from a 7,000-cow dairy farm located in Changchun City, Jilin Province, China. All cows received a routine physical examination to ensure absence of other co-morbidities. Cows had ad libitum access to the same diet (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15726>). We chose lactating Holstein cows with a similar number of lactations (median = 3, range = 2 to 4) and DIM (median = 6 d, range = 3 to 10 d). The cows were classified as suspected clinical ketosis by veterinarians if feed intake, milk yield, or both were reduced and a nitroprusside test for ketone bodies in milk was positive (Du et al., 2018). Subsequently, the blood concentration of BHB in these cows was measured. According to the clinical symptoms and serum BHB concentrations (Itle et al., 2015; Du et al., 2018), 15 cows with serum BHB concentrations greater than 3 mM and 15 control cows with serum BHB concentrations below 0.6 mM were selected. The basic description of the cows is reported in Table 1.

Table 1. Body weight; milk production; DMI; and serum glucose, nonesterified fatty acids (NEFA), and BHB of healthy and clinically ketotic cows

Item	Median; IQR ¹	
	Control (n = 15)	Clinical ketosis (n = 15)
BW (kg)	(657; 22.41)	(665; 28.33)
Milk production (kg of milk/cow per day)	(31.2; 2.5)	(26.4; 2.1)*
DMI (kg/d)	(21.4; 1.6)	(20.6; 1.9)
Serum glucose (mM)	(4.19; 0.32)	(2.16; 0.19)**
Serum NEFA (mM)	(0.21; 0.013)	(1.22; 0.13)**
Serum BHB (mM)	(0.37; 0.025)	(3.57; 0.325)**

¹IQR = interquartile range.

*P < 0.05; **P < 0.01.

Blood samples were collected on 3 consecutive days at the same hour every day via jugular venipuncture (no anticoagulant). The first blood sample was collected at the moment of clinical examination and then the other 3 samples harvested every 24 h. The blood samples were collected between 0700 and 0800 h before feeding and the serum was separated after centrifugation at $3,500 \times g$ for 15 min at 4°C and stored at -80°C until analysis. Cows had access to a constant supply of fresh water and were milked twice daily at 0800 and 1530 h. Milk yield was recorded electronically.

Subcutaneous adipose tissue (approximately 3 g) biopsies from each of the 15 cows were taken according to published procedures (Zachut et al., 2017). Briefly, the biopsy site, a 5×5 cm area of skin at the tailhead, was prepared by clipping, washing, and disinfecting. The biopsy site was anesthetized and aseptic techniques were used in making a 1.5- to 2.5-cm scalpel incision through the skin and subcutaneous tissue. Adipose samples were captured with tweezers and cut off with scissors, tissue samples were washed with saline, and then snap-frozen in liquid nitrogen and stored at -80°C .

After 3 d of sample collection, the ketotic cows receive combined therapy with glucocorticoids and oral glucogenic supplements, propylene glycol (PG), according to published procedures (van der Drift et al., 2015). Briefly, after collection of samples, cows were all treated with 250 mL of PG (Eurovet Animal Health) orally twice daily for 3 d and single intramuscular injections of 1 mg/50 kg of BW dexamethasone-21-isonicotinate (Voren Suspension; Boehringer Ingelheim). Metabolic blood variables were monitored for 6 d. The BHB concentrations in blood decreased in all cows after treatment, indicating a good therapeutic effect.

Determination of Blood Biomarkers

Concentrations of glucose, BHB, and NEFA in serum were determined using a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan) with commercially available kits (BHB: cat. no. RB1008; NEFA: cat. no. FA115; glucose: cat. no. GL3815; Randox Laboratories, Crumlin, UK).

