Cytoprotection of vitamin E on hyperthermia-induced damage in bovine mammary epithelial cells

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ABSTRACT

The protective effects of vitamin E (VE) against hyperthermia-induced damage in bovine mammary epithelial cells (BMEC) were studied. The structure of BMEC membrane was damaged by hyperthermia treatment. The VE (25 nmol/ml) efficiently increased cell viability and attenuated morphological damages in hyperthermia-treated BMEC. Compared with the control, VE significantly reduced lactate dehydrogenase leakage and malondialdehyde formation in hyperthermia-treated BMEC. Meanwhile, superoxide dismutase activity was increased significantly in the presence of VE. It is inferred that VE displayed cytoprotective effects on hyperthermia-induced damage in BMEC through increasing intracellular antioxidant levels and decreasing lipid peroxidation.

1. Introduction

Under heat stress conditions in the tropics and subtropics during the summer, lactating dairy cows suffer from reduced dry matter intake and lactation performance (Kadzere et al., 2002; West, 2003). The effects of heat stress on oxidative status in lactating cows have extensively studied (Gupta et al., 2005; Padilla et al., 2006). It is observed that heat stress enhances production of reactive oxygen species (ROS) and induces deleterious effects on dairy cows (Padilla et al., 2006). When the ROS production is faster than their neutralization by antioxidative mechanisms, oxidative stress is induced. Therefore, heat stress probably affects antioxidative mechanisms.

Environmental and nutritional strategies to alleviate heat stress usually improve lactation performance of dairy cows (Kendall et al., 2006; Bruno et al., 2009). Because of its potential role as an antioxidant, vitamin E (VE) is able to prevent free-radical mediated tissue damage, and as a consequence to prevent or delay the development of certain degenerative and inflammatory diseases (Moorthi et al., 2006; Rahman et al., 2008). The reported benefits of vitamin supplementation to dairy cows include the reduction in the prevalence of retained fetal membranes, reduction in the incidence and severity of mastitis, and improvement in reproductive and lactation performance (Brozos et al., 2009; Baldi, 2005; Moeini et al., 2009). However, little information is available on the effects and mechanisms of dietary VE supplementation to mammary epithelia cells of dairy cows during heat stress.

As a unique gland, the mammary gland attracts the attention of scientists for over a century because of its special function for milk synthesis and secretion. Mammary epithelia cells are sensitive to the exogenous nutriments and environment changes and could be used as a model for evaluating actions of nutriments on hyperthermia-induced damage in vitro (Liu et al., 2007; Du et al., 2008; Lauzon et al., 2005). In the present study, VE was used to investigate the protective effect against hyperthermia-induced damage in bovine mammary epithelial cells (BMEC) by analysis of lactate dehydrogenase (LDH) leakage, malondialdehyde (MDA) formation and superoxide dismutase (SOD) activities. The results of the study would help provide dietary means against heat stress in lactating dairy cows.

2. Materials and methods

2.1. Culture of bovine mammary epithelial cells

Mammary tissues were obtained from a health Holstein diary cow at the middle stage of lactation. Tissues were cut into 1 mm³ pieces and incubated at 37 ºC in a water-saturated atmosphere of 95% air and 5% CO₂. The procedures of purification and culture of BMEC were as described elsewhere (Liu et al., 2007). Briefly, after the cells covered 80% of the bottom, the tissues were removed and cells were digested with 0.25% trypsin and 0.15% trypsin plus 0.02% EDTA. Fibroblast cells and BMEC were separated according to their different sensitivity to trypsin. The dispersed cells were seeded at a density of 5 x 10⁴ cells/ml in DMEM/F12 medium (Gibco BRL Life Technologies). The basal medium was replenished with 5 µg/ml transferrin, 5 µg/ml insulin, 5 µg/ml prolactin, 1 µg/ml
hydrocortisone (Sigma-Aldrich, St. Louis, MO), 1% glutamine, 1% penicillin, 1% streptomycin and 10% FCS (Sangon, Shanghai).

