Heat stress induces apoptosis through disruption of dynamic mitochondrial networks in dairy cow mammary epithelial cells

Kun-Lin Chen [1,2] · Hui-Li Wang [1,2] · Lin-Zheng Jiang [3] · Yong Qian [1,2] · Cai-Xia Yang [4] · Wei-Wei Chang [4] · Ji-Feng Zhong [1,2] · Guang-Dong Xing [1,2]

Abstract

Heat stress–induced reductions in milk yield and the dysfunction of mammary glands are economically important challenges that face the dairy industry, especially during summer. The aim of the present study is to investigate the effects of heat stress on mitochondrial function by using dairy cow mammary epithelial cells (DCMECs) as an in vitro model. Live cell imaging shows that the mitochondria continually change shape through fission and fusion. However, heat stress induces the fragmentation of mitochondria, as well as the decreased of ATP level, membrane potential, and anti-oxidant enzyme activity and the increased of respiratory chain complex I activity. In addition, the cytosolic Ca^{2+} concentration and cytochrome c expression (Cyto-c) were increased after heat stress treatment. Both qRT-PCR and western blot analysis indicate that mitofusin1/2 (Mfn1/2) and optic atrophy protein-1 (Opa-1) are downregulated after heat stress, whereas dynamin-related protein 1 (Drp1) and fission 1 (Fis-1) are upregulated, which explains the observed defect of mitochondrial network dynamics. Accordingly, the present study indicated that heat stress induced the dysfunction of DCMEC through disruption of the normal balance of mitochondrial fission and fusion.

Keywords Heat stress · Mitochondria fission · Mitochondria fusion · Oxidative stress · Dairy cow mammary epithelial cells

Introduction

Heat stress, one of the major problems in tropical and subtropical regions, adversely affects large-size livestock farming production (Tao et al. 2018; Liu et al. 2019). Dairy cattle are one of the most sensitive livestock to high temperature, with excessive heat reducing their both reproductive performance and production potential (Herbut et al. 2018). Previous studies have reported that heat stress reduces the feed intake, milk yield and quality, and fertility and induces the metabolic disorders of dairy cattle. It also increases respiratory rates, heart rates, and peripheral blood flow (Takahashi 2012; Polsky and von Keyserlingk 2017; Tao et al. 2018). In addition, heat stress also affects the development of mammary glands, which is the main reason underlying observed reductions in milk yield during dry periods (Tao et al. 2018).

The negative effect of heat stress on mammary gland function is well known, and the resulting dysfunction is accompanied by apoptosis and autophagy, as well as reductions in mammary epithelial cells. Heat stress also reduces immunity and eventually increases the susceptibility of dairy cattle to mastitis (Wohlgemuth et al. 2016). Histological studies have shown that heat stress reduces levels of macrophages and lymphocytes and increases neutrophil count, as well as the increase of apoptotic cells in mammary gland, which is identified by the morphological changes (e.g., nuclear fragmentation, nuclear and cytoplasmic condensation, and the formation of apoptotic bodies) (Lindsay 2004).

Mammary epithelial cells (MECs) are the essential secretory cells of mammary glands, which involved in milk

✉ Ji-Feng Zhong
zhongjifeng64@sina.cn

✉ Guang-Dong Xing
xing_gd@aliyun.com

[1] Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base, Ministry of Science and Technology, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China

[2] Key Laboratory of Crop and Animal Integrated Farming, Ministry of Agriculture, Nanjing 210014, China

[3] Youyuan Research Institute of Dairy Industry Co., Ltd, Nanjing 211100, China

[4] College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China

production (Boutinaud et al. 2015). Previous studies have reported that heat stress is associated with swollen mitochondria with broken cristae, low matrix density, and reduced myocardium ATP content, suggesting that the mitochondrial function is disrupted after heat stress (Qian et al. 2004). Mitochondria are double membrane–bound subcellular organelles that are the main source of cellular energy, owing to their generation of ATP through respiration and regulation of cellular metabolism. The morphology and population size of mitochondria were dynamically changed through continuous fission and fusion (Scott and Youle 2010). The imbalance of mitochondria fission and fusion can cause the dysfunction of mitochondria.

Mitochondrial fission and fusion processes are mediated by the dynamin family of large GTPases. The outer membrane protein Fis1 and the cytosolic protein Drp1, which are responsible for fission, are recruited to form spirals around mitochondria, thereby severing both inner and outer membranes (Knott et al. 2008). Meanwhile, the fusion of mitochondrial outer membranes is regulated by the mitochondrial outer membrane protein Mfn1/2, whereas fusion of mitochondrial inner membranes is mediated by Opa1, which located in the inner mitochondrial membrane (Hoppins et al. 2007). Depletion of Mfn1/2 results in severe mitochondrial fragmentation (Chen et al. 2003). A recent study showed that mitochondrial fusion and fission could eliminate the damaged material and maintain a healthy mitochondrial population (Liesa and Shirihai 2013). Furthermore, the skeletal muscle of Mfn1/2 transgenic mice exhibits several hallmarks of mitochondrial dysfunction, including mitochondrial swelling, loss of respiratory complex activity, and accumulation of mtDNA mutations (Chen et al. 2010). Meanwhile, mutation of Drp1 in mammalian cells has been reported to increase mitochondrial interconnectivity, which has similar mitochondrial morphology phenotype compared with Fis1-depleted cells, whereas overexpression of Drp1 or Fis1 increases mitochondrial fragmentation (Yoon et al. 2003). Overexpression of Mfn1 and Mfn2 could delay the occurrence of apoptosis (Sugioka et al. 2004), thereby mitochondrial fusion which regulated by Mfn1/2 may involve in apoptotic process. A previous study reported that heat stress could induce swollen mitochondria in bovine mammary epithelial cells (Du et al. 2008). However, the mechanism underlying the effects of heat stress on mitochondrial morphology and function in DCMEC remains unclear. Accordingly, the aim of the present study is to investigate the effects of high temperature on mitochondrial function by using DCMEC as an in vitro model.