Isolation of Primary Preadipocytes

Adipocytes were isolated according to published procedures (Yin et al., 2015; Zhang et al., 2018). Five healthy Holstein calves (1 d old, female, 30–40 kg) were anesthetized with xylazine and ketamine and maintained with halothane and oxygen in a semi-closed circle system. Lactated Ringer's solution was administered intravenously during the procedure. The calves were positioned in dorsal recumbency and prepared for

aseptic surgery, a 15-cm abdominal incision was made extending caudally from the costal margin. The adipose tissue from the peritoneal omentum and mesentery were captured with tweezers and cut off with scissors under sterile conditions. The peritoneal omentum and mesentery were sutured with surgical gut sutures. After sample collection, the incision was closed in 5 layers (internal rectus sheath and peritoneum, rectus abdominus muscle, external rectus sheath, subcutaneous tissue, and skin), each in a simple continuous suture pattern. Additional postoperative management included ampicillin (20 mg/kg i.m. twice daily) for 3 d and daily examination including incisional inspection, behavior, appetite, urination, and defecation. Calves were confined until suture removal 10 to 14 d following surgery and then returned to the farm and routine management in dairy farm. The resulting tissue was rinsed in sterile PBS containing penicillin (2,500 U/mL) and streptomycin (2,500 mg/mL) to remove adherent blood. The fascia and blood vessels visible in the tissue were carefully peeled away, and the resulting tissue was cut into small pieces of approximately 1 mm and digested using Dulbecco's modified Eagle medium (DMEM)/F12 digestion solution containing collagenase type I (1 mg/mL, Sigma-Aldrich, St. Louis, MO) at 37°C in a slightly shaking water bath for 1 h. The mixture was removed from the digested tissue fluid through a $80\text{-}\mu\text{m}$ cell filter and the filtrate was centrifuged at $175 \times g$ at room temperature for 10 min. The residual erythrocytes were removed by adding ACK lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) into the precipitate and centrifuging at $175 \times g$ at room temperature for 10 min. The supernatant was discarded, and the pellet was resuspended with basic culture medium (BCM), which was DMEM/F12 with 10% fetal bovine serum and 1% penicillin/streptomycin. After cell counting, the cell suspension was adjusted to a concentration of $1 \times 10^4/\text{cm}^2$ and inoculated in a cell culture flask. The culture was then incubated at 37°C and 5% CO_2 in a cell incubator for 24 h. The medium was replaced to remove nonadherent cells and tissue residues. The BCM was replaced every other day.

Cell Culture and Treatment

Adipocytes were differentiated as described previously (Yin et al., 2015; Zhang et al., 2018). To induce differentiation of preadipocytes into mature adipocytes, primary cells were seeded in cell culture plates. The BCM was discarded after cell aggregation reached $\sim 70\%$. Then, freshly prepared differentiation culture medium 1, which was a final concentration of 0.5 mM IBMX (I-7018 Sigma-Aldrich), 1 μM dexamethasone (D-4902

Sigma-Aldrich), and 1 $\mu\text{g/mL}$ insulin (I-5500 Sigma-Aldrich) in BCM, was used to induce differentiation. After 48 h of culture, differentiation culture medium 1 was discarded and replaced by differentiation culture medium 2, which was a final concentration of 1 $\mu\text{g/mL}$ insulin in BCM, to maintain the differentiation culture. After 48 h of culture, differentiation culture medium 2 was discarded and replaced by BCM (DMEM/F12 with 10% fetal bovine serum and 1% penicillin/streptomycin), the fresh BCM was replaced every other day to keep the culture until visible lipid droplets appeared in the cell, indicating that the cells have completed differentiation. This period lasted approximately 10 d.

Adipogenic differentiation was evaluated using oil red O staining as an indication of intracellular lipid accumulation (Park et al., 2010). After induction of adipogenic differentiation as described above, cells were washed 3 times in PBS, fixed in 10% formalin for 15 min, and washed a further 3 times in PBS. Subsequently, cells were washed in 60% (vol/vol) isopropanol for 2 min, stained with 0.5% (wt/vol) oil red O solution for 15 min, washed with 60% isopropanol and subsequently with PBS, and then counter-stained with hematoxylin before microscopy. After induction of adipogenic differentiation, the adipogenic efficiency was 96.2% and the representative images are shown in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2018-15726>).