2.2. Hyperthermia treatment of cultured cells

After 5 d culture of BMEC at 37°C in a water-saturated atmosphere, hyperthermia treatment was carried out by incubating the cells at 42°C for 1 h in a humidified atmosphere of 95% air and 5% CO2. Then, the cells were immediately incubated at 37°C in 5% CO2 for a range of periods. Finally, cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT, Sigma) assay and compared to that of non-pretreated cells.

2.3. Cytoprotection of VE on hyperthermia-induced damage of BMEC

At the beginning of hyperthermia treatment, BMEC in 6-well culture plates were treated with VE (Sigma-Aldrich, St. Louis, MO, USA) at 5–50 nmol/ml. The control received the vehicle only. Then, cell viability was assessed by MTT assay. Finally, the medium and cultured cells were used for determination of SOD, MDA and LDH in hyperthermia and optimal VE-treated groups.

2.4. Determination of cell viability by MTT assay

Cell viability was quantified by measurement of the mitochondrial reduction of MTT to produce a dark-blue formazan product. Briefly, MTT (0.25 mg/ml, Sigma) was added to cultured cells. After incubating for 4 h at 37°C, the medium was removed and 100 μl dimethylsulfoxide was added to solubilize the formazan crystals. The color developed was measured at 570 nm by a microplate reader (Tecan sunrise, USA). Cell viability was expressed as the proportion of absorbance values to the control.

2.5. Determination of LDH release, MDA formation and SOD activity

Cytotoxicity was estimated by quantification of LDH activity in the culture medium versus total LDH activity in the samples after treatment. Cultured cells were treated with 10% Triton X-100, and LDH activity was assayed by absorbance change at a wavelength of 440 nm with an LDH kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Lipid peroxidation was evaluated indirectly by MDA formation from the breakdown of polyunsaturated fatty acids, measured by the thiobarbituric acid reactive substances (TBARS) at 532 nm (Agostinho et al., 1997). Total SOD activity was evaluated using the xanthine-xanthine oxidase system, and the absorbance at 550 nm was determined. One unit of enzyme activity was defined as the quantity of SOD required for 50% inhibition of SOD reduction (Flohe´ and Otting, 1984).

2.6. Analysis of Morphological changes

Morphological changes of BMEC were observed under an IX70 phase contrast microscope (Olympus, Japan). Five different regions were selected randomly in each well and the images were captured with a video camera (Pixera Pro 150 ES, USA) to a computer. Analysis was achieved by using Simple PCI Advanced Imaging Software (Compix, Inc., USA).

2.7. Statistical analysis

The experiment was repeated three times and each treatment included 4 wells. All data were expressed as the mean ± SD and analyzed by ANOVA and Duncan’s multiple range tests using the SAS software system (SAS, 2000). P < 0.05 was considered as significantly different.

3. Results and discussion

3.1. Effects of hyperthermia on cell morphology and cell viability

The structure of BMEC membrane was severely damaged by hyperthermia treatment. Condensed nuclei and vacuolated cytoplasm occurred, many cell pieces were released into the medium, and cytolysis and disorganization of cells were found (Fig. 1A). Cell viability of BMEC changed in response to the time at 37°C.

Fig. 1. Morphological changes of bovine mammary epithelial cells (BMEC) after hyperthermia (42°C, 1 h) and vitamin E (VE, 25 nmol/ml) treatment for 8 h. A and B represent BMEC of hyperthermia (42°C, 1 h) and hyperthermia (42°C, 1 h) + VE (25 nmol/ml) treated group, respectively. Condensed nuclei and vacuolated cytoplasm appeared in BMEC and many cell pieces were released into the medium in A. While in B, the adverse effects of hyperthermia on BMEC were attenuated by VE. Scale bar: 10 μm.

