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Retinoic Acid Attenuates Oxidative Injury in Bovine Mammary Epithelial Cells Induced by Hydrogen Peroxide

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ABSTRACT

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The objective of this study was to explore how retinoic acid (RA) attenuates oxidative injury induced by hydrogen peroxide (H_2O_2) in bovine mammary epithelial cells (BMEC). Subconfluence BMEC were randomly divided into four groups with six replicates: the control group (incubated in serum-free medium without RA or H_2O_2 for 30 h), H_2O_2 group (pre-incubated for 24 h without RA, then for another 6 h with 600 μM H_2O_2), RA group (incubated with 1 mg/ml RA for 30 h without H_2O_2), and RA + H_2O_2 group (RA prevention group, pre-incubated with 1 mg/ml RA for 24 h and then for another 6 h with 600 mM H_2O_2). The results showed that the H_2O_2 treatment significantly decreased several measured traits, including the cell viability, glutathione peroxidase (GPX) and thioredoxin reductase (TRXR) activities, selenoprotein P (SELP) content, catalase and superoxide dismutase activities, total antioxidant capacity, and *GPX1*, *TRXR1,* and *SELP* gene expression, as well as GPX1 and TRXR1 protein expression. H_2O_2 treatment also increased the malondialdehyde and reactive oxygen species contents and induced a marked increase of several measured traits, including the arachidonic acid (ARA) concentration, cytosolic phospholipase A2 and 5-lipoxygenase gene expression and activity, and 15-hydroxy twenty-four arachidonic acid and hydroxy peroxide tetracosenic arachidonic acid contents. RA pre-treatment prevented corresponding increases in parameters related to ARA metabolism and increased the activity of TRXR. Moreover, RA pre-treatment attenuated the phosphorylation levels of p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase and effectively decreased the ARA content. These results suggest that RA protected BMEC from oxidative stress by elevating TRXR activity, which inhibited the MAPK signaling pathway and led to a decreased concentration of ARA.

Keywords: vitamin A; thioredoxin reductase; oxidative injury; arachidonic acid; p38 mitogen-activated protein kinase

List of abbreviations: RA = retinoic acid, ROS = reactive oxygen species, VA = vitamin A, BMEC = bovine mammary epithelial cells, *GPX1* = glutathione peroxidase 1, *TRXR1* = thioredoxin reductases 1, ARA = arachidonic acid, CPLA2 = cytosolic phospholipase A2, MAPK = mitogen-activated protein kinase, H_2O_2 = hydrogen peroxide, DMSO = dimethyl sulfoxide, MTT = 5-diphenyltetrazolium bromide, DMEM/F12 = Dulbecco's Modified Eagle's Medium/F12, 5-LOX = 5-lipoxygenase, PGE2 = prostaglandin E2, COX-2 = cyclooxygenase-2, LTB4 = leukotriene B4, 12- or 15-HETE = 12- or 15-hydroxyeicosatetraenoic acid, 12- or 15-HPETE = 12- or 15-hydroperoxyeicosatetraenoic acid, ERK1/2 = extracellular signal-regulated kinase ½, JNK = c-Jun N-terminal kinase, PBS = phosphate buffered solution, RGR = relative growth rate, T-AOC = total antioxidant capacity, SOD = superoxide dismutase, CAT = catalase, MDA = malondialdehyde, SELP = selenoprotein P, DTNB = dithiobis-nitrobenzoic acid, BCA = bicinchoninic acid, TMB = tetramethylbenzidine, ASK1 = apoptosis signal-regulating kinase-1, NO = nitric oxide, RT = reverse transcription, RT-PCR = reverse transcription polymerase chain reaction

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Dairy cattle undergo dramatic metabolic changes during lactation periods that likely contribute to the production of a large amount of reactive oxygen species (ROS) and lipid peroxides. If these excessive oxygen free radicals are not cleared in time, oxidative stress occurs, leading to an imbalance between the production of ROS and host antioxidant capabilities. Oxidative stress in the mammary gland not only contributes to dysfunctional immune responses and increased disease susceptibility, but is also a major problem for ensuring the quality of milk in the dairy industry (Sordihho and Aitken 2009). Therefore, regulating and attenuating oxidative injury in bovine mammary tissue is an important measure for guaranteeing the health of mammary glands and enhancing the milk performance of dairy cows. All-*trans* retinoic acid (RA), a derivative of vitamin A (VA), has a profound effect on the mRNA levels of selenoproteins, which are involved in antioxidant functions within mammary cells (Jin et al. 2016). Bruzelius et al. (2010) observed that supplementing bovine mammary epithelial cell (BMEC) cultures with RA could up-regulate the gene expression of selenoproteins, including glutathione peroxidases 1 (*GPX1*) and thioredoxin reductases 1 (*TRXR1*). Our previous *in vivo* study indicated that adding a higher dose of VA than currently recommended to the diet of dairy cows could improve serum TRXR activity in dairy cows (Jin et al. 2014a).