After the adipogenic induction, mature adipocytes were treated with H_2O_2 (0, 25, 50, 100, 200, and 400 μM ; 323381 Sigma-Aldrich) for 2 h. For the transfection experiments, to overexpress or silence HSPB7, differentiated adipocytes were transfected with HSPB7 overexpression adenovirus (**Ad-HSPB7**), GFP negative control adenovirus (**Ad-GFP**), small interfering RNA (**siRNA**) for HSPB7, a negative siRNA control for 48 h, and cells were then treated with 200 μM H_2O_2 for 2 h. The detailed grouping is described in the figure legends. The empty adenovirus vector (Ad-GFP) and HSPB7 overexpression adenovirus (Ad-HSPB7) were constructed by Gemma Genes (Shanghai, China). The siRNA was designed and synthesized by Gemma Genes based on the bovine HSPB7 mRNA sequence. The siRNA sequence was sense: 5'-CCUUCGGACG-GAGAUCAATT-3', and antisense: 5'-UUGAUCUCC-GUCCGGAAGGTT-3'.

Cell Viability Assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (**MTT**) cell proliferation and cytotoxicity assay kit (C0009, Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's protocol. Briefly, adipocytes were

plated on 96-well multiplates at a density of 2×10^3 cells/mL, and then treated as aforementioned in Cell Culture and Treatment section. And then followed by treatment with 5 mg/mL of MTT for 4 h at 37°C. Optical density was measured at 570 nm on a microplate reader (Tecan, Grödig, Austria). Results are presented as a percentage of the control values.

Determination of ROS Generation

The levels of intracellular ROS were confirmed using the peroxide sensitive fluorescent probe 2'7'-dichlorofluorescein diacetate (Beyotime Institute of Biotechnology). The adipocytes were exposed to serum-free DMEM/F12 medium containing 25 μM 2'7'-dichlorofluorescein diacetate at 37°C in the dark for 20 min and then washed 3 times with DMEM/F12. Cells were detached from the dish with 0.25% trypsin and washed twice with PBS. Then, the cells were resuspended with BCM in 1.5-mL tubes and centrifuged at $1,000 \times g$ for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended with fresh serum-free DMEM/F12. Fluorescence was measured by flow cytometry (FACSCalibur, Becton Dickinson, Sunnyvale, CA).

Detection of Oxidative Stress Indicators

The activities of GSH-Px and SOD as well as the contents of malondialdehyde (**MDA**) in adipose tissue and adipocytes were determined using spectrophotometric diagnostic kits (GSH-Px, S0058; SOD, S0109; MDA, S0131; Beyotime Institute of Biotechnology), according to the manufacturer's protocols. In brief, adipose tissue specimens were crushed with a microthermal (4°C) tissue grinder (Shanghai Gino Tech & Trading Co. Ltd., Shanghai, China), diluted with ice-cold PBS, and then centrifuged at $12,000 \times g$ for 10 min at 4°C. Adipocytes were collected with ice-cold PBS by scraping, and then centrifuged at $1,000 \times g$ for 10 min at 4°C. Absorbance was detected at 340 nm (GSH-Px), 560 nm (SOD), and 532 nm (MDA) and was recorded with a 722N spectrophotometer (Scientific Instrument Co. Ltd., Shanghai, China).

RNA Isolation and Quantitative Reverse-Transcription PCR

Total RNA was extracted with RNAiso Plus (TaKaRa Biotechnology Co. Ltd., Dalian, China) from calf adipocytes and adipose tissue. The RNA concentration and quality were measured using a K5500 MicroSpectrophotometer (Beijing Kaiiao Technology Development Ltd., Beijing, China) and electrophoresis (1% agarose

gels). The RNA analysis by electrophoresis showed that the total RNA was of high quality, evident by the appearance of clear 28S, 18S, and 5S bands without significant degradation. According to MIQE guidelines (Bustin et al., 2009), the 1.8 to 2.0 of optical density (OD) at 260 nm/OD₂₈₀ ratio is the threshold for RNA quality. In our study, the OD₂₆₀/OD₂₈₀ ratio of the total RNA was determined to be 1.9 and met the specified purity requirements. Then 1 µg of total RNA in each sample was reverse-transcribed to cDNA (TaKaRa Biotechnology Co. Ltd.) according to the supplier's protocol. The primers used were designed by Primer Express software 5.0 and the primer sequences are shown in Supplemental Table S2 (<https://doi.org/10.3168/jds.2018-15726>). The SYBR green plus reagent kit (Roche, Norwalk, CT) was used to prepare a 20-µL mixture and mRNA abundance was detected with a 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA). The reaction conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene expression was determined by the $2^{-\Delta\Delta CT}$

