ABSTRACT

During the periparturient period, both oxidative stress and inflammation of adipose tissue are considered high risk factors for metabolic disorder of dairy cows. Oxidative stress can activate transcription factor nuclear factor kappa B (NF-κB), which lead to the upregulation of genes involved in inflammatory pathways. Thioredoxin-2 (TXN2) is a mitochondrial protein that regulates cellular redox by suppressing mitochondrial reactive oxygen species (ROS) generation in nonruminant, whereas the function of TXN2 in bovine adipocytes was unclear. Thus, the objective of this study was to evaluate how or by which mechanisms TXN2 regulates oxidative stress and NF-κB signaling pathway in bovine adipocytes. Bovine pre-adipocytes isolated from 5 healthy Holstein cows were differentiated and used for 1) treatment with different concentrations of hydrogen peroxide (H2O2; 0, 25, 50, 100, 200 or 400 μM) for 2 h; 2) transfection with or without TXN2 small interfering RNA (si-TXN2) for 48 h and then treated with or without 200 μM H2O2 for 2 h; 3) transfection with scrambled negative control siRNA (si-control) or si-TXN2 for 48 h, and then treatment with or without 10 mM N-acetylcysteine (NAC) for 2 h; 4) transfection with or without TXN2-overexpressing plasmid for 48 h and then treatment with or without 200 μM H2O2 for 2 h. High concentrations of H2O2 (200 and 400 μM) decreased protein and mRNA abundance of TXN2, reduced total antioxidant capacity (T-AOC) and adenosine triphosphate (ATP) content in adipocytes. Moreover, 200 and 400 μM H2O2 reduced protein abundance of inhibitor of kappa B α (IkBα), increased phosphorylation of NF-κB and upregulated mRNA abundance of tumor necrosis factor-α (TNFA) and interleukin-1β (IL-1B), suggesting that H2O2-induced oxidative stress and activated NF-κB signaling pathway. Silencing of TXN2 increased intracellular ROS content, phosphorylation of NF-κB and mRNA abundance of TNFA and IL-1B, decreased ATP content and protein abundance of IkBα in bovine adipocytes. Knockdown of TXN2 aggravated H2O2-induced oxidative stress and inflammation. In addition, treatment with antioxidant NAC ameliorated oxidative stress and inhibited NF-κB signaling pathway in adipocytes transfected with si-TXN2. In bovine adipocytes treated with H2O2, overexpression of TXN2 reduced the content of ROS and elevated the content of ATP and T-AOC. Overexpression of TXN2 alleviated H2O2-induced inflammatory response in adipocytes, as demonstrated by decreased expression of phosphorylated NF-κB, TNFA, IL-1B, as well as increased expression of IkBα. Furthermore, the protein and mRNA abundance of TXN2 was lower in adipose tissue of dairy cows with clinical ketosis. Overall, our studies contribute to the understanding of the role of TXN2 in adipocyte oxidative stress and inflammatory response.

Key words: TXN2, NF-κB, oxidative stress, periparturient period

INTRODUCTION

Oxidative stress refers to the imbalance between oxidative and antioxidative processes in cells (Agarwal et al., 2003). In adipocytes, lipolysis generates reactive oxygen species (ROS), including hydroxyl radical, superoxide anion, and hydrogen peroxide (H2O2; Rains and Jain, 2011). Of note, in conditions with intense and protracted lipolysis, the production of ROS rapidly depletes antioxidant systems, and oxidative stress develops (Pizzino et al., 2017). During the periparturient period, lipolysis causes a reduction of adipose tissue (AT) mass, which may exceed 30% of the volume (i.e., weight) of adipose depots in dairy cows (Akter et al., 2011). Thus, dairy cows experience oxidative stress of AT around the time of calving, especially in dairy cows with ketosis or fatty liver (Sahoo et al., 2009; Li et al., 2016).
The intracellular ROS are mostly derived from mitochondrial superoxide, which can be converted to \( \text{H}_2\text{O}_2 \) via superoxide dismutase (McArdle et al., 2004). Key proteins involved in scavenging \( \text{H}_2\text{O}_2 \) are members of the thioredoxin (\text{TXN}) and the glutathione systems (Lu and Holmgren, 2012). Thioredoxin-2 (\text{TXN2}), one of the indispensable components of mitochondrial-specific TXN system, is a small mitochondrial redox protein essential for the control of mitochondrial ROS homeostasis (Yoshioka et al., 2006). A previous study demonstrated that heterozygous \text{TXN2}-deficient mice exhibit decreased mitochondrial electron transport chain activity and adenosine triphosphate (\text{ATP}) production and increased mitochondrial ROS generation in the liver (Pérez et al., 2008). In addition, overexpression of \text{TXN2} decreased ROS level in rat embryonic ventricular myocytes (Li et al., 2017). Kweh et al. (2021) reported that vitamin D increased antioxidant activity in bovine monocytes via enhancing TXN system. Adipocyte-specific \text{TXN2} deletion increased mitochondrial ROS generation in AT and elevated blood levels of free fatty acids in mice (He et al., 2021). However, the role of \text{TXN2} in regulating oxidative stress of bovine adipocyte is not well known.