Materials and Methods

Cell lines and culture The DCMEC was provided by Prof. Gen-Lin Wang (Nanjing Agricultural University, Nanjing, China), and the separation of DCMEC was performed as described previously (Chen et al. 2019). In brief, the breast tissues were cut into 1.0 mm × 1.0 mm × 1.0 mm pieces and washed in PBS. Then, the tissue samples were treated with enzyme mixture (1.5 g/L type I collagenase, 1.5 g/L type II collagenase, and 1.5 g/L trypsin) (Sigma-Aldrich, St, Louis, MO; cat.: C0130 and C6885) and incubated in an oscillation incubator for 3 h (100 r/min). After filtration and centrifugation, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Morgan Hill, CA;cat: 10438026) and 1% antibiotic-antimycotic solution in a humidified incubator with 5% CO_2 at 37°C. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution in a humidified incubator with 5% CO_2 at 37°C. After 1 h, the DCMEC was selected based on means of differential attachment technique and identified with Cytokeratin 18 antibody staining (Abcam, Cambridge, MA;cat: ab52459, 1:300; Supplementary Figure 1).

Heat stress treatment Heat stress treatment refers to previous studies. In briefly, DCMECs were seeded in a six-well plate and cultured for 24 h until they reached 80% confluence at 37°C in a 5% CO_2 atmosphere, and the DCMECs were incubated at a high-temperature humidified incubator (42°C) for 2 h to induce heat stress (Collier et al. 2006) and then immediately for the next experiment.

Assessment of mitochondrial morphology The DCMECs were seeded onto coverslips and incubated with MitoTracker Green (25 nM, Molecular Probes, Waltham, MA) in a cell culture incubator with 5% CO_2 at 37°C. After 30-min incubation at 37°C, the cells were visualized using a confocal microscope, and mitochondrial morphology was quantified by ImageJ, according to previously reported methods (Merrill et al. 2017).

Measurement of mitochondrial membrane potential (JC-1) The mitochondrial membrane potential of the DCMEC was measured using the JC-1 kit (Beyotime, Wuhan, China; cat.: c2006) according to the manufacturer's instructions. Images were acquired using a fluorescence microscope (Olympus, Tokyo, Japan), and the fluorescence intensity was analyzed by ImageJ.

Measurement of the ATP content, Mn-SOD activity, and complex I activity The DCMECs were homogenized, and the ATP content, Mn-SOD activity, and complex I activity of the supernatant were measured using an ATP kit (Beyotime, cat.: S0026), Mn-SOD kit (Beyotime, cat.: S0103), and complex I kit (Solarbio, Beijing, China; cat.: BC0515), respectively.

Total RNA extraction and qRT-PCR The total RNA was extracted using TRIzol reagent (Invitrogen, Waltham, MA; cat.:
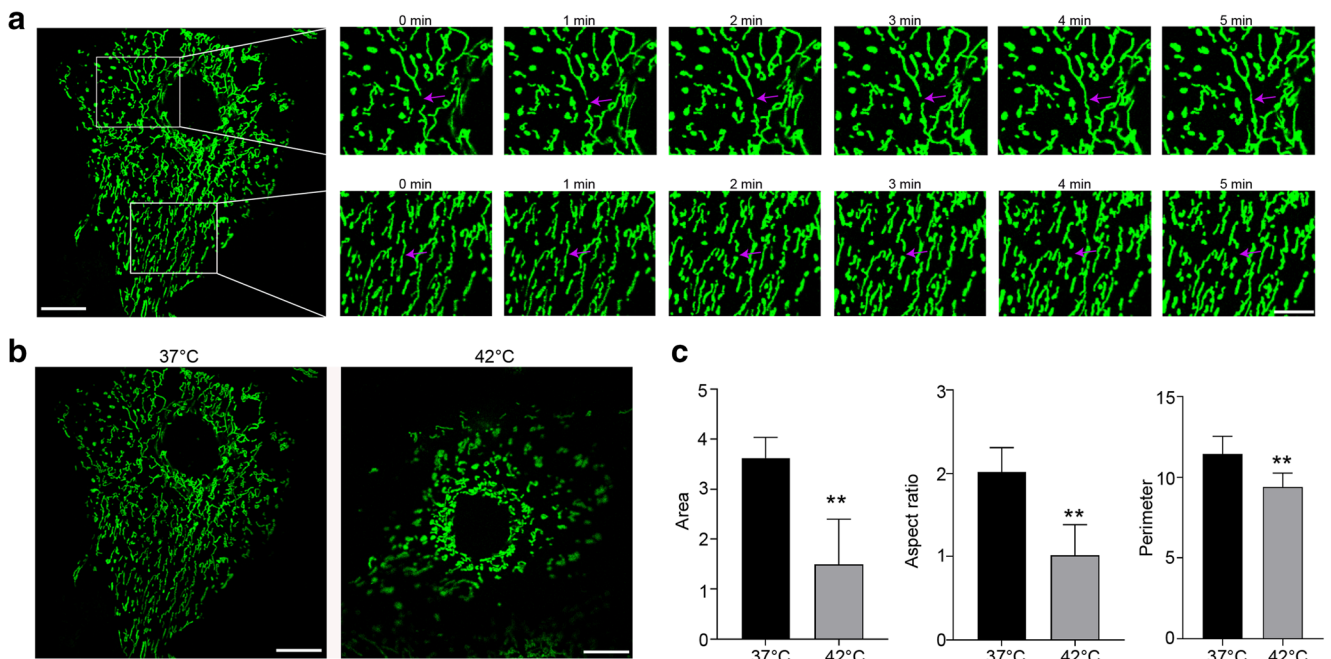
Figure 1. Effect of heat stress on the mitochondrial morphology in DCMEC. *A* Representative time-lapse images of mitochondria undergoing dynamic fusion (upper panels) and fission (lower panels) in wild-type DCMEC. Green, mitochondria, scale bar 10 μm. *B* Representative morphology of mitochondria from 37°C- and 42°C-treated cells. Green, mitochondria; scale bar, 10 μm. *C* Quantification of mitochondrial morphology, based on the average area (*C*), aspect ratio, and perimeter of mitochondria from 37°C- and 42°C-treated cells. Values and error bars represent means ± SD. Two-tailed unpaired *t* test, *$p < 0.05$, **$p < 0.001$.