method. The cycles-to-threshold values of GAPDH, ubiquitin, and β -actin were not affected by different treatments (Supplemental Figure S2; <https://doi.org/10.3168/jds.2018-15726>); therefore, the relative expression of target genes was normalized to GAPDH, ubiquitin, and β -actin. For in vivo quantitative real-time PCR experiments, the PCR reaction was performed in triplicate for each sample (n = 15 cows per group). For in vitro quantitative real-time PCR experiments, the PCR reaction was performed in triplicate from each of the 3 individual cell preparations.

Western Blot Analysis

Total protein was extracted from the biopsy adipose samples and cultured cells using a commercial protein extraction kit [250 mM Tris (pH 7.5), 40 mM NaCl, 10 mM EDTA, 5 mM NP-40, protease and phosphatase inhibitors; C510003, Sangon Biotech Co. Ltd., Shanghai, China] according to the manufacturer's instructions. The protein concentration was determined using the

Figure 1. Dairy cows with ketosis displayed oxidative stress in adipose tissue, and increased abundance of heat-shock protein B7 (HSPB7) and nuclear factor, erythroid 2 like 2 (NFE2L2). (A) Superoxide dismutase (SOD) activity, (B) glutathione peroxidase (GSH-Px) activity, (C) malondialdehyde (MDA) content, (D) relative mRNA expression levels of HSPB7 and NFE2L2, (E) Western blot analysis of HSPB7 and NFE2L2, and (F) relative protein expression levels of HSPB7 and NFE2L2. The mRNA and protein expression levels of the ketosis group were normalized to the control group. Panels A–C were analyzed with Wilcoxon signed-rank test and expressed as the mean $\pm$ SEM (n = 15 per group). Panels D–F were analyzed with paired *t*-tests and expressed as the mean $\pm$ SEM (n = 15 per group). ***P* < 0.01.

BCA Protein Assay Kit (P1511, Applygen Technologies). Thirty micrograms of protein was separated using SDS-PAGE with known molecular weight markers (Sangon Biotech Co. Ltd.). Subsequently, the protein was transferred onto 0.45 μm polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were incubated with primary antibodies against GAPDH (internal control; sc-59540, Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:5,000), HSPB7 (cat. no. Ab150390, Abcam, Cambridge, MA; 1:1,000), and NFE2L2 (cat. no. Ab31163, Abcam; 1:1,000) at 4°C overnight. Subsequently, the PVDF membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Boster, Wuhan,

China). Last, the immunoassay was performed using an enhanced chemiluminescent reagent (Pierce Biotechnology Inc., Chicago, IL). Densitometry analysis was performed with Image-Pro Plus 6.0 (Media Cybernetics Inc., Warrendale, PA) and target protein expression was normalized to GAPDH expression.

Statistical Analysis

The results are expressed as means $\pm$ standard error of the mean of at least 3 independent experiments. Statistical analysis was conducted using SPSS 19.0 software (SPSS Inc., Chicago, IL). In the *in vivo* studies, data from quantitative real-time PCR and Western

Figure 2. H_2O_2 -induced oxidative stress in bovine adipocytes. Cells were treated with 0, 25, 50, 100, 200, and 400 μM H_2O_2 for 2 h. (A) Superoxide dismutase (SOD) activity, (B) glutathione peroxidase (GSH-Px) activity, (C) malondialdehyde (MDA) content, and (D–E) reactive oxygen species (ROS) content. M1 = proportion of positive cells in 10,000 cells. Comparisons among groups were calculated using a 1-way ANOVA with subsequent Bonferroni correction. The data presented are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

blotting were normally distributed and analyzed with paired *t*-tests; other data were nonnormally distributed and analyzed with Wilcoxon signed-rank test. In the vitro studies, comparisons among groups were analyzed using the 1-way ANOVA with subsequent Bonferroni correction. A *P*-value <0.05 was considered statistically significant, and a *P*-value <0.01 was considered highly significant.