Studies on nonruminants revealed that localized inflammation plays an important role in coordinating the lipid metabolism and adipokine secretion of adipocytes (Toczyłowski et al., 2019; Trayhurn, 2022). Dairy cows experience localized inflammation of AT during the transition period, as evidenced by infiltration of macrophages and upregulation of proinflammatory biomarkers (Vailati-Riboni et al., 2016; Depreester et al., 2018). Furthermore, it was reported that nuclear factor kappa B (\text{NF}-\kappa\text{B}) signaling was overactivated in AT of ketotic cows (Fan et al., 2021). In rodents, \text{TXN2} deficiency induced macrophage infiltration in AT (He et al., 2021). In contrast, overexpression of \text{TXN2} significantly reduced the production of interleukin-1\text{B} (\text{IL}-1\text{B}) and tumor necrosis factor-\alpha (\text{TNF-\alpha}) in murine macrophage (Wang et al., 2020). Because ROS act as central regulators of inflammatory response (Park et al., 2015), we hypothesized that \text{TXN2} affects mitochondrial ROS generation and contributes to inflammatory response of bovine adipocytes. Thus, the objective of this study was to evaluate the effects of oxidative stress inducer \( \text{H}_2\text{O}_2 \) on \text{TXN2} function and \text{NF}-\kappa\text{B} inflammatory pathway, as well as the role of \text{TXN2} in regulating oxidative stress and inflammation of bovine adipocytes.

### Materials and Methods

**Ethics**

The use of animals and experimental procedures were approved by the Ethics Committee on the Use and Care of Animals of Jilin University (Changchun, China, SY202208003).

**Isolation of Bovine Pre-adipocytes**

All cows received routine physical examinations by the attending veterinarian to ensure there were no clinical diseases, such as hyperketonemia, laminitis, mastitis, hypocalcemia, and endometritis. Five healthy Holstein cows with similar BCS (median = 3.25, range = 3–3.5), DIM (median = 14, range = 10–18) and number of lactations (median = 3, range = 2–4) were enrolled in this study. The values (mean ± SEM) for serum concentrations of free fatty acids, \text{BHB} and glucose in cows were 0.43 ± 0.07 mM, 0.69 ± 0.13 mM and 3.75 ± 0.14 mM, respectively. Subcutaneous AT (1 to 2 g) was collected at 14 (±4) d postpartum from the tailhead depot of all cows on the same day through the methods described previously (Xu et al., 2019). The resulting tissue was rinsed in sterile PBS containing penicillin (2,500 \text{U/mL}) and streptomycin (2,500 \mu\text{g/mL}). Then, the resulting tissue was cut into small pieces of approximately 1 mm\(^3\) and digested using 50 mL of Dulbecco’s modified Eagle medium (\text{DMEM})/F12 digestion solution containing collagenase type I (1 mg/mL; SH30023.01; HyClone, Logan, Utah, USA) at 37°C in a slightly shaking water bath for 1 h. The mixture was removed from the digested tissue fluid through 80- and 40- \mu m cell strainers and the filtrate was centrifuged at 175 \times g at room temperature for 10 min. The residual erythrocytes were removed by adding ammonium-chloride-potassium lysis buffer (C3702; Beyotime Biotechnology Inc., Jiangsu, China, SY202208003). All cows received routine physical examinations by the attending veterinarian to ensure there were no clinical diseases, such as hyperketonemia, laminitis, mastitis, hypocalcemia, and endometritis. The use of animals and experimental procedures were approved by the Ethics Committee on the Use and Care of Animals of Jilin University (Changchun, China, SY202208003)

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Cell Differentiation

To induce differentiation of pre-adipocytes into mature adipocytes, primary cells were seeded in culture plates. The BCM was discarded after cell aggregation reached ~70%. Then, freshly prepared differentiation culture medium (DCM) 1, which was a final concentration of 0.5 mM 3-isobutyl-1-methylxanthine (I-7018; Sigma-Aldrich, MO, USA), 1 μM dexamethasone (D-4902; Sigma-Aldrich), and 1 μg/mL insulin (I-5500; Sigma-Aldrich) in BCM, was used to induce differentiation. As adipogenesis progresses, the adipocytic phenotype is determined by the appearance of lipid droplets or by the expression of adipocyte-specific genes. In the present study, adipogenesis was defined by the appearance of visible lipid droplets in the cells, a sign that adipocytes were differentiated. The number of mature adipocytes was 4 × 10^5 per well of a 6-well plate.