Arachidonic acid (ARA) is present in the form of a phospholipid as a precursor of eicosanoids. In the normal physiological state, there are very low levels of free ARA in the body, which maintain optimal growth and physiological activities (Astudillo et al. 2012). However, ARA is freed from phospholipids by cytosolic phospholipase A2 (CPLA2) when cell membrane in BMEC is stimulated by ROS, lipopolysaccharide, cytokines, or nitric oxide (NO) (Piotrowska-Tomala et al. 2012). Metabolites of excess ARA can cause cell toxicity and injury (Shimizu et al. 2008). CPLA2 is a substrate for mitogen-activated protein kinase (MAPK) and can be phosphorylated and activated by MAPK under oxidative stress, resulting in the production of large amounts of ARA (Zhu et al. 2009), further aggravating oxidative stress. Therefore, regulating the release and metabolism of ARA is the key to protecting cells from oxidative stress-induced damage. Kurosawa et al. (2009) investigated the role of TRXR in inhibiting the release of ARA, and their results suggested that TRXR suppressed the activity of CPLA2 and release of ARA, avoiding toxic effects in L929 mouse fibrosarcoma cells. Therefore, we propose that RA may protect BMEC from oxidative stress by promoting TRXR activity, which results in inhibition of the MAPK signaling pathway, leading to suppression of ARA release. There is, however, very little data regarding the mechanisms by which RA protects BMEC from oxidative injury, and considerable research has focused on mice tumour cells. Hence, the present study was conducted to induce BMEC oxidative injury with hydrogen peroxide (H_2O_2) and explore the mechanism by which RA induces TRXR to protect BMEC against oxidative injury.

MATERIAL AND METHODS

Materials. RA, H_2O_2, dimethyl sulfoxide (DMSO), 5-diphenyltetrazolium bromide (MTT), and an antibody against GPX1 were purchased from Sigma-Aldrich (USA). Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) and an antibody against TRXR1 were obtained from Gibco (USA). ELISA kits for 5-lipoxygenase (5-LOX) and CPLA2 were purchased from MyBiosource (USA). Kits for prostaglandin E2 (PGE2) were purchased from R&D Systems (USA). Kits for cyclooxygenase-2 (COX-2), leukotriene B4 (LTB4), and 15-hydroxyeicosatetraenoic acid (15-HETE) were purchased from Cayman Chemicals (USA). Kits for ARA and 15-hydroperoxyeicosatetraenoic acid (15-HPETE) were acquired from Shanghai Hengyuan Biotechnology (China). The p38 MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK) phosphorylation kits were obtained from eBioscience (USA). All other chemicals and reagents were standard, commercially available biochemical-quality products.

Cell culture. The modified procedures for BMEC cultures were previously described (Sheng et al. 2015). In brief, bovine mammary tissues were collected from 4 late-lactating Holstein cows from a slaughterhouse. Several approximately 1-cm^3 pieces of mammary gland tissue were aseptically removed and washed with cold phosphate buffered solution (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin. Then, small mammary tissues were thoroughly minced into 1-mm^3 pieces and connective tissues were carefully removed.

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The mammary tissue fragments were digested by type-II collagenase for 60 min at 37°C and gently shaken every 20 min. The digested, cell-containing liquid was filtered through a 150-mm filter to remove large tissue fragments, and centrifuged at 800 *g* for 5 min. The supernatant was removed, and the precipitate was rinsed with PBS and centrifuged at 800 *g* for 3 min. Cells were cultured in DMEM (5 μg/ml ovine prolactin, 10 ng/ml epidermal growth factor, 1 mg/ml hydrocortisone, 0.5% insulin-transferrin-selenium, 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B) containing 10% foetal bovine serum (Gibco Laboratories, USA). Then, adherent cells were cultured until they were confluent at 37°C in 5% CO_2/95% air, and the medium was replaced every other day.