15596026). Reverse transcription was performed using PrimeScript RT Master Mix (TaKaRa, Shiga,Japan; cat.: RR036A), and mRNA expression was quantified using real-time PCR. The expression levels of all target genes were normalized to those of the endogenous reference gene β-actin using an optimized comparative Ct ($2^{-\Delta\Delta Ct}$) value method, where $\Delta\Delta Ct = \Delta Ct_{target} - \Delta Ct_{\beta\text{-actin}}$. Primer sequences are listed in Supplementary Material Table S1.

Protein extraction and western blot analysis To extract total proteins, cells were incubated with lysis buffer and centrifuged at 15,000×*g* for 15 min at 4°C. The mitochondrial and cytoplasmic protein fractions in supernatant were isolated using an extraction kit (Beyotime, cat.: c3601) according to the manufacturer's instructions. The protein concentration was quantified using a BCA protein assay kit (Beyotime, cat.: P0010), and the western blot procedures were performed as previously described (Chen et al. 2018). The intensity of each band was normalized against that of the respective β-actin or VDAC loading control. The following commercially available antibodies were used: anti-cytochrome C (ProteinTech, Wuhan, China, cat.: 10993-1-AP, 1:3000), anti-Mn-SOD (ProteinTech, cat.: 24127-1-AP, 1:3000), anti-β-actin (ProteinTech, cat.: 10494-1-AP, 1:4000), anti-HSP70 (ProteinTech, cat.: 10995-1-AP, 1:4000), anti-Mfn1 (Abcam, cat.: ab104274, 1:2000), anti-Mfn2 (ProteinTech, cat.: 12186-

1-AP, 1:2000), anti-Fis1 (ProteinTech, cat.: 10956-1-AP, 1:2000), anti-Drp1 (Abcam, cat.: ab154879, 1:2500).

Ca^{2+} concentration assay The levels of calcium in the cell cytoplasm were measured by using a cell-permeable calcium-sensitive fluorescent dye (Fluo-3/AM, Solarbio, cat.: F8841). DCMECs were incubated with 5 μM Fluo-3/AM for 30 min at 37°C. Fluorescent images were acquired by using a fluorescence microscope (Olympus), and the fluorescence intensity of five randomly selected visual fields was analyzed using ImageJ.

Statistical analysis Statistical analysis was performed using GraphPad Prism 8.0.1 (La Jolla, CA). Data were compared using one-way analysis of variance (ANOVA) with two-tailed unpaired *t* tests, and *p* values < 0.05 were considered significant.

Results

Effect of heat stress on mitochondrial morphology in DCMEC
The present study found that mitochondria exhibit dynamic networks in DCMEC and undergo dynamic fusion and fission processes (Fig. 1A). In contrast, after heat stress treatment, the mitochondria became more fragmented, which indicated that heat stress could affect mitochondrial function by disrupting