RESULTS

Increased Oxidative Stress and HSPB7 and NFE2L2 Abundance in Adipose Tissue of Cows with Clinical Ketosis

The activity of the antioxidant enzymes SOD and GSH-Px was lower in adipose tissue of cows with clinical ketosis than in control cows (*P* < 0.01, Figure 1A and B). Conversely, the level of MDA in adipose tissue of cows with clinical ketosis was greater than in control cows (*P* < 0.01, Figure 1C). The mRNA and protein abundance of HSPB7 and NFE2L2 was greater in adipose tissue of the cows with clinical ketosis than in control cows (*P* < 0.01, Figure 1D-F).

Effects of H₂O₂ on Oxidative Stress and the Abundance of the HSPB7 and NFE2L2 Pathway in Bovine Adipocytes

As shown in Supplemental Figure S3 (<https://doi.org/10.3168/jds.2018-15726>), 25 to 200 μ M H₂O₂ did not have significantly decreased cell viabilities, whereas 400 μ M H₂O₂ significantly decreased cell viabilities (*P* < 0.05, Supplemental Figure S3A; <https://doi.org/10.3168/jds.2018-15726>). The SOD and GSH-Px activity decreased in a H₂O₂ dose-dependent manner and was lower in the 50 to 400 μ M cultures than in the control (*P* < 0.05 and *P* < 0.01, respectively, Figure 2A and B). In contrast, the levels of MDA and ROS had an opposite trend and were greater in the 50 to 400 μ M cultures (*P* < 0.05 and *P* < 0.01, respectively, Figure 2C-E). The mRNA and protein abundance of HSPB7 and NFE2L2 was greater with 25, 50, and 100 μ M H₂O₂ but was lower with 200 and 400 μ M than in the control (*P* < 0.05 and *P* < 0.01, respectively, Figure 3A-E). Furthermore, the mRNA abundance of NFE2L2 downstream target genes *HMOX1* and *NQO1* had a similar trend (*P* < 0.05 and *P* < 0.01, respectively, Figure 3F and G).

Figure 3. Effect of H₂O₂ on heat-shock protein B7 (HSPB7) and nuclear factor, erythroid 2 like 2 (NFE2L2) abundance in bovine adipocytes. Cells were treated with 0, 25, 50, 100, 200, and 400 μ M H₂O₂ for 2 h. (A) Relative mRNA expression level of HSPB7, (B) relative mRNA expression level of NFE2L2, (C) Western blot analysis of HSPB7 and NFE2L2, (D) relative protein expression levels of HSPB7, (E) relative protein expression levels of NFE2L2, (F) relative mRNA expression level of heme oxygenase-1 (HMOX1), and (G) relative mRNA expression level of NADH quinone oxidoreductase 1 (NQO1). The data of the 0 μ M H₂O₂ group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a 1-way ANOVA with subsequent Bonferroni correction. The data presented are the mean $\pm$ SEM. **P* < 0.05, ***P* < 0.01.

Effects of HSPB7 Overexpression on Oxidative Stress in Bovine Adipocytes

The mRNA and protein abundance of HSPB7 in the Ad-HSPB7 group was greater than in the control group ($P < 0.01$, Figure 4A, C, and D). The MTT assay showed that adenovirus and $200 \mu\text{M}$ H_2O_2 did not alter adipocyte viability (Supplemental Figure S3B; <https://doi.org/10.3168/jds.2018-15726>). Overexpression of HSPB7 upregulated the protein and mRNA abundance of NFE2L2, and attenuated the effect of H_2O_2 on the protein and mRNA abundance of NFE2L2 ($P < 0.05$ and $P < 0.01$, respectively, Figure 4B, C, and E). Similar trends in mRNA abundance of NFE2L2 downstream target genes *HMOX1* and *NQO1* were also observed ($P < 0.05$ and $P < 0.01$, respectively, Figure 4F and G). Furthermore, HSPB7 overexpression increased SOD and GSH-Px activity, and attenuated the decrease of SOD and GSH-Px activity induced by H_2O_2 ($P < 0.01$,

Figure 5A and B). Importantly, HSPB7 overexpression markedly decreased MDA and ROS production, and attenuated the increase of MDA and ROS induced by H_2O_2 ($P < 0.05$ and $P < 0.01$, respectively, Figure 5C–E).