Treatment of the BMEC culture. All cells were cultured in serum-free medium for 1 day. Then, cells were randomly divided into four groups: a control group, H_2O_2 group, RA group, and RA + H_2O_2 group. Cells in the control group were incubated in serum-free medium without RA or H_2O_2 for 30 h. In the H_2O_2 group, cells were pre-incubated for 24 h in serum-free medium without RA and then incubated for another 6 h following the addition of H_2O_2 (600 mM, the concentration was established by a previous study to be optimally toxic by inducing oxidative damage of BMEC) (Jin et al. 2014b). All cells in the RA group were incubated for 30 h in serum-free medium containing 1 mg/ml RA (a concentration that was established on the basis of our previous study). In the RA + H_2O_2 group (RA prevention group), cells were pre-incubated for 24 h in serum-free medium containing 1 mg/ml RA, followed by a 6-h incubation after addition of 600 mM H_2O_2.

Viability of cells. Cells were seeded in 96-well culture plates (1×10^4 cells/well), and the cell viability was measured using the MTT assay. Briefly, after the various incubation times indicated, cells were exposed to 20 ml of MTT (5 mg/ml in 1 × PBS) and incubated at 37°C for 4 h. After MTT removal, the coloured formazan was dissolved in 100 ml of DMSO. The absorbance at 490 nm in each well was recorded immediately using a microplate reader (BioTek, USA). To determine the cell viability, the cell relative growth rate (RGR) was calculated using the following formula:

$$RGR = OD490_{test\ group}/OD490_{control\ group}$$

where:
OD490 = absorbance value at the wavelength of 490 nm.

Preparation of cell lysates. The culture supernatant was collected for analysis of the total antioxidant capacity (T-AOC), superoxide dismutase (SOD) and catalase (CAT) activities, and selenoprotein P (SELP), ARA, 15-HETE, 15-HPETE, PGE2, and LTB4 contents. Cells from different treatment groups were lysed on ice for 30 min in lysis buffer (Beyotime, China). Lysates were centrifuged at 1200 *g* for 10 min to remove cell debris, and the supernatant was used for analysis of the GPX, TRXR, CPLA2, 5-LOX, and COX-2 activities, malondialdehyde (MDA) and ROS contents, and p38 MAPK, ERK1/2, and JNK phosphorylation levels.

Measurement of the enzyme activity and phosphorylation levels. The activities of GPX, SOD, CAT, and T-AOC and the content of MDA were measured with a commercially available colorimetric assay kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The assay of TRXR activity was based on a method by which the reduction of dithiobis-nitrobenzoic acid (DTNB) was monitored at 412 nm (Hill et al. 1997). The concentration of SELP was measured by radioimmunoassay as described earlier (Hill et al. 1996). The ROS concentration was estimated by a FLx800™ Fluorescence Reader (BioTek) using the chemical fluorometry enzyme immunoassay described by Kim et al. (2004). The values of GPX, TRXR, and MDA were expressed as IU per mg of protein. The protein concentration was measured with the bicinchoninic acid (BCA) protein assay kit (Pierce, USA) using bovine serum albumin as a standard.

ELISA kits for the CPLA2, 5-LOX, and COX-2 activities; ARA, 15-HETE, 15-HPETE, PGE2, and LTB4 contents; and p38 MAPK, ERK1/2, and JNK phosphorylation levels were used according to the manufacturer's protocol. Briefly, samples, including standards, were incubated in 96-well plates coated with the respective antibodies for 2 h. Then, samples were treated in subsequent steps with enzyme working reagent (30 min) and tetramethylbenzidine (TMB) One-Step substrate reagent (30 min in the dark). The reaction plates were read within 30 min in a microplate reader at different wavelengths. The phosphorylation levels of p38 MAPK, ERK1/2, and JNK were determined using stand-by absorbance.

RNA extraction and real time polymerase chain reaction (RT-PCR) assay. Total cellular RNA was extracted using TRIzol solution (TaKaRa, Inc.,

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China) according to the manufacturer's instructions. For reverse transcription (RT), the reaction mixture contained 2 µl of 5 × PrimeScript Buffer, 0.5 µl of Prime Script RT Enzyme Mix I, 0.5 µl of Oligo dT Primer (50 µM), 0.5 µl of Random 6 mers (100 µM), and 6.5 µl of RNA reverse transcriptase (TaKaRa) at a total volume of 10 µl. The RT reaction parameters were as follows: RT at 37°C for 15 min and RT inactivation at 85°C for 5 s. The RT products (cDNA) were stored at –20°C.