Figure 2. Effect of heat stress on the mitochondrial respiratory chain activity and oxidative stress in DCMEC. *A–C* Effect of heat stress on the ATP content (*A*), complex I activity (*B*), and Mn-SOD (*C*) activity of DCMEC. Values and error bars represent means ± SD. Two-tailed unpaired *t* test, **p* < 0.05. *D* Effect of heat stress on the relative expression of *HSP70*, *Mn-SOD*, and *PGC-1α*. Values and error bars represent means ± SD. Two-tailed unpaired *t* test, **p < 0.001, ***p < 0.0001. *E, F* Effect of heat stress on the levels of HSP70 and Mn-SOD proteins. Values and error bars represent means ± SD. Two-tailed unpaired *t* test, **p* < 0.05.
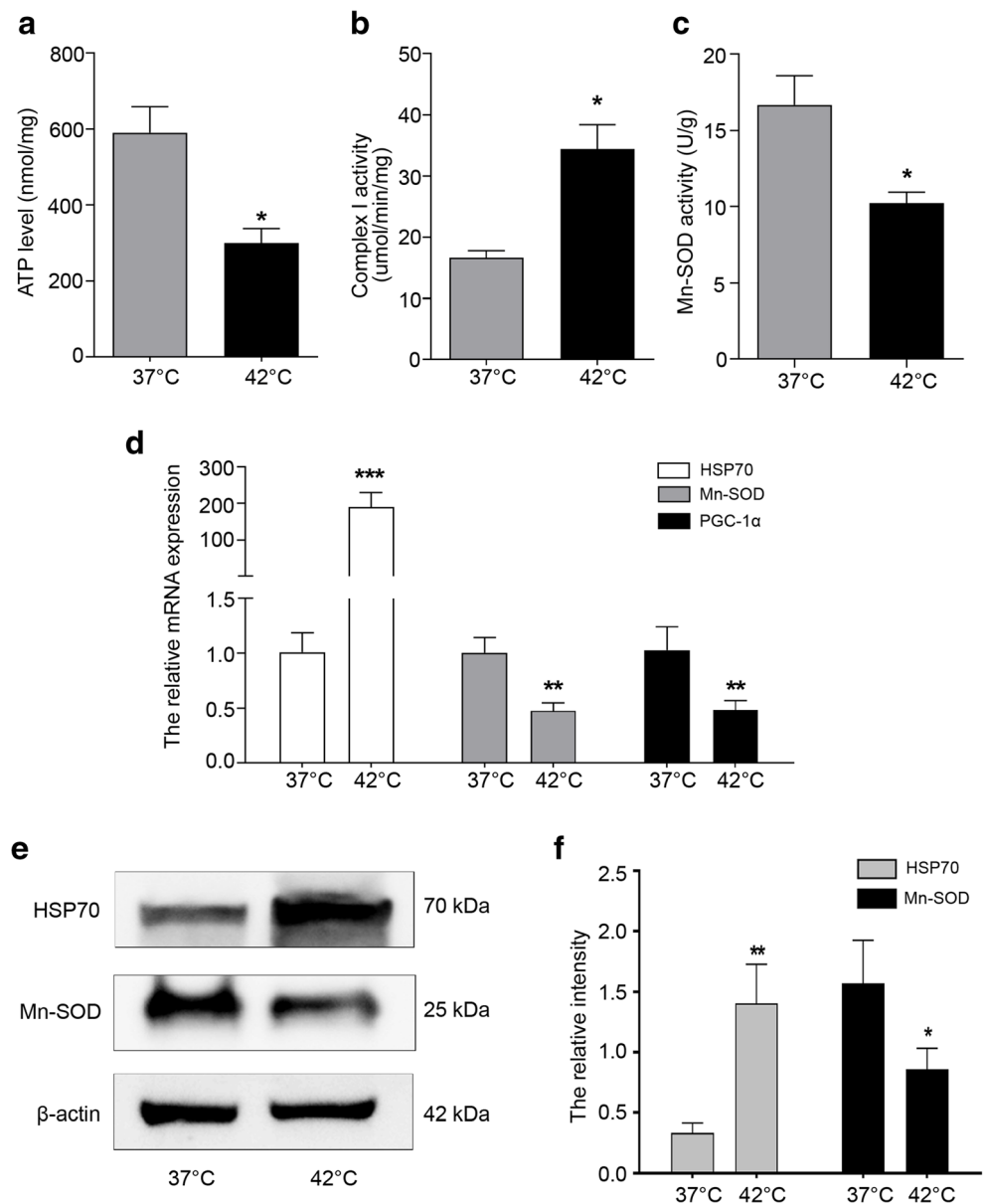

mitochondrial morphology (Fig. 1*B*). Indeed, the quantification data confirmed that the average mitochondrial area, aspect ratio, and perimeter were significantly reduced after heat stress treatment (Fig. 1*B, C*).

Effect of heat stress on mitochondrial respiratory chain activity and oxidative stress in DCMEC The change of mitochondrial morphology suggested that the mitochondrial function of DCMEC was disrupted under heat stress condition. In the meanwhile, the present study found that heat stress significantly reduced the ATP level (Fig. 2*A*) of DCMEC and significantly increased *HSP70* expression (Fig. 2*D–F*). Complex I is the largest of the five enzyme supercomplexes in the mitochondrial electron transport chain. The present study found that heat stress significantly reduced complex I activity (Fig.

2*B*), as well as manganese superoxide dismutase (Mn-SOD) transcription, translation, and activity, thereby suggesting that heat stress causes defects in the mitochondrial electron transport chain (ETC) and oxidative phosphorylation system (OXPHOS; Fig. 2*C–E*). Interesting, heat stress also reduced the expression of *PGC-1a*, which indicated the disruption of mitochondrial biogenesis in DCMEC.