Fig. 2. Effect of hyperthermia (42°C, 1 h) on cell viability in different 37°C incubation period. Cell viability was quantified by measurement of MTT reduction and expressed as the proportion of absorbance values to the control. Values are the means ± SD (n=4). Bars with different superscripts are statistically different (P < 0.05).
incubation, with the lowest value at 8 h of hyperthermia treatment ($P < 0.05$; Fig. 2). The non-linear response of the cell viability to hyperthermia may be due to the cell's self-recovery and thermotolerance (Hildebrandt et al., 2002; Park et al., 2005; Breen et al., 1999). It has been shown that heat shock transcriptional response attenuates against return to physiological temperature (Santos-Marques et al., 2006; Heydari et al., 2000). With prolonging of 37 °C incubation time, more focal adhesions were formed, intercellular connections enhanced and multiple growth factor receptors activated (Chen et al., 2005; Park et al., 2005). The series reactions helped cells keep their original shape and proliferation after heating. As different cell types have their own characteristic temperature sensitivities, thermotolerances and self-recovery abilities (Chen et al., 2008; Santos-Marques et al., 2006; Argov et al., 2005), we chose 8 h of hyperthermia as the treatment time in the latter study.

### 3.2. Cytoprotection of VE on hyperthermia-induced damage of BMEC

Morphological damages induced by hyperthermia were significantly attenuated, indicated by relative integral of cell membranes in BMEC (Fig. 1B). An increased trend in cell viability of hyperthermia-treated BMEC was observed following addition of VE (5–50 nmol/ml). Adding VE at 25 nmol/ml markedly increased cell viability of hyperthermia-treated BMEC ($P < 0.05$; Fig. 3). These data are consistent with reports that VE increased reproductive and lactation performance in dairy cows (Baldi, 2005; Moenini et al., 2009) and alleviated negative effects of heat stress on Japanese quails (Sahin et al., 2002). Based on current literatures and our results, it is presumed that VE attenuated hyperthermia-induced damage on BMEC.

#### 3.3. Assessment of cytotoxicity by LDH release assay

The VE significantly reduced LDH leakage from hyperthermia-treated BMEC ($P < 0.05$, Fig. 4A), consistent with the results obtained by the morphological observations (Fig. 1B). The leakage of the cytosolic enzyme LDH correlates well with cellular viability, thus being a useful indicator of plasmatic membrane damage (Davila et al., 1998). Our results complied with the findings that VE could defend hepatocytes against oxidative stress by inhibited LDH leakage (Zhou and Zhang, 2005). It is indicated that VE may effectively protect the integrity of cellular membrane of hyperthermia-treated BMEC.

#### 3.4. Measurement of oxidative damage by MDA formation

The MDA formation was markedly reduced by VE (25 nmol/ml) in hyperthermia-treated BMEC ($P < 0.05$, Fig. 4B). The MDA is widely used as an index of lipid peroxidation. Vitamin E was considered to play an important role in protecting highly unsaturated fatty acids in cellular and subcellular membrane phospholipids of cells against ROS-induced lipid peroxidation (Clavines et al., 1971). Determinations of the lipid peroxidation status in cultured BMEC after hyperthermia and VE treatments indicated that toxicity induced by heat stress through lipid peroxidation might be alleviated by antioxidant VE.

#### 3.5. Determination of intracellular antioxidant status by SOD activity

In hyperthermia-treated BMEC, SOD activity was decreased, but no such decrease was observed by the presence of VE (25 nmol/ml) ($P < 0.05$, Fig. 4C). The SOD is a family of antioxidant enzymes that act as a first cell defense against oxidative stress. The SOD activity is regulated by different means such as oxidative stress and UVB radiation (Okamoto et al., 2001). Vitamin E significantly increased SOD activities on A1254-induced cytotoxicity of hepatocytes (Zhou and Zhang, 2005), which was consistent with our study. These results further proved the antioxidant action of VE in hyperthermia-induced toxicity in BMEC.

In conclusion, cultured BMEC represented a convenient sensitive and visible in vitro model for assessment of hyperthermia-induced damage. Hyperthermia may interfere with the integrity of cell membrane and cause cytotoxicity in cultured BMEC. However, VE could block these deleterious effects through...
a reduction in MDA and an increase in SOD. It is suggested that the dietary antioxidant VE may attenuate the damage caused by heat stress through increasing intracellular antioxidant levels and decreasing lipid peroxidation in lactating dairy cows.

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References