For quantitative PCR assays, all primers (see Table 1) were designed using Oligo software (National Biosciences, USA) and custom-synthesized (Sangon Biological Technologies, China). The RT-PCR analysis was performed using a Bio-Rad iCycler IQ5 detector system (PerkinElmer/Applied Biosystems, USA).

The relative levels of specific gene mRNA were quantified using a SYBR® Prime Script™ RT-PCR Kit (TaKaRa, Inc.) following manufacturer's instructions. Briefly, the PCR reaction was performed using 2 µl of cDNA template, 10 µl of SYBR® *Premix Ex Taq*™ (2 ×), 7.2 µl of dH_2O, and 0.4 µl (10 mM) of forward and reverse gene-specific primers at a final volume of 20 µl. The reaction was cycled 40 times for 5 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The level of *GAPDH* gene expression served as an internal control. The quantitative real-time PCR data were calculated by the $2^{-\Delta\Delta Ct}$ method (Pan et al. 2015). Amplified products were separated by electrophoresis on a 2% agarose gel and detected under UV light.

Western blotting. BMEC from the experimental conditions were collected and homogenized in lysis buffer (Beyotime). Cell lysates containing 20–50 µg of protein were separated by SDS-PAGE. Following separation, proteins were transferred to a polyvinylidene fluorine membrane (Millipore, Canada). Membranes were blocked with blocking buffer for 1 h before incubation with the appropriate specific antibodies.

Membranes were then incubated with polyclonal rabbit anti-GPX1 (22 kDa; 1 µg/ml), anti-TRXR1 (55 kDa; 1 : 500), or anti-GAPDH (36 kDa; 1 : 2000) overnight at 4°C. The secondary antibody, goat anti-rabbit IgG, was used at a 1 : 1000 dilution. Immunoreactive proteins were detected by autoradiography utilizing Enhancer ChemiLuminescence (Beyotime). The autoradiographs were scanned and quantified using Image Quant software (GE Healthcare, UK).

Statistical analysis. Data were evaluated using analysis of variance and Duncan's multiple range

Table 1. Primers for the real-time RT-PCR assay

Genes	GenBank Acc. No.	Primer sequences (5'-3')	Length (bp)	Annealing temperature (°C)
GAPDH	XM_001252479	F: GCGCTCTARATGTTCACCTTCC R: AGCATCACCCARACTTTATGTT	314	60
GPX1	NM_174076.3	F: AGTGCGAGGTGARATGGCGAGARA R: TGGGCARAARATCCCTGGAGAGCA	328	60
GPX4	NM_174770.3	F: ATCARAAGAGTTCGCCGCTGGCT R: TCGGARACACAGGCARACAGGCTT	295	60
TRXR1	NM_174625.3	F: AGGAGARAAGCTGTGGAGARAA R: TTATCCCTTGATGGARATCGT	94	60
SELP	NM_174459.3	F: CTTCATCACCACCACCACAG R: GAGGCARAACGTCACTGTCARA	331	60
CPLA2	NM_001075864.1	F: ATTGCCCGACTATCATTCAC R: ATGCTGTGGGTTTGCTTAG	351	60
5-LOX	NM_001192792.1	F: ACTGGCAGGARAGACCGCATGTT R: ACCTGGTTGAGCTGGATGGCARA	316	60
COX-2	NM_174445.2	F: TGTGCCTGATGACTGCCCARACA R: TCCTGGCCCACAGCARARACTTCA	408	60

GPX1 = glutathione peroxidase 1, *GPX4* = glutathione peroxidase 4, *TRXR1* = thioredoxin reductase 1, *SELP* = selenoprotein P, *CPLA2* = cytosolic phospholipase A2, *5-LOX* = 5-lipoxygenase, *COX-2* = cyclooxygenase-2, F = forward primer, R = reverse primer

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Table 2. Effect of retinoic acid (RA) on the cell viability in H_2O_2-induced bovine mammary epithelial cells

Item	Control	H_2O_2 group	RA group	RA + H_2O_2 group	SEM	*P*-value
RGR	1.000^{c}	0.765^{b}	1.416^{a}	1.212^{a}	0.0611	0.0009

H_2O_2 = hydrogen peroxide, SEM = standard error of the mean, RGR = relative growth rate
$^{a–c}$means within a row without the same superscripts differ significantly ($P < 0.05$)

test by SAS software (Statistical Analysis System, Version 9.0, 2003). Differences of $P < 0.05$ were considered significant, while differences of $0.05 < P < 0.10$ were regarded as a statistical trend.