Effect of heat stress on mitochondrial membrane potential in DCMEC Because the generation of mitochondrial membrane potential is essential to oxidative phosphorylation, JC-1 dye was used to determine the effect of heat stress on mitochondrial membrane potential. As shown in Fig. 3*A*, the mitochondria exhibited a heterogeneous pattern of fluorescence, and most of the mitochondria exhibited red fluorescence in
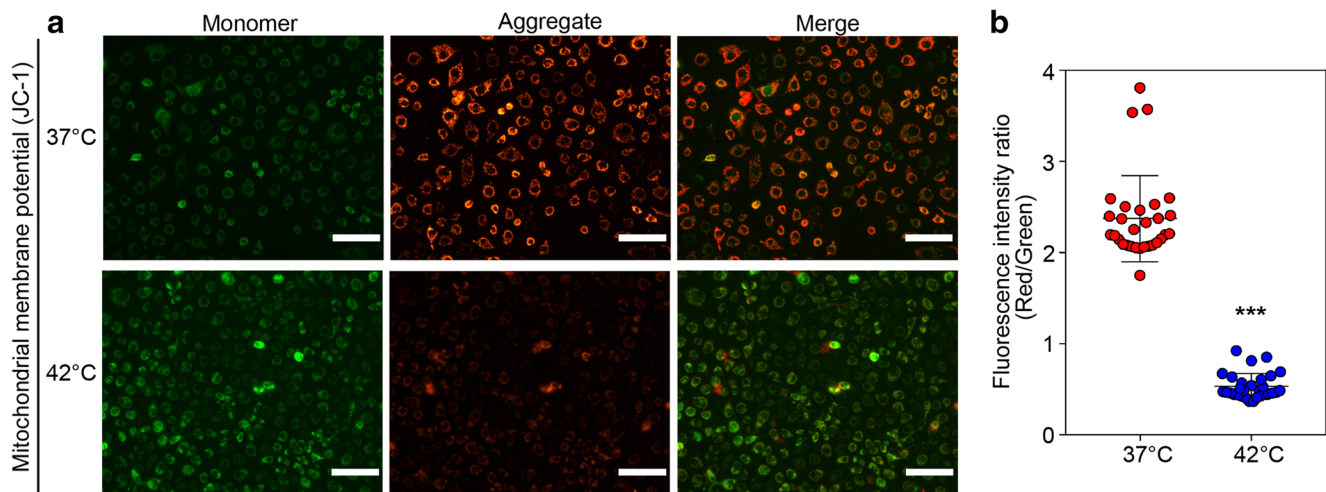
Figure 3. Effect of heat stress on the mitochondrial membrane potential in DCMEC. *A*, *B* JC-1 staining (*A*) and red/green ratio (*B*) of 37°C- and 42°C-treated dairy cow mammary epithelial cells. Red, aggregate; green, monomer. The high red/green ratio indicates high membrane potential in cells. Scale bar 100 μm. Values and error bars represent means ± SD. Two-tailed unpaired *t* test, ***$p < 0.0001$.

DCMEC. However, most of the heat-stressed DCMEC mitochondria exhibited high levels of green fluorescence and low levels of red fluorescence, which indicated the formation of mitochondrial monomers and the reduced membrane potential. Indeed, the quantification data showed that the ratio of red/green was reduced after heat stress in DCMEC (Fig. 3B).

Effect of heat stress on the balance of mitochondrial fission and fusion in DCMEC Because mitochondria continually change morphology through dynamic fission and fusion, the expression of *Drp1* and *Fis1*, which are involved in mitochondrial fission, were examined to evaluate the mechanism of heat stress–induced mitochondrial fragmentation. The results showed that the expression of *Drp1* and *Fis1* were upregulated after heat stress treatment (Fig. 4A, C), which is consistent with the result that the mitochondria are fragmented in heat-stressed cells. In contrast, Mfn1 and Mfn2, which are important in the fusion of outer mitochondrial membranes, were significantly downregulated by heat stress, thereby suggesting that defects in Mfn1 and Mfn2 function can promote the fragmentation of mitochondria after heat stress (Fig. 4B, D). In addition, the negative effect of heat stress on the expression of *Opa1* further demonstrated the conclusion that the occurrence of mitochondrial fusion defects is the main mechanism underlying the fragmentation of mitochondria in heat-stressed DCMEC (Fig. 4B).

Effect of heat stress on mitochondrial calcium uptake in DCMEC

Previous studies have suggested that disruption of calcium homeostasis could induce mitochondrial fragmentation (Xu et al. 2013). To investigate whether the heat stress–induced mitochondrial fragmentation was associated with calcium homeostasis, DCMECs were incubated with Fluo-3/AM for 30 min after heat stress. The increased fluorescence intensity of Fluo-3/AM demonstrated that the calcium homeostasis was disrupted after heat stress treatment in DCMEC, and the quantification data further confirmed that heat stress increased Ca^{2+} concentration in DCMEC (Fig. 5A, B).

Effect of heat stress on the release of cytochrome c from mitochondria in DCMEC Cytochrome c is a component of the electron transport chain, which is involved in the initiation of apoptosis. It has been shown that inhibition of Drp1-mediated mitochondrial fission could prevent the release of cytochrome c and, thus, delay the apoptosis (Estaquier and Arnoult 2007). The present study found that heat stress significantly increased levels of cytochrome c in the cytoplasm, but not in the mitochondria, suggesting that heat stress induced the release of cytochrome c from mitochondria and, eventually, induced apoptosis (Fig. 6).

Discussion

The results of the present study provide mechanistic insight into the effects of heat stress on mitochondrial dysfunction in DCMEC. The results demonstrate that heat stress causes mitochondrial fragmentation by disrupting the balance of mitochondrial fission and fusion, thus contributing to mitochondrial disorders and dysfunction, which further induce the apoptosis of DCMEC and the dysfunctional of dairy mammary glands.