Effects of HSPB7 Knockdown on Oxidative Stress in Bovine Adipocytes

The MTT assay showed that siRNA and $200 \mu\text{M}$ H_2O_2 did not alter adipocyte viability (Supplemental Figure S3C; <https://doi.org/10.3168/jds.2018-15726>). In contrast to HSPB7 overexpression, HSPB7 knockdown decreased the mRNA and protein abundance of NFE2L2 and mRNA abundance of *HMOX1* and *NQO1* and further exacerbated the downregulation of *NFE2L2*, *HMOX1*, and *NQO1* in H_2O_2 -treated adipocytes ($P < 0.05$ and $P < 0.01$, respectively, Figure 6A–G). Furthermore, HSPB7 knockdown sharply de-

Figure 4. Effect of overexpression of heat-shock protein B7 (HSPB7) on HSPB7 and nuclear factor, erythroid 2 like 2 (NFE2L2) abundance in bovine adipocytes. The cells were divided into 4 groups as follows: an Ad-GFP group (transfected with empty adenovirus vector for 48 h), an Ad-GFP + $200 \mu\text{M}$ H_2O_2 group (transfected with empty adenovirus vector for 48 h and then treated with $200 \mu\text{M}$ H_2O_2 for 2 h), an Ad-HSPB7 group (transfected with HSPB7 overexpression adenovirus for 48 h), and an Ad-HSPB7 + $200 \mu\text{M}$ H_2O_2 group (transfected with HSPB7 overexpression adenovirus for 48 h and then treated with $200 \mu\text{M}$ H_2O_2 for 2 h). (A) Relative mRNA expression level of HSPB7, (B) relative mRNA expression level of NFE2L2, (C) Western blot analysis of HSPB7 and NFE2L2, (D) relative protein expression levels of HSPB7, (E) relative protein expression levels of NFE2L2, (F) relative mRNA expression level of heme oxygenase-1 (HMOX1), and (G) relative mRNA expression level of NADH quinone oxidoreductase 1 (NQO1). The data of the Ad-GFP group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a 1-way ANOVA with subsequent Bonferroni correction. The data presented are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

creased SOD and GSH-Px activity but increased MDA and ROS production, and further exacerbated H_2O_2 -induced downregulation of SOD and GSH-Px activity and overproduction of MDA and ROS ($P < 0.05$ and $P < 0.01$, respectively, Figure 7A–E).

DISCUSSION

Severe NEB, which initiates fat mobilization, induces stress in adipose tissue and renders periparturient cows susceptible to ketosis (White, 2015; Wankhade et al., 2017; Zhang et al., 2018). In humans and mice, heat shock protein and NFE2L2 act as antioxidant molecules maintaining redox homeostasis in human and mice (Gomer et al., 1996; Sherif, 2018). In this study, the abundance of HSPB7 and NFE2L2 and oxidative stress was greater in adipose tissue of clinically ketotic cows than healthy cows and our *in vitro* data revealed that HSPB7 protected bovine adipocytes against oxidative stress via the NFE2L2 pathway.

Heat shock proteins can protect cells against oxidative stress by inhibiting ROS production (Chong et al., 1998). Thus, the high levels of HSPB7 gene expression detected in adipose tissues of obese humans (González-

Muniesa et al., 2013) suggested an antioxidant role for HSPB7. In this study, the higher HSPB7 abundance in adipose tissue of clinically ketotic dairy cows may be a compensatory adaptation mechanism to curtail the NEB-induced metabolic stress. The NFE2L2 signaling pathway is a key mechanism of protection of the cell against oxidative stress (Sherif, 2018). Importantly, Zachut et al. (2017) reported that NFE2L2 abundance was upregulated in adipose tissue of heat-stressed dairy cows. Activation of NFE2L2 occurs when the concentrations of electrophiles or ROS increase in the cell. Thus, the fact that we detected a marked upregulation of NFE2L2 abundance in adipose tissue of clinically ketotic dairy cows strongly suggests an upregulation of cytoprotective responses. Pedernera et al. (2010) found that severe NEB was an important factor in a high level of oxidative stress in dairy cows. Ketosis occurs when physiologic mechanisms for the adaptation to NEB fail (Herd, 2000). A clinical investigation revealed that cows with ketosis displayed systemic signs of oxidative stress (Sahoo et al., 2009). In the present study, the greater MDA content coupled with the lower activity of GSH-Px and SOD, 2 of the most frequently used antioxidant indicators, in clinically ketotic cows confirmed