Cell Treatment

After the adipogenic induction, experiments with differentiated adipocytes were divided into 4 sections: (1) adipocytes were treated with different concentrations of H_2O_2 (0, 25, 50, 100, 200 or 400 μM; H1009; Sigma-Aldrich) for 2 h; (2) adipocytes were transfected with or without TXN2 small interfering RNA (si-TXN2) for 48 h and then treated with or without 200 μM H_2O_2 for 2 h; (3) adipocytes were transfected with scrambled negative control siRNA (si-control) or si-TXN2 for 48 h, and then treated with or without 10 mM N-acetylcysteine (NAC; A9165; Sigma-Aldrich) for 2 h; (4) adipocytes were transfected with or without TXN2-overexpressing plasmid for 48 h and then treated with or without 200 μM H_2O_2 for 2 h. Before H_2O_2 treatment, adipocytes were serum-starved in DCM1, which was a final concentration of 1 μg/mL insulin in BCM, to maintain the differentiation culture. As adipocytes were differentiated, the number of mature adipocytes was 4 × 10^5 per well of a 6-well plate.

Detection of Total Antioxidant Capacity (T-AOC)

The T-AOC of the adipocytes is defined as the total levels of various antioxidants. In the present study, T-AOC was measured according to the instructions of the T-AOC Assay Kit with a rapid 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) method (S0121; Beyotime Biothechnology Inc.). Briefly, adipocytes were disrupted by using ultrasonication. The cell lysate was collected by centrifuging at 4°C, 12,000 × g for 5 min. The protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay Kit (P0012; Beyotime Biothechnology Inc.). Subsequently, 10 μL of each sample were mixed with 170 μL ABTS detection solution, which was prepared according to the manufacturer’s protocol. After incubation at room temperature for 6 min, the absorbance at 414 nm was recorded. Trolox was used as a standard antioxidant reagent to generate a standard curve. According to the manufacturer’s protocol, the T-AOC was calculated and expressed as the trolox-equivalent antioxidant capacity (mmol/g of protein).

Determination of ROS Content

The intracellular ROS concentrations were measured using the peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate (S0033S; Beyotime Biothechnology Inc.). The cells were exposed to serum-free medium containing 10 μM DCFH-DA in the dark for 30 min and then washed 3 times with cold PBS. The fluorescence was measured by flow cytometry (BD FACSCalibur, Becton Dickinson, Mountain View, USA).

Adenosine Triphosphate (ATP) Content Determination

The ATP content was determined using an ATP analysis kit (A095; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. The ATP values were corrected by protein content. Total protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (P0012; Beyotime Biothechnology Inc.). After the adipogenic induction, experiments with differentiated adipocytes were divided into 4 sections: (1) adipocytes were treated with different concentrations of H_2O_2 (0, 25, 50, 100, 200 or 400 μM; H1009; Sigma-Aldrich) for 2 h; (2) adipocytes were transfected with or without TXN2 small interfering RNA (si-TXN2) for 48 h, and then treated with or without 200 μM H_2O_2 for 2 h; (3) adipocytes were transfected with scrambled negative control siRNA (si-control) or si-TXN2 for 48 h, and then treated with or without 10 mM N-acetylcysteine (NAC; A9165; Sigma-Aldrich) for 2 h; (4) adipocytes were transfected with or without TXN2-overexpressing plasmid for 48 h and then treated with or without 200 μM H_2O_2 for 2 h. Before H_2O_2 treatment, adipocytes were serum-starved in DCM1 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h. The choice of H_2O_2 dose was based on previous in vitro experiments with dairy cow or calf adipocytes and bovine mammary epithelial cells, demonstrating that adipocytes were serum-starved in DMEM/F12 medium for 12 h.
estimated by the BCA method (P1511; Applygen Technologies Inc., Beijing, China).