Mitochondria are dynamic organelles that constantly undergo fission and fusion processes, which are essential for mitochondrial inheritance and maintenance, when cells are
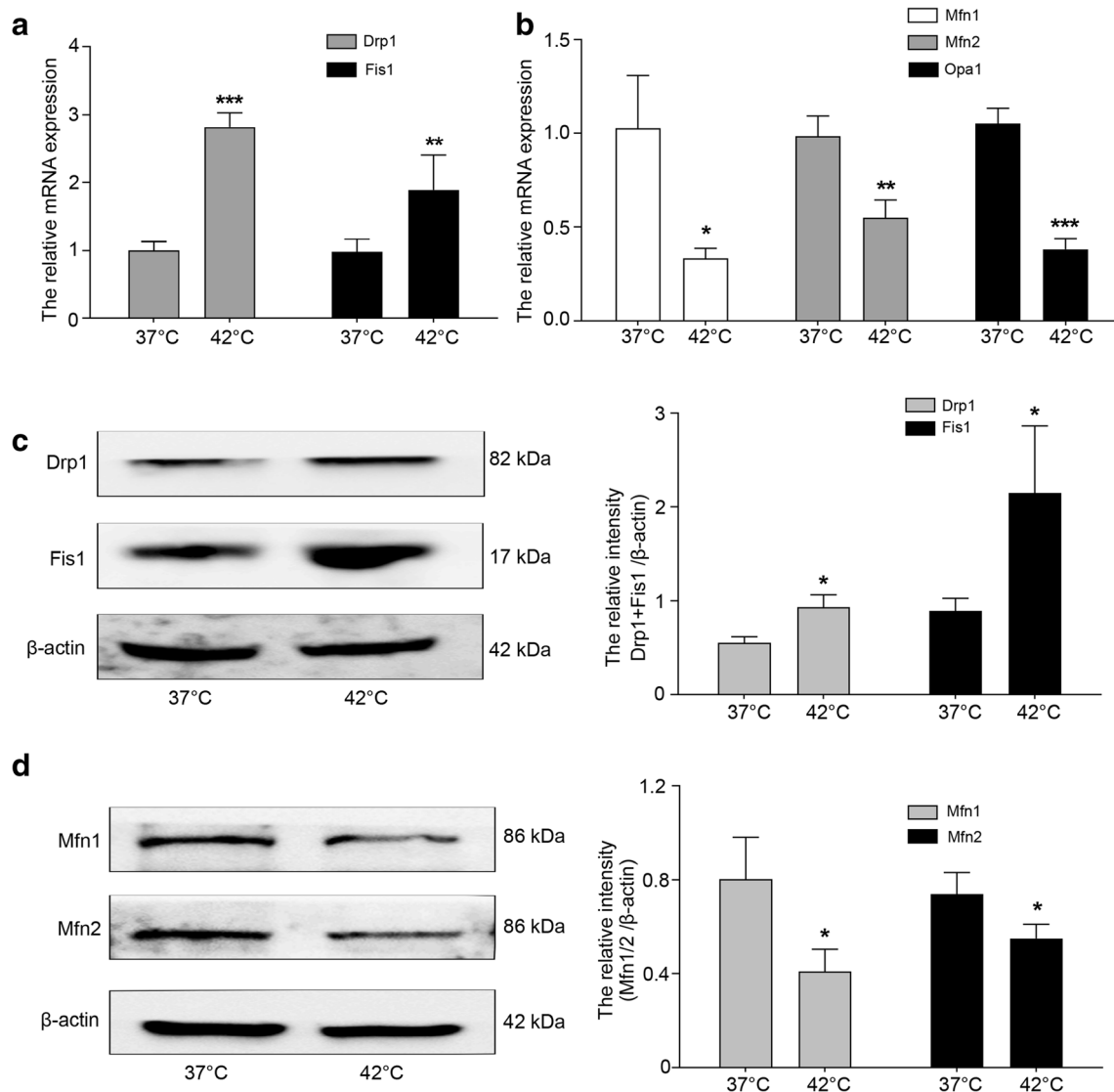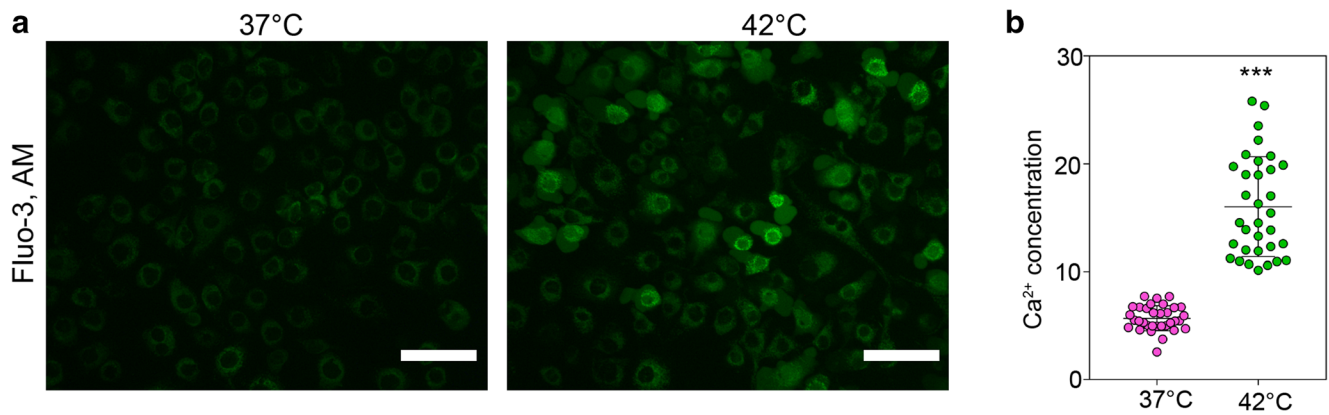
Figure 4. Effect of heat stress on the mitochondrial fission-fusion balance in DCMEC. *A*, *C* Effect of heat stress on the relative mRNA and protein levels of Drp1 and Fis1. Values and error bars represent means ± SD. Two-tailed unpaired *t* test, **$p < 0.001$, ***$p < 0.0001$. *B* Effect of heat stress on the relative mRNA levels of *Mfn1*, *Mfn2*, and *Opa1*. Values and error bars represent means ± SD. Two-tailed unpaired *t* test, *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$. *D* Effect of heat stress on the relative level of Mfn1 and Mfn2 proteins. Values and error bars represent means ± SD. Two-tailed unpaired *t* test, *$p < 0.05$.