RESULTS

Effects of RA on the viability of BMEC induced by H_2O_2. The effect of RA pre-treatment on the viability of H_2O_2-induced BMEC was evaluated by the MTT assay. As shown in Table 2, there was a significant improvement in the viability of cells treated with RA alone compared with the control ($P < 0.05$). After exposure to H_2O_2, the survival rate of BMEC decreased markedly compared with the control ($P < 0.05$). However, pre-incubation with RA (RA prevention group) dramatically increased the viability of H_2O_2-treated BMEC ($P < 0.05$).

Effect of RA on the antioxidant parameters of H_2O_2-induced BMEC. The addition of 1 mg/ml RA to the culture medium significantly up-regulated T-AOC as well as the activities of GPX, TRXR, CAT, and SOD compared with the control ($P < 0.05$) (Table 3). H_2O_2 treatment caused a remarkable decrease in the T-AOC and SELP contents as well as the GPX, TRXR, CAT, and SOD activities, but the intracellular MDA and ROS contents were found to be significantly increased compared with the control ($P < 0.05$). However, pre-incubation with RA noticeably attenuated the changes induced by H_2O_2 in BMEC, including the T-AOC and SELP contents, activities of TRXR, CAT, and SOD, and intracellular MDA and ROS contents ($P < 0.05$).

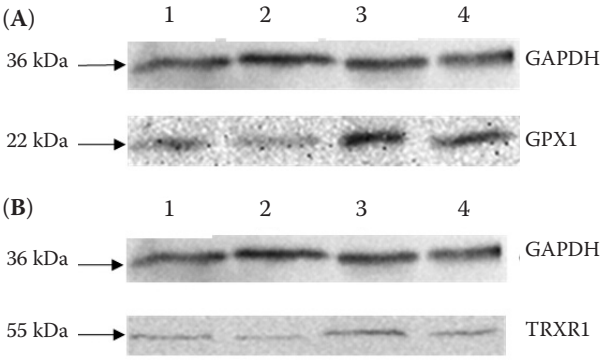

Figure 1. Effect of retinoic acid (RA) on GPX1 protein expression (**A**) and TRXR1 protein expression (**B**) in hydrogen peroxide (H_2O_2)-induced bovine mammary epithelial cells
1 = control group, 2 = H_2O_2 group, 3 = RA group, 4 = RA + H_2O_2 group

Table 3. Effect of retinoic acid (RA) on the antioxidant parameters in H_2O_2-induced bovine mammary epithelial cells

Item	Control	H_2O_2 group	RA group	RA + H_2O_2 group	SEM	*P*-value
CAT (IU/ml)	1.43^{b}	0.85^{c}	2.38^{a}	1.64^{b}	0.15	< 0.0001
SOD (IU/ml)	16.71^{b}	13.34^{c}	20.21^{a}	19.21^{ab}	0.86	0.0013
T-AOC (IU/ml)	1.68^{b}	1.09^{c}	2.22^{a}	1.83^{b}	0.10	< 0.0001
GPX (IU/mg prot)	135.47^{b}	72.68^{cd}	195.6^{a}	159.82^{ab}	17.21	0.0053
TRXR (U/g prot)	3.05^{bc}	1.94^{d}	4.49^{a}	3.31^{bc}	0.21	< 0.0001
SELP (mg/l)	0.34^{bcd}	0.16^{e}	0.47^{ab}	0.33^{bcd}	0.04	0.0051
MDA (nmol/mg prot)	1.74^{cd}	4.65^{b}	1.19^{d}	2.50^{cd}	0.49	0.0023
ROS (fluorescence intensity/ml)	133.39^{de}	187^{b}	115.52^{e}	137.86^{de}	6.98	< 0.0001

H_2O_2 = hydrogen peroxide, CAT = catalase, SOD = superoxide dismutase, T-AOC = total antioxidant capacity, GPX = glutathione peroxidase, TRXR = thioredoxin reductase, SELP = selenoprotein P, MDA = malondialdehyde, ROS = reactive oxygen species, prot = protein, SEM = standard error of the mean
$^{a–e}$means within a row without the same superscripts differ significantly ($P < 0.05$)

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Table 4. Effect of retinoic acid (RA) on the mRNA expression of selenoproteins in H_2O_2-induced bovine mammary epithelial cells ($2^{-\Delta\Delta Ct}$)