Figure 5. Effect of overexpression of heat-shock protein B7 (HSPB7) on oxidative stress in bovine adipocytes. Cells were treated as in Figure 4. (A) Superoxide dismutase (SOD) activity, (B) glutathione peroxidase (GSH-Px) activity, (C) malondialdehyde (MDA) content, and (D–E) reactive oxygen species (ROS) content. M1 = proportion of positive cells in 10,000 cells. Comparisons among groups were calculated using a 1-way ANOVA with subsequent Bonferroni correction. The data presented are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

the greater degree of oxidative stress within adipose tissue. The lower activity of antioxidant may be due to the impairment of antioxidant system in clinically ketotic cows (Du et al., 2017). Furthermore, it is possible that severe metabolic lipolysis-induced oxidative stress exceeded the antioxidant ability in adipose tissue of clinically ketotic cows.

The transcription factor NFE2L2 is a key factor that protects cells from oxidative stress by regulating the transcription of the antioxidant genes (NQO1 and HMOX1; Zachut et al., 2017). The NQO1 is a key oxidative stress response gene under the control of NFE2L2, and has a role in protecting against oxidative stress by scavenging superoxides, preserving various endogenous antioxidants, and catalyzing reductive metabolism of chemicals (Nioi and Hayes, 2004). It has been shown that the induction of NQO1 by some stimulating factors is fully dependent on NFE2L2 (Zhang et al., 2011). HMOX1 is another downstream antioxidant gene of the NFE2L2 that plays an important role in defense mecha-

nisms against oxidative damage by metabolizing heme into bilirubin and biliverdin, both of which are important antioxidants in liver (Sherif, 2018) and adipocytes (Wang et al., 2017).

In this study, we found that overexpression of HSPB7 in cultured adipocytes increased the abundance of NFE2L2 and its target genes HMOX1 and NQO1 in bovine adipocytes. Furthermore, HSPB7 overexpression increased the activity of SOD and GSH-Px but inhibited ROS and MDA production. These data indicate that HSPB7 overexpression can activate the NFE2L2 pathway to further increase the cellular antioxidant capacity and maintain redox balance in adipocytes. In contrast to HSPB7 overexpression, HSPB7 knockdown decreased the abundance of NFE2L2, HMOX1, and NQO1 as well as SOD and GSH-Px activity but promoted ROS and MDA production in bovine adipocytes. These data underscored a positive association between HSPB7 and the NFE2L2 pathway. Although the exact mechanisms responsible for this positive associa-

Figure 6. Effect of knockdown of heat-shock protein B7 (HSPB7) on HSPB7 and nuclear factor, erythroid 2 like 2 (NFE2L2) abundance in bovine adipocytes. The cells were divided into 4 groups as follows: an si-Control group [transfected with negative control of small interfering RNA (siRNA) for 48 h], an si-Control + 200 μ M H_2O_2 group (transfected with negative control of siRNA for 48 h and then treated with 200 μ M H_2O_2 for 2 h), an si-HSPB7 group (transfected with siRNA for HSPB7 for 48 h), and an Ad-HSPB7 + 200 μ M H_2O_2 group (transfected with siRNA for HSPB7 for 48 h and then treated with 200 μ M H_2O_2 for 2 h). (A) Relative mRNA expression level of HSPB7, (B) relative mRNA expression level of NFE2L2, (C) Western blot analysis of HSPB7 and NFE2L2, (D) relative protein expression levels of HSPB7, (E) relative protein expression levels of NFE2L2, (F) relative mRNA expression level of heme oxygenase-1 (HMOX1), and (G) relative mRNA expression level of NADH quinone oxidoreductase 1 (NQO1). The data of the si-Control group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a 1-way ANOVA with subsequent Bonferroni correction. The data presented are the mean $\pm$ SEM. * P < 0.05, ** P < 0.01.

tion could not be discerned, the downregulation of the NFE2L2 pathway suggests that HSPB7 can mediate its activation in cow adipocytes.