subject to metabolic or environmental stresses (Westermann 2010). Recent studies have reported that the change of mitochondrial morphology is associated with disease occurrence. For example, the expression of *Mfn1*, *Mfn2*, and *Opa1* promote the elongation of mitochondria, and further protect cells during embryonic development or nutrient starvation (Chen et al. 2003; Rambold et al. 2011). In contrast, expression of *Drp1* promoted mitochondrial fragmentation, cell death, and apoptosis (Frank et al. 2001; Liot et al. 2009). However, it remains unclear whether mitochondrial morphology is associated with heat stress–induced mitochondrial dysfunction. The present study demonstrated that heat stress induces mitochondrial fragmentation, through mediating the expression levels of *Mfn1/2*, *Opa1*, *Drp1*, and *Fis1*. Previous studies have

reported that mitochondrial fusion is important for maintaining healthy mitochondrial populations and that the process can ameliorate environment–induced stress by allowing functional mitochondria to complement dysfunctional mitochondria and sharing the components (Youle and van der Bliek 2012).

The destruction of dynamic mitochondrial networks is associated with mitochondrial dysfunction, including reductions in mitochondrial membrane potential and respiration and increases in the formation of mitochondrial reactive oxygen species (ROS) (Nagdas and Kashatus 2017; Jezek et al. 2018). Depletion of Mfn1 and Mfn2 altered mitochondrial metabolism along with the loss of mitochondrial membrane potential and reduction in endogenous respiration (Chen et al. 2005), which coincides with the present study's observation

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Figure 5. Effect of heat stress on the calcium uptake by the mitochondria in DCMEC. *A*, *B* Representative images of intracellular free Ca²⁺ (*A*) and quantification of Ca²⁺ concentrations (*B*) in 37°C- and 42°C-treated dairy cow mammary epithelial cells. Values and error bars represent means ± SD. Two-tailed unpaired *t* test, ***$p < 0.0001$.

that heat stress reduces complex I activity and mitochondrial membrane potential, along with *Mfn1* and *Mfn2* expression. Heat stress has also been reported to modulate the activity of anti-oxidant enzymes and heat stress response, which is characterized by reduced Mn-SOD activity and increased *HSP70* expression, and that is coupled with increased ROS production (Slimen et al. 2014). Several studies have shown that increased ROS production is correlated with mitochondrial fission, suggesting that oxidative stress can induce mitochondrial fragmentation. However, when cells are under hyperglycemic conditions, mitochondria undergo Drp1-dependent fission, which further increases the release of ROS. Therefore, there is a reciprocal relationship between oxidative stress and mitochondrial morphology dynamics (Yu et al. 2006; Jendrach et al. 2008; Fan et al. 2010; Wu et al. 2011).

Calcium ions (Ca²⁺) function as a primary intracellular second messenger that directly or indirectly regulate a variety of cellular processes. In fact, it has been reported that heat stress induces large increases in intracellular Ca²⁺ concentrations (Han et al. 2010), which could potentially disrupt the

balance between mitochondrial fission and fusion (Hom et al. 2010). Conditions that reduce cytosolic Ca²⁺ levels are sufficient to prevent mitochondrial fragmentation, and this could explain the result of the present study that heat stress–induced increases in cytoplasmic Ca²⁺ could contribute to mitochondrial fragmentation in DCMEC. However, the characterization of the mechanisms underlying this process will still need to be explored (Pinton et al. 2001).