Item	Control	H_2O_2 group	RA group	RA + H_2O_2 group	SEM	*P*-value
GPX1	1.048^{c}	0.705^{d}	2.872^{a}	2.309^{b}	0.182	< 0.0001
GPX4	1.014^{a}	1.013^{a}	1.122^{a}	0.939^{a}	0.150	0.7285
TRXR1	1.023^{d}	0.655^{e}	1.965^{ab}	1.649^{bc}	0.124	< 0.0001
SELP	1.015^{b}	0.629^{cd}	1.672^{a}	1.181^{b}	0.077	< 0.0001

H_2O_2 = hydrogen peroxide, *GPX1* = glutathione peroxidase 1, *GPX4* = glutathione peroxidase 4, *TRXR1* = thioredoxin reductase 1, *SELP* = selenoprotein P, SEM = standard error of the mean

$^{a–e}$means within a row without the same superscripts differ significantly ($P < 0.05$)

Table 5. Effect of retinoic acid (RA) on the protein expression of selenoproteins in H_2O_2-induced bovine mammary epithelial cells

Item	Control	H_2O_2 group	RA group	RA + H_2O_2 group	SEM	*P*-value
GPX1	1.071^{c}	0.682^{d}	2.114^{a}	1.57^{b}	0.059	< 0.0001
TRXR1	1.126^{c}	0.613^{d}	1.938^{a}	1.29^{b}	0.049	< 0.0001

H_2O_2 = hydrogen peroxide, GPX1 = glutathione peroxidase 1, TRXR1 = thioredoxin reductase 1, SEM = standard error of the mean

$^{a–d}$means within a row without the same superscripts differ significantly ($P < 0.05$)

The results also showed that the addition of RA alone markedly increased T-AOC as well as the activities of TRXR and CAT compared with the RA + H_2O_2 group ($P < 0.05$).

Effect of RA on mRNA and protein expression of selenoproteins in H_2O_2-induced BMEC. As shown in Tables 4 and 5 as well as in Figure 1, the addition of RA alone caused a significant increase in mRNA expression of *GPX1*, *TRXR1,* and *SELP* as well as protein expression of GPX1 and TRXR1 compared to the control ($P < 0.05$). The H_2O_2 group significantly decreased the above parameters compared to the control ($P < 0.05$). However, the RA prevention group markedly inhibited the H_2O_2-

Table 6. Effect of retinoic acid (RA) on the content of arachidonic acid (ARA) and the parameters related to ARA metabolism in H_2O_2-induced bovine mammary epithelial cells

Item	Control	H_2O_2 group	RA group	RA + H_2O_2 group	SEM	*P*-value
ARA content (pmol/l)	2.04^{e}	3.329^{ab}	2.207^{e}	2.889^{d}	0.07	< 0.0001
CPLA2 activity (IU/ml)	78.60^{c}	101.25^{a}	82.20^{c}	91.20^{b}	2.48	< 0.0001
5-LOX activity (pg/ml)	187.57^{c}	229.48^{a}	199.24^{bc}	209.48^{b}	6.09	0.0008
COX-2 activity (ng/ml)	3.18^{a}	3.69^{a}	3.48^{a}	3.51^{a}	0.16	0.2782
LTB4 content (pg/ml)	60.58^{a}	68.1^{a}	62.65^{a}	61.21^{a}	2.37	0.1787
15-HETE content (ng/ml)	40.46^{c}	54.61^{a}	41.85^{c}	47.80^{b}	1.32	< 0.0001
15-HPETE content (ng/ml)	104.00^{c}	140.33^{a}	107.30^{c}	112.33^{b}	2.47	< 0.0001
PGE2 content (ng/l)	127.83^{a}	133.00^{a}	127.00^{a}	131.50^{a}	4.22	0.7226
CPLA2 mRNA expression	1.024^{d}	3.463^{a}	1.066^{d}	1.348^{d}	0.193	< 0.0001
5-LOX mRNA expression	1.018^{c}	2.931^{a}	1.418^{c}	1.925^{b}	0.131	< 0.0001
COX-2 mRNA expression	1.085^{a}	1.525^{a}	1.493^{a}	1.587^{a}	0.132	0.0688

H_2O_2 = hydrogen peroxide, *CPLA2* = cytosolic phospholipase A2, *5-LOX* = 5-lipoxygenase, *COX-2* = cyclooxygenase-2, ARA= arachidonic acid, PGE2 = prostaglandin E2, LTB4 = leukotriene B4, 15-HETE = 15-hydroxyeicosatetraenoic acid, 15-HPETE = 15-hydroxy peroxide tetracosenic arachidonic acid, SEM = standard error of the mean

$^{a–e}$means within a row without the same superscripts differ significantly ($P < 0.05$)