**RNA Isolation and Quantitative Reverse-Transcription PCR**

Total RNA was isolated from adipocytes using RNAiso Plus (9109; TaKaRa Biotechnology Co. Ltd., Dalian, China) according to the manufacturer’s instructions. The RNA concentration and quality were measured using a K5500 MicroSpectrophotometer (Beijing Kaiao Technology Development Ltd., Beijing, China) and electrophoresis (1% agarose gels). The OD260/OD280 ratio of the total RNA was determined to be 1.83 to 1.96 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 SYBR green plus reagent kit (RR420A; TaKaRa Biotechnology Co. Ltd.) was used to prepare a 20 μL mixture and mRNA abundance was detected with a 7500 Real-Time PCR System (Applied Biosystems Inc., 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. Relative transcription of each target gene was normalized against the geometric mean of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB) both of which were stably expressed across treatment groups (Supplemental Figure S1). Relative gene expression was calculated with the 2-ΔΔCT method. The primer pairs used in this study were designed with Primer Express software 3.0 (Applied Biosystems Inc.) according to bovine reference sequences from the National Center for Biotechnology Information (Supplemental Table S1).

**Western Blot Analysis**

Total protein was extracted from the adipose tissue and mature adipocytes using a commercial protein extraction kit (C510003, Sangon Biotech Co. Ltd., Shanghai, China) according to the manufacturer’s instructions. Briefly, adipose tissue dissolved in precooled (4°C) lysis buffer containing phosphatase inhibitors, protease inhibitors, and phenylmethylsulfonyl fluoride was homogenized by cryo-milling using a tissue mixer mill at 30 Hz for 2 min (MM400; Retsch, Haan, Germany). Then, tissue homogenates were centrifuged 15,000 × g for 15 min at 4°C, to obtain total protein from adipose tissue. The total protein concentration of adipose tissue and adipocytes was determined using the BCA Protein Assay Kit (P1511, Applygen Technologies). Twenty micrograms of protein were separated using SDS-PAGE with known molecular weight markers (Sangon Biotech Co. Ltd.). Subsequently, the protein was transferred onto 0.45 μm polyvinylidene difluoride membranes. Membranes were blocked in 3% BSA and Tris-buffered saline solution with 0.01% Tween-20 (TBST) for 4 h at room temperature. Subsequently, blocked membranes were incubated with primary antibodies against phosphorylated-NF-κB Ser536 (p-NF-κB; 1:1,000; 3033; Cell Signaling Technology, Danvers, MA), NF-κB (1:1,000; 4764; Cell Signaling Technology), inhibitor of kappa B α (IκBα; 1:1,000; 4814; Cell Signaling Technology), TXN2 (1:500–2,000; 4256; Bios, Beijing, China), β-actin (1:2,000; ab8226; Abcam, Cambridge, MA) at 4°C overnight, followed by washing 3 times with TBST. Membranes were then incubated at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody for 45 min. After washing with TBST for 3 times, the immunoassay was performed using an enhanced chemiluminescence reagent (WBKLS0500; Millipore, Bedford, MA) to visualize bands on the membrane. Lastly, all bands were analyzed using Image-Pro Plus 6.0 (Media Cybernetics Inc., Warrendale, PA).

**Animal and AT Collection**

Detailed information on the healthy and ketotic cow selection and AT collection was reported in our previous study (Fan et al., 2021).

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 8.0 (Graph Pad Software) or SPSS 23.0 software (IBM Corp.). Results are presented as the means ± standard error of the mean (SEM). All data were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene tests, respectively. Linear and quadratic contrasts were conducted to evaluated dose-dependent effects. Data were analyzed by t test in cases of 2 comparison groups. One or 2-way ANOVA was performed for multiple comparisons with Bonferroni correction for data meeting homogeneity of variance. P < 0.05 was considered significant and P < 0.01 markedly significant.

**RESULTS**

**Effects of H2O2 on TXN2 Abundance, Oxidative Stress and NF-κB Signaling in Bovine Adipocytes**

Compared with the control group, 50 and 100 μM H2O2 treatment increased, whereas 200 and 400 μM H2O2 treatment decreased protein (P < 0.05, Figure 1A and B; liner and quadratic, Supplemental Table S2) and mRNA abundance (P < 0.05, Figure 1A and C;
linear and quadratic, Supplemental Table S3) of TXN2 in adipocytes. The intracellular ROS (P < 0.01, Figure 1D; liner and quadratic, Supplemental Table S4) and ATP content (P < 0.01, Figure 1E; liner and quadratic, Supplemental Table S4) was elevated and reduced by H2O2 treatment in adipocytes, respectively. The T-AOC (P < 0.01, Figure 1F; liner, Supplemental Table S4) was decreased by 200 and 400 μM H2O2 treatment. Moreover, the protein abundance of p-NF-κB (P < 0.01, Figure 1G; liner, Supplemental Table S3) and mRNA abundance of TNFA (P < 0.01, Figure 1H; liner and quadratic, Supplemental Table S2) were higher, but protein abundance of IκBα (P < 0.01, Figure 1G and I; linear, Supplemental Table S3) and IL-1B (P < 0.01, Figure 1J) and mRNA abundance of TNFA (P < 0.01, Figure 1J) were lower in 200 and 400 μM H2O2 treatment group than control group.