The morphology of mitochondria is known to change during the early stages of apoptosis, resulting in small and fragmented mitochondria (Youle and Karbowski 2005). Mitochondrial fragmentation generally occurs at the same time that Bax, a pro-apoptotic member of the BCL-2 family, translocates from the cytoplasm to mitochondria, but before caspase activation (Frank et al. 2001; Capano and Crompton 2002; Karbowski et al. 2004). Inhibition of Drp1 or Fis1 before the induction of apoptosis blocks mitochondrial fission and delays the activation of caspase and apoptosis (Frank et al. 2001; Karbowski et al. 2002; Breckenridge et al. 2003; Lee et al. 2004). Downregulation of *Mfn1/2* has been reported to
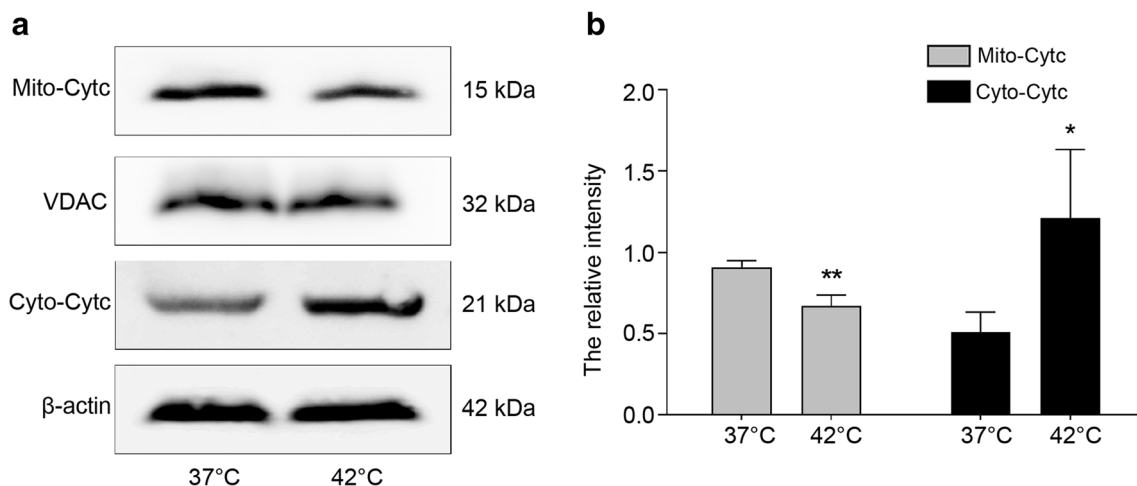
Figure 6. Effect of heat stress on the release of cytochrome c from mitochondria in DCMEC. *A*, *B* Cropped immunoblotting images (*A*) and cytochrome c quantification (*B*). Values and error bars represent means ± SD. Two-tailed unpaired *t* test, *$p < 0.05$, **$p < 0.001$.

Figure 7. Possible mechanism of heat stress–induced mitochondrial dysfunction in DCMEC.
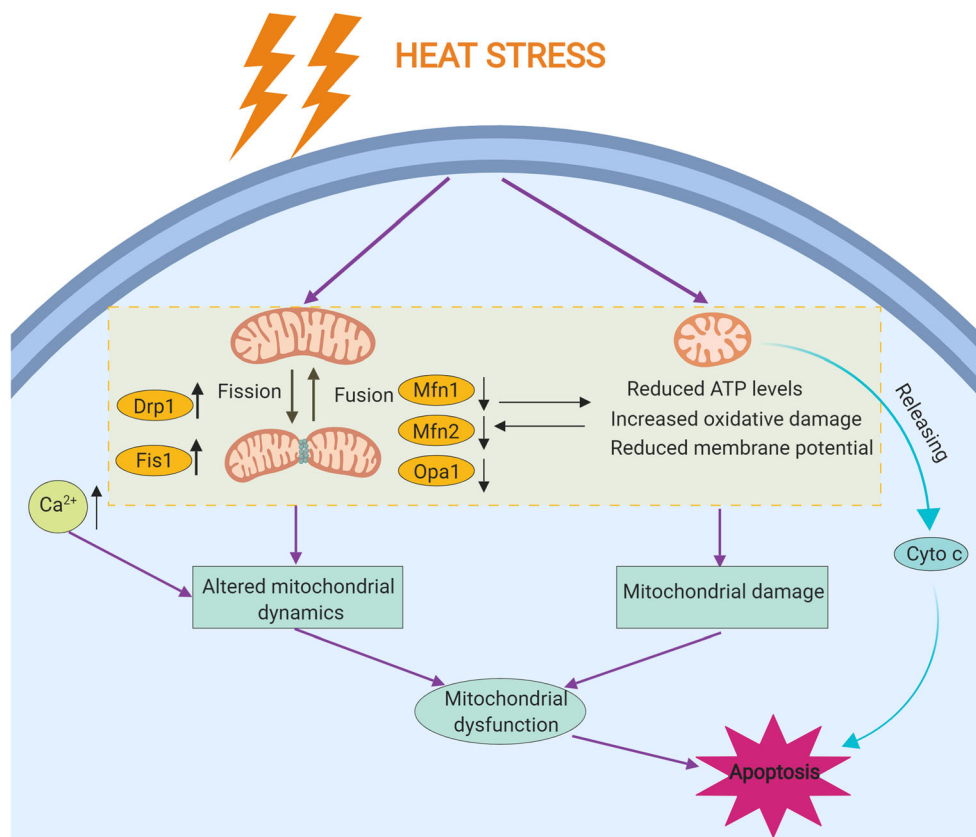
promote the development of shorter mitochondria and to enhance apoptosis (Sugioka et al. 2004); meanwhile, depletion of Opa1 has been reported to induce mitochondrial fragmentation and the spontaneous release of cytochrome c (Olichon et al. 2003). The present study revealed that heat stress can induce the release of cytochrome c from mitochondria to the cytosol, thereby confirming that mitochondrial fusion and fission machinery participates in heat stress–induced apoptosis. Under stress conditions, Bax and Bak coalesce on the surface of the outer mitochondrial membrane (OMM) into large foci (Nechushtan et al. 2001; Valentijn et al. 2003) and that these foci co-localize with Mfn2 and Drp1 at mitochondrial fission sites (Karbowski et al. 2002), suggesting that Bax and Bak are involved in Drp1- or Mfn2-regulated mitochondrial fission during early apoptosis.

Conclusion

Taken together, the present study demonstrates that heat stress induces both oxidative stress and apoptosis was related with the mitochondrial fission- and fusion-regulated network (Fig. 7), thereby suggesting that mitochondrial network dynamics are important for physiological homeostasis and disease states. Accordingly, the results of the present study indicate that maintaining the normal balance of mitochondrial

fission and fusion could help to attenuate the heat stress–induced oxidative stress and apoptosis in DCMEC.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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