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Table 7. Effect of retinoic acid (RA) on the H_2O_2-induced phosphorylation level of mitogen-activated protein kinase

Item	Control	H_2O_2 group	RA group	RA + H_2O_2 group	SEM	*P*-value
p38 MAPK	0.335[b]	0.924[a]	0.295[b]	0.555[b]	0.083	0.001
JNK	0.481[b]	0.698[a]	0.475[b]	0.531[b]	0.049	0.038
ERK1/2	0.321[a]	0.421[a]	0.347[a]	0.341[a]	0.040	0.407

H_2O_2 = hydrogen peroxide, p38 MAPK = p38 mitogen-activated protein kinase, JNK = c-jun N-terminal kinase, ERK1/2 = extracellular signal-regulated kinase, SEM = standard error of the mean

[a, b]means within a row without the same superscripts differ significantly ($P < 0.05$)

induced decrease in the corresponding parameters ($P < 0.05$). In addition, the RA treatment led to slightly higher mRNA expression of *GPX1* and *SELP* as well as protein expression of GPX1 and TRXR1 than the RA + H_2O_2 treatment.

Inhibitory effect of RA on the ARA content and parameters related to ARA metabolism in H_2O_2-induced BMEC. As shown in Table 6, RA alone had no effect on the content of ARA nor on the parameters related to ARA metabolism ($P > 0.10$). The activities and mRNA expression of *CPLA2* and *5-LOX* as well as the amount of ARA, 15-HETE, and 15-HPETE were minimal in the control and were sharply increased after exposure to H_2O_2 ($P < 0.05$). Contrary to this result, pre-treatment with RA for 24 h markedly attenuated the H_2O_2-induced increase in these parameters ($P < 0.05$).

Effect of RA on the phosphorylation levels of p38 MAPK, JNK, and ERK1/2 in H_2O_2-induced BMEC. The RA-alone treatment did not affect the phosphorylation levels of p38 MAPK and JNK ($P > 0.10$) (Table 7). Stimulation of BMEC with H_2O_2 resulted in activation of p38 MAPK and JNK pathway signaling molecules ($P < 0.05$). By contrast, RA pre-treatment attenuated the H_2O_2-induced phosphorylation levels of p38 MAPK and JNK ($P < 0.05$). Phosphorylation of ERK1/2 was unaffected by either RA or H_2O_2 ($P > 0.10$).

DISCUSSION

Several selenoproteins, such as GPX, TRXR, and SELP, have antioxidant activities, play key roles in removing potentially damaging lipid hydroperoxides and hydrogen peroxides, and protect mammalian breast cells from oxidative stress-induced damage (Papp et al. 2007). However, little is known about the effects of RA on selenoproteins in BMEC, and considerable research has focused on studies of human breast cancer cells. The present research investigated the protective effects of RA on BMEC oxidative injury induced by H_2O_2 to explore the probable anti-oxidative mechanism of RA in the mammary gland. The results showed that addition of H_2O_2 alone to the BMEC culture, without RA, caused a remarkable depression of the GPX and TRXR activities, *SELP* and *TRXR1* expression, and SELP content compared to the control, but the intracellular ROS content had the opposite trend. The results also showed that pre-incubation with RA noticeably attenuated the changes in gene and protein expression, activities of the above selenoproteins, and intracellular contents of ROS in H_2O_2-treated BMEC. All of these results indicated that H_2O_2 could induce cell oxidative stress and depress the anti-oxidative function of cells as well as that RA prevention improved anti-oxidative function of BMEC, promoted production of selenoproteins, and protected BMEC from the oxidative injury induced by H_2O_2.

CPLA2 esterification of the sn-2 position of phospholipids is very important for hydrolytic cleavage and is responsible for the release of ARA from the phospholipid form to serve as a precursor of eicosanoids. In the normal physiological state, there are very low levels of ARA in the body, which maintain optimal growth and physiological activities (Li et al. 2012). However, excessive ARA from the LOX and COX pathways causes accumulation of prostaglandins, thromboxanes, leukotrienes, and peroxidised lipids, such as 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and 15-hydroperoxyeicosatetraenoic acid (15-HPETE) (Sordillo et al. 2008). Moreover, oxidative stress and peroxidation of membrane phospholipids have been shown to enhance CPLA2 activity and lead to the release of ARA in various cells (Balboa and Balsinde 2006).