Effects of Silencing of TXN2 on Oxidative Stress and NF-κB Signaling in Bovine Adipocytes

The silence efficiency of si-TXN2 was 75% (P < 0.01, Figure 2A and B). In adipocytes transfected with si-control, treatment with 200 μM H2O2 decreased protein abundance of TXN2 (P < 0.05, Figure 2A and B). In adipocytes transfected with si-TXN2, treatment with 200 μM H2O2 further lowered protein abundance of TXN2 (P < 0.05, Figure 2A and B). Compared with the control group, silencing of TXN2 increased ROS content (P < 0.01, Figure 2C) and decreased ATP content (P < 0.05, Figure 2D) and T-AOC (P < 0.01, Figure 2E) in adipocytes. Moreover, the protein abundance of p-NF-κB (P < 0.05, Figure 2F and G) was greater in si-TXN2 group, but IκBα (P > 0.05, Figure 2F and H) was lower. Compared with the si-control group, silencing of TXN2 increased mRNA abundance of TNFA (P < 0.01, Figure 2I) and IL-1B (P < 0.01, Figure 2J).

In the presence of H2O2, treatment with si-TXN2 further exacerbated oxidative stress, as evidenced by increased ROS content (P < 0.01, Figure 2C) and decreased ATP content (P < 0.01, Figure 2D) and T-AOC (P < 0.01, Figure 2E). Moreover, silencing of TXN2 further aggravated H2O2-induced inflammatory response in adipocytes, as demonstrated by elevated protein abundance of p-NF-κB (P < 0.05, Figure 2F and G) and mRNA abundance of TNFA (P < 0.01, Figure 2I) and IL-1B (P < 0.05, Figure 2J), as well as reduced protein abundance of IκBα (P < 0.01, Figure 2F and H).

NAC Attenuates Oxidative Stress and Inflammatory Response in Bovine Adipocytes Transfected with si-TXN2

In adipocytes transfected with si-TXN2, antioxidant NAC reduced ROS content (P < 0.01, Figure 3A) and increased ATP content (P < 0.01, Figure 3B) and T-AOC (P < 0.01, Figure 3C). Moreover, NAC decreased protein abundance of p-NF-κB (P < 0.05, Figure 3D and E) and mRNA abundance of TNFA (P < 0.01, Figure 3G) and IL-1B (P < 0.01, Figure 3H), but increased protein abundance of IκBα (P < 0.01, Figure 3D and F) in si-TXN2-transfected adipocytes.

Overexpression of TXN2 Alleviates Oxidative Stress and Inflammatory Response in H2O2-Treated Bovine Adipocytes

The overexpression efficiency of pCMV-FLAG-TXN2 was 192% (P < 0.01, Figure 4A and B). In the absence of H2O2, overexpression of TXN2 slightly decreased ROS content (P < 0.05, Figure 4C) and marginally increased ATP content (P < 0.05, Figure 4D) and T-AOC (P < 0.05, Figure 4E) in adipocytes. In addition, overexpression of TXN2 did not alter the protein abundance of p-NF-κB (Figure 4F and G) and IκBα (Figure 4F and H) and the mRNA abundance of TNFA (Figure 4I) and IL-1B (Figure 4J).

In the presence of H2O2, overexpression of TXN2 reduced the content of ROS (P < 0.01, Figure 4C) and elevated the content of ATP (P < 0.01, Figure 4D) and T-AOC (P < 0.01, Figure 4E) in adipocytes. Moreover, overexpression of TXN2 alleviated H2O2-induced inflammatory response in adipocytes, as demonstrated by decreased expression of p-NF-κB (P < 0.05, Figure 4F and G), TNFA (P < 0.01, Figure 4I) and IL-1B (P < 0.01, Figure 4J), as well as increased expression of IκBα (P < 0.05, Figure 4F and H).