An imbalance between ROS production and antioxidant defenses can alter normal cell function and results in oxidative stress in dairy cows (Sordillo et al., 2009). In the present study, the increased content of ROS and MDA coupled with the decreased activity of GSH-Px and SOD induced by H_2O_2 exceeded the ability of adipocytes to scavenge ROS to prevent oxidative stress. The NFE2L2 pathway is at the forefront of cellular antioxidant defenses (Zhang et al., 2011). Several studies have confirmed that the expression of NFE2L2 and its target antioxidant genes are increased in several types of cells challenged with various oxidants (Ding et al., 2018; Li et al., 2018). In this study, our results showed that a low concentration of H_2O_2 increased the abundance of HSPB7, NFE2L2, HMOX1, and NQO1 in bovine adipocytes, which is consistent with the high abundance of HSPB7 and NFE2L2 in adipose tissue of clinically ketotic cows with oxidative stress. These data indicated that activation the HSPB7-NFE2L2

pathway in adipose tissue of clinically ketotic dairy cows augments antioxidant capacity to scavenge ROS. In addition, we found that a high concentration of H_2O_2 decreased the abundance of HSPB7, NFE2L2, HMOX1, and NQO1 indicating that high concentrations of H_2O_2 inactivated the HSPB7-NFE2L2 pathway. This result may be due to severe oxidative injury caused by high concentrations of H_2O_2 exceeding the adipocyte's adaptive ability.

CONCLUSIONS

In summary, we demonstrated that activation of HSPB7 protects adipocytes from preruminant calves against H_2O_2 -induced oxidative stress via the NFE2L2 pathway. Our data indicated that overexpression HSPB7 in adipocytes attenuated the decrease of NFE2L2, HMOX1, and NQO1 abundance, and SOD and GSH-Px activity. Furthermore, it attenuated the increase of MDA and ROS induced by H_2O_2 , thus alleviating H_2O_2 -induced oxidative stress. Silencing HSPB7 further exacerbated the H_2O_2 -induced downregulation

Figure 7. Effect of knockdown of heat-shock protein B7 (HSPB7) on oxidative stress in bovine adipocytes. The cells were divided into 4 groups as follows: an si-Control group [transfected with negative control of small interfering RNA (siRNA) for 48 h], an si-Control + 200 μM H_2O_2 group (transfected with negative control of siRNA for 48 h and then treated with 200 μM H_2O_2 for 2 h), an si-HSPB7 group (transfected with siRNA for HSPB7 for 48 h), and an Ad-HSPB7 + 200 μM H_2O_2 group (transfected with siRNA for HSPB7 for 48 h and then treated with 200 μM H_2O_2 for 2 h). (A) Superoxide dismutase (SOD) activity, (B) glutathione peroxidase (GSH-Px) activity, (C) malondialdehyde (MDA) content, and (D-E) reactive oxygen species (ROS) content. M1 = proportion of positive cells in 10,000 cells. Comparisons among groups were calculated using a 1-way ANOVA with subsequent Bonferroni correction. The data presented are the mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$.

of the NFE2L2 pathway, increasing ROS and MDA concentrations and reducing GSH-Px and SOD activity, which further exacerbated H₂O₂-induced oxidative stress. Thus, these data further support the role of the HSPB7-NFE2L2 pathway in the antioxidant response of the adipocytes from preruminant calves. Together, our data indicate that overactivation of HSPB7, in response to oxidative stress, activates the NFE2L2 signaling pathway and enhances antioxidant capacity in adipocytes from preruminant calves. These findings provide new avenues of investigation for the prevention and treatment of cows with clinical ketosis. However, there may be many differences in metabolic and physiological characteristics between the newborn calf adipogenic model and adipocytes of ketosis cows. Thus, results from this study should be translated to adipocytes from transition cows and ketotic cows. Further research should be focused on the role of HSPB7 in the transition cow adipogenic model as well as in adipose tissue of ketotic cows to better address the relevance of the present findings.

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