The present study indicated that the activities and mRNA expression of *CPLA2* and *5-LOX* as well

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as the contents of ARA, 15-HETE, and 15-HPETE were sharply increased after exposure to H_2O_2 compared to the control. The current findings indicated that cells treated with H_2O_2 had higher CPLA2 activity and gene expression as well as higher concentrations in ARA and 15-HPETE compared to the control, and the results also show that stimulation of BMEC with H_2O_2 leads to activation of the p38 MAPK and JNK pathway signaling molecules, whereas pre-treatment with RA attenuates the changes of the above parameters. It was previously reported that H_2O_2 directly induces phosphorylation of MAPK, leading to activation of MAPK (Zhu et al. 2009). Phosphorylated MAPK transfers a phosphate group to CPLA2 by phosphotransferase, activating CPLA2, which then releases ARA. Therefore, the present research suggested that oxidative stress was associated with activation of the MAPK signaling pathway, which elevated the activity of CPLA2, increasing the release of ARA. Few results are available regarding the regulation of CPLA2 activity and ARA release by RA in BMEC. However, Hope et al. (1990) showed that retinoids inhibited CPLA2 activity in human synovial fluid and inhibited ARA release from rat peritoneal macrophages.

Soga et al. (2012) reported that apoptosis signal-regulating kinase-1 (ASK1), which belongs to a family of MAPK kinase kinases, activates downstream JNK and p38 MAPK. In our trial, the survival rate of BMEC decreased markedly when the BMEC was exposed to H_2O_2. In general, the reduced form of thioredoxin binds to the inactive region of ASK1 playing an important role in inhibiting ASK1 kinase activity. However, when the oxidized form of thioredoxin dissociates from ASK1 after exposure to ROS, ASK1 is activated by the autophosphorylation of Thr845 in its kinase domain (Tobiume et al. 2002). Then, MAPK is phosphorylated and activates CPLA2, resulting in the release of ARA. In our study, compared with the H_2O_2 group, the RA prevention group significantly increased TRXR activity, decreased the p38 MAPK and JNK phosphorylation levels as well as the ARA content. Taking all of these factors into consideration, RA-induced protection from H_2O_2-induced oxidative damage may be associated with lower ARA production resulting from increased TRXR activity. Kurosawa et al. (2009) suggested that TRXR inhibited the activity of CPLA2 and release of ARA as well as prevented toxic effects in L929 mouse fibrosarcoma cells. Kelavkar et al. (2001) indicated that TRXR could directly reduce the lipid hydroperoxide 15-HPETE and potentially limit its accumulation in cells that express 15-LOX. In addition, 15-HPETE was previously shown to inhibit the activity of mammalian TRXR1 (Yu et al. 2004). Therefore, we can infer that the protective effect of RA against oxidative damage is likely related to TRXR regulation of the release of ARA. Randall et al. (2013) found that siRNA silencing of TRXR1 significantly induced activation of p38 MAPK and JNK in human bronchial epithelial cells. However, the present research did not detect the effects of inhibiting TRXR1 on the activation of p38 MAPK and JNK by using siRNA silencing, so more detailed mechanisms need to be further explored.

Some studies have shown that GPX is capable of inhibiting the activity of LOX enzymes (Conrad et al. 2007). Specifically, intracellular reduced glutathione protected endothelial cells against 15-HPETE-induced cell injury and stimulated the conversion of 15-HPETE to 15-HETE. Rock and Moos (2010) have reported that SELP can decrease peroxidized lipid production derived from 15-HPETE in human embryonic kidney cells. Our present results show that pre-treatment with RA improved the GPX activity and SELP content, which were accompanied by down-regulation of the parameters that are involved in ARA metabolism, such as the CPLA2 and 5-LOX activities as well as the 15-HPETE content, compared with the H_2O_2 group. These results implied that RA protected BMEC from oxidative stress by regulation of the GPX activity and SELP content, which caused a decrease in the concentration of ARA. Moreover, a large amount of NO could also result in oxidative injury. Ferret et al. (2000) showed that TRXR may have important preventative capacities against NO-mediated cellular injury in human monocytic macrophage cells. Therefore, the effect of RA on antioxidant function may be that elevating TRXR activity inhibited the concentration of NO. However, little relevant information is available for BMEC, so further investigation is required to examine the exact mechanism.

CONCLUSION

Our data provided a possible mechanism for how RA protects BMEC from oxidative stress. By up-regulating TRXR activity and down-regulating

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phosphorylation of p38 MAPK and JNK, RA caused a decrease in the concentration of ARA. Further investigation is required to examine the exact mechanism by which RA attenuates oxidative injury in BMEC.

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