Expression of TXN2 in AT

Protein abundance of TXN2 in AT used for pre-adipocytes isolation was similar (Figure 5A). The protein (P < 0.01, Figure 5B and C) and mRNA (P < 0.01, Figure 5D) abundance of TXN2 were lower in AT from clinical ketosis cows than in healthy cows.

DISCUSSION

During early lactation, oxidative stress and inflammatory response of AT increase the risk of metabolic diseases of dairy cows (Contreras et al., 2017; Zachut and Contreras, 2022). The main sources of cellular ROS are mitochondria (Piao et al., 2019). Meanwhile,
mitochondria are equipped with several antioxidant enzyme systems posed to detoxify ROS (Lismont et al., 2015; Mailloux, 2018). In nonruminants, TXN2 is the main ROS-scavenging enzyme in mitochondria that maintains mitochondrial function (Sugano et al., 2013; Chen et al., 2017). In the present study, decreased TXN2 gene expression was accompanied by ROS overproduction and NF-κB signaling pathway overaction in bovine adipocytes. Moreover, overexpression of TXN2 attenuated oxidative stress and inflammatory response in H2O2-treated adipocytes, suggesting that TXN2 is a potential target for modulation of redox homeostasis in bovine adipocytes.

In the AT of healthy cows, excessive ROS can be scavenged by various antioxidative defense mechanisms (Yu et al., 2023). However, in dairy cows with prolonged and sustained lipolysis, the production of ROS depletes antioxidant systems, and oxidative stress develops (Mavangira and Sordillo, 2018). As a mitochondrial member of the TXN antioxidant defense system, TXN2 interacts with TXN reductase 2 and peroxiredoxin 3 to scavenge H2O2 and offer protection against oxidative stress in nonruminant (Pérez et al., 2008; He et al., 2021). In the present study, protein and mRNA abundance of TXN2 was lower in AT of dairy cows with clinical ketosis. Moreover, we observed that incubation of bovine adipocytes with 50 and 100 μM H2O2 increased intracellular ROS content and TXN2 expression. In addition, other studies with bovine mammary epithelial cells (Ma et al., 2018) and dairy cow (Xu et al., 2021) or calf adipocytes (Sun et al., 2019) have revealed no deleterious effects on cell viability with 100 μM H2O2 treatment. Although speculative at this time, enhanced TXN2 protein expression could be the

Figure 1. Effects of hydrogen peroxide (H2O2) on thioredoxin-2 (TXN2) expression, oxidative stress and nuclear factor kappa B (NF-κB) signaling pathway in bovine adipocytes. Cells were treated with 0, 25, 50, 100, 200 or 400 μM H2O2 for 2 h. (A) Representative blot of TXN2. (B) Quantification of protein level of TXN2. (C) Relative mRNA abundance of TXN2 in bovine adipocytes. (D) Relative reactive oxygen species (ROS) content in bovine adipocytes. (E) Adenosine triphosphate (ATP) content in bovine adipocytes. (F) Total antioxidant capacity (T-AOC) in bovine adipocytes. (G) Representative blots of p-NF-κB, NF-κB and inhibitor of kappa B α (IκBα) in bovine adipocytes. (H) Quantification of protein level of p-NF-κB/NF-κB. (J, K) Relative mRNA abundance of tumor necrosis factor-α (TNFA) and interleukin-1B (IL-1B) in bovine adipocytes. Data are expressed as the means ± SEM. Data were analyzed by one-way ANOVA with subsequent Bonferroni correction. *P < 0.05; **P < 0.01.
adaptation mechanism to combat oxidative stress and maintain cell viability. This idea is partly supported by the observation that enforced TXN2 expression reduced ROS content and enhanced antioxidant capacity of bovine adipocytes treated with high concentration of \( \text{H}_2\text{O}_2 \) (200 \( \mu \text{M} \)). Similarly, Huang et al. (2015) reported that TXN2 inhibited mitochondrial ROS generation to maintain mouse cardiac function. Thus, our results combined with previous study suggest a role for TXN2 in the modulation of ROS homeostasis in ruminants and TXN2 may be a novel target for preventing oxidative stress in adipocytes.

Because mitochondria are both a generator of and target for ROS, oxidative stress is closely linked to mitochondria dysfunction (Silwal et al., 2020). In our study, high concentration of \( \text{H}_2\text{O}_2 \) (200 and 400 \( \mu \text{M} \)) not only increased intracellular ROS content but reduced TXN2 expression, ATP content and antioxidant capacity in bovine adipocytes. Moreover, knockdown of TXN2 induced oxidative stress and decreased ATP content in bovine adipocytes. In line with these observations, deletion of TXN2 in murine adipocytes resulted in severe mitochondrial damage, as evidenced by fragmentation and cristae disruption of mitochondria, as well as decreased mitochondrial DNA copy number and ATP production (He et al., 2021). These studies suggest that dysregulation of TXN2 contributes to development of mitochondrial dysfunction and oxidative stress in adipocytes. Given that oxidative stress of AT is one of the risk factors leading to metabolic dysfunction (Abou-Rjeileh and Contreras, 2021; Zachut and Contreras, 2022), more research is needed to identify

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**Figure 2.** Effects of knockdown of thioredoxin-2 (TXN2) on oxidative stress and nuclear factor kappa B (NF-κB) signaling pathway in bovine adipocytes. Adipocytes were transfected with or without TXN2 small interfering RNA (si-TXN2) for 48 h and then treated with or without 200 \( \mu \text{M} \) hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) for 2 h. (A) Representative blot of TXN2. (B) Quantification of protein level of TXN2. (C) Relative reactive oxygen species (ROS) content in bovine adipocytes. (D) Adenosine triphosphate (ATP) content in bovine adipocytes. (E) Total antioxidant capacity (T-AOC) in bovine adipocytes. (F) Representative blots of p-NF-κB, NF-κB and inhibitor of kappa B α (IκBα) in bovine adipocytes. (G) Quantification of protein level of p-NF-κB/NF-κB. (H) Quantification of protein level of IκBα. (I, J) Relative mRNA abundance of tumor necrosis factor-α (TNFA) and interleukin-1B (IL-1B) in bovine adipocytes. Data are expressed as the means ± SEM. Data were analyzed by 2-way ANOVA with subsequent Bonferroni correction. *\( P < 0.05 \); **\( P < 0.01 \).
the role of TXN2 in regulating mitochondrial function of AT in dairy cows during the transition period.

It is well established that oxidative stress can cause inflammation in ruminants (Zachut and Contreras, 2022) and nonruminants (Mittal et al., 2014). In humans and rodents, ROS induces the degradation of the inhibitor IkB, which holds NF-κB in an inactive state in the cytoplasm, freeing NF-κB to translocate to the nucleus and drive the expression of proinflammatory genes (Morgan and Liu, 2011). We observed that H2O2 activated NF-κB inflammatory signaling pathway in bovine adipocytes, as evidenced by decreased expression of IkB and increased expression of phosphorylated NF-κB and mRNA expression of TNFA and IL-1B. Thus, the greater phosphorylation of NF-κB along with upregulation of TNFA and IL-1B in AT of ketotic cows might be associated with oxidative stress (Fan et al., 2021).

An activation of NF-κB signaling could induce the upregulation of NLRP3 and IL-1B leading to formation of NLRP3 inflammasome (Bauernfeind et al., 2009). Notably, ROS are shown to be critical mechanism triggering NLRP3 inflammasome formation and activation (Abais et al., 2015). Moreover, in mice, TNF-α and IL-1B produced during activation of the NF-κB and NLRP3 signaling pathways can lead to mitochondria dysfunction and oxidative stress (Zhou et al., 2011; Kastl et al., 2014). This leads to a vicious circle where ROS activates NF-κB pathway and NLRP3 inflammasome, which induces proinflammatory cytokines overproduction and further promotes oxidative stress. Thus, such as vicious circle may promote development of metabolic disorder of AT in dairy cows during early lactation.

In this study, knockdown of TXN2 induced oxidative stress and inflammatory response whereas antioxidant NAC attenuated this effect in bovine adipocytes. Consistently, treatment with mitochondrial ROS scavenger mito-TEMPO abrogated NF-κB and NLRP3 inflammasome activation in TXN2 knockout mouse adipocytes (He et al., 2021; Huang et al., 2022). Thus, current and previous data indicate that ROS overproduction induced by H2O2 treatment or downregulation of TXN2 may contribute to the occurrence of overt inflammatory responses in bovine adipocyte.

Although our previous study demonstrated that the NF-κB signaling pathway was overactivated in adipose tissues of dairy cows with ketosis (Fan et al., 2021), the

Figure 3. Effects of antioxidant N-acetylcysteine (NAC) on oxidative stress and nuclear factor kappa B (NF-κB) signaling pathway in thioredoxin-2 (TXN2) knockdown bovine adipocytes. Adipocytes were transfected with scrambled negative control siRNA (si-control) or TXN2 small interfering RNA (si-TXN2) for 48 h, and then treated with or without 10 mM NAC for 2 h. (A) Relative reactive oxygen species (ROS) content in bovine adipocytes. (B) Adenosine triphosphate (ATP) content in bovine adipocytes. (C) Total antioxidant capacity (T-AOC) in bovine adipocytes. (D) Representative blots of p-NF-κB, NF-κB and inhibitor of kappa B α (IkBα) in bovine adipocytes. (E) Quantification of protein level of p-NF-κB/NF-κB. (F) Quantification of protein level of IkBα. (G, H) Relative mRNA abundance of tumor necrosis factor-α (TNFA) and interleukin-1B (IL-1B) in bovine adipocytes. Data are expressed as the means ± SEM. Data were analyzed by 2-way ANOVA with subsequent Bonferroni correction. *P < 0.05; **P < 0.01.
inflammatory phenotype remained unclear. Furthermore, adipose tissue is heterogeneous; it is comprised of an array of cell types including adipocytes, preadipocytes, immune system cells, endothelial cells and stromal cells. Depreester et al. (2018) reported infiltration of macrophages in adipose tissue was associated with the proinflammatory status of transition dairy cows. Thus, whether TXN2 regulate the inflammatory response of adipocyte in adipose tissue of ketotic cows needs further investigation.

In AT, sustained lipolysis produces excessive ROS and leads to a remodeling process characterized by an inflammatory response (Zachut and Contreras, 2022). In this study, knockdown of TXN2 aggravated while overexpression of TXN2 alleviated H2O2-induced oxidative stress and inflammatory response in bovine adipocytes. However, potential associations between TXN2 function and the resulting effects on adipocyte lipolysis have been neglected in the present study. Furthermore, the total ROS content and T-AOC, not specific mitochondrial-derived ROS and antioxidant capacity, were measured. Thus, based on the present data, we cannot fully evaluate the status of oxidative stress in mitochondrion of adipocyte. Those are limitations in present study.

CONCLUSIONS

High concentration of H2O2 inhibited TXN2 expression, induced oxidative stress and activated NF-κB signaling pathway in bovine adipocytes. Knockdown of TXN2 aggravated, whereas overexpression of TXN2

![Figure 4. Effects of overexpression of thioredoxin-2 (TXN2) on oxidative stress and nuclear factor kappa B (NF-κB) signaling pathway in bovine adipocytes. Adipocytes were transfected with or without TXN2-overexpressing plasmid for 48 h and then treated with or without 200 μM hydrogen peroxide (H2O2) for 2 h. (A) Representative blot of TXN2. (B) Quantification of protein level of TXN2. (C) Relative reactive oxygen species (ROS) content in bovine adipocytes. (D) Adenosine triphosphate (ATP) content in bovine adipocytes. (E) Total antioxidant capacity (T-AOC) in bovine adipocytes. (F) Representative blots of p-NF-κB, NF-κB and inhibitor of kappa B α (IκBα) in bovine adipocytes. (G) Quantification of protein level of p-NF-κB/NF-κB. (H) Quantification of protein level of IκBα. (I, J) Relative mRNA abundance of tumor necrosis factor-α (TNFA) and interleukin-1B (IL-1B) in bovine adipocytes. Data are expressed as the means ± SEM. Data were analyzed by 2-way ANOVA with subsequent Bonferroni correction. *P < 0.05; **P < 0.01.](image-url)
alleviated H$_2$O$_2$-induced oxidative stress and NF-κB activation in bovine adipocytes. Our study opens the possibility of regulating TXN2 expression as a potential therapeutic target for the modulation of oxidative stress and NF-κB signaling pathway in AT of periparturient dairy cows.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Beijing, China; grant nos. 32002349 and 32172927) and the Fundamental Research Funds for the Central Universities (Changchun, China; grant no. 2022-JCXK-21). The authors have not stated any conflicts of interest.

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ORCID

Mingchao Liu https://orcid.org/0000-0001-5183-5719
Zhiyuan Fang https://orcid.org/0000-0003-4377-0972
Wenwen Gao https://orcid.org/0000-0003-4702-7596
Lin Lei https://orcid.org/0000-0001-5740-1508
Xinwei Li https://orcid.org/0000-0002-0765-677X
Guowen Liu https://orcid.org/0000-0002-2672-6104
Xiliang Du https://orcid.org/0000-0002-1467-6998

Journal of Dairy Science Vol. TBC No. TBC, TBC

https://doi.org/10.1038/s12967-019-2060-7

https://doi.org/10.1089/ars.2006.8.2143

https://doi.org/10.1038/nature09663.