Selenomethionine increases proliferation and reduces apoptosis in bovine mammary epithelial cells under oxidative stress

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ABSTRACT

The decline in mammary epithelial cell number as lactation progresses may be due, in part, to oxidative stress. Selenium is an integral component of several antioxidant enzymes. The present study was conducted to examine the effect of oxidative stress and selenomethionine (SeMet) on morphology, viability, apoptosis, and proliferation of bovine mammary epithelial cells (BMEC) in primary culture. Cells were isolated from mammary glands of lactating dairy cows and grown for 3 d in a low-serum gel system containing lactogenic hormones and 0 or 100 μ M H_2O_2 with 0, 10, 20, or 50 nM SeMet. Hydrogen peroxide stress increased intracellular H_2O_2 to 3 times control concentrations and induced a loss of cuboidal morphology, cell–cell contact, and viability of BMEC by 25%. Apoptotic cell number more than doubled during oxidative stress, but proliferating cell number was not affected. Supplementation with SeMet increased glutathione peroxidase activity 2-fold and restored intracellular H_2O_2 to control levels with a concomitant return of morphology and viability to normal. Apoptotic BMEC number was decreased 76% below control levels by SeMet and proliferating cell number was increased 4.2-fold. These findings suggest that SeMet modulated apoptosis and proliferation independently of a selenoprotein-mediated reduction of H_2O_2 . In conclusion, SeMet supplementation protects BMEC from H_2O_2 -induced apoptosis and increased proliferation and cell viability under conditions of oxidative stress.

Key words: mammary epithelial cell, selenomethionine, apoptosis, cell proliferation

INTRODUCTION

Selenium (Se) has a pivotal role in the synthesis and activity of several antioxidant enzymes, particularly

those of the glutathione peroxidase (GPx) superfamily. The best known biological function of this selenoenzyme family is the reduction of hydrogen peroxide (H_2O_2) and organic peroxides into water or their respective alcohols using glutathione as a reductant. Numerous degenerative diseases in different species are associated with the disruption of this antioxidant mechanism. In dairy cows, retained fetal membranes (Brezinska-Slebozinska et al., 1994), white muscle disease, and increased risk of mastitis (Kincaid, 1995) are linked to plasma Se deficiency and low GPx activity. Although evidence associating Se and GPx activity with mammary health in dairy animals was first detailed more than 2 decades ago (Smith et al., 1984), it is only now becoming clear that milk production in dairy cows is positively related to Se supplementation in areas where soil Se concentration is low (Wang et al., 2009). Nevertheless, the mechanism by which these Se supplements enhance milk yield is unclear.

The decline in daily milk yield as lactation progresses is the consequence of a rate of cell death that is faster than cell renewal by proliferation and differentiation (Capuco et al., 2001). Oxidative damage to secretory cells due to their high biosynthetic activity was proposed as contributing to the lactational decline of secretory cell numbers and milk yield (Hadsell et al., 2007). Reactive oxygen species (ROS), including H_2O_2 , damage biomolecules such as DNA, causing DNA fragmentation and cell death (Burdon, 1995). Indeed, a significant increase in apoptotic cell number was detected after human breast adenocarcinoma cells (MCF-7) were treated with 150 μ M H_2O_2 (Dasari et al., 2006). In addition, morphological modifications and decreased proliferation of cells exposed to 10 to 200 μ M H_2O_2 have been documented (Burdon, 1995; Mori et al., 2004), although not in bovine mammary epithelial cells (BMEC).

The role of selenoproteins in removal of ROS provides a hypothetical link between Se status and secretory cell number. We previously showed that GPx expression and activity, and number and viability of BMEC in culture, were improved by the addition of 10 to 50 nM

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selenomethionine (**SeMet**) to the media (Miranda et al., 2009). Whether changes in cell proliferation or death were responsible for the stimulatory effect of Se on BMEC number remains unknown. In other cell lines, addition of nanomolar concentrations of Se to the culture media decreased basal and H_2O_2 -stimulated apoptosis (Yoon et al., 2002; Yeo and Kang, 2007). Effects of low concentrations of Se on proliferation have been studied less; however, it has been reported that 250 nM Se stimulated division of leukemia cells in vitro (Zeng, 2002). These results suggest that cellular antioxidant status may modulate apoptosis and proliferation of BMEC under oxidative stress. In this context, an in vitro model of oxidative stress was employed in which H_2O_2 was added directly to determine (1) the effect of oxidative stress on BMEC morphology, viability, apoptosis, and proliferation; (2) the effect of SeMet on H_2O_2 -stressed BMEC; and (3) whether proliferation or apoptosis is modulated by SeMet.

MATERIALS AND METHODS

Chemicals, Materials, and Antibodies

Collagenase type III from *Clostridium histolyticum* (400 U/mL) and hyaluronidase (200 U/mL) were obtained from Worthington Biochemicals (Freehold, NJ). Colloidal silica (Percoll) was purchased from Pharmacia Fine Chemical (Piscataway, NY) and fetal bovine serum (**FBS**) was acquired from PAA Laboratories Inc. (Etobicoke, Ontario, Canada). An ApopTag kit was obtained from Chemicon (Teluca, CA) and the 5-bromo-2'-deoxyuridine (**BrdU**) cell proliferation assay kit was purchased from Roche Diagnostics (Laval, Quebec, Canada). CellTiter 96 commercial kit was acquired from Promega (Madison, WI) and a GPx commercial kit from Cayman Chemical (Ann Arbor, MI). Bicinchoninic acid assay was obtained from Pierce (Rockford, IL) and 2'-7'-dihydrodichlorofluorescein diacetate (**DCFH-DA**) from Molecular Probes Inc. (Eugene, OR). All other chemicals were purchased from Sigma Chemical Co. (Oakville, Ontario, Canada).

Collagen-coated culture slides (2-chamber), 24-well culture plates, 35-mm and 100-mm culture dishes coated with collagen type I from rat tail tendon were purchased from BD Biosciences BioCoat (Bedford, MA), and 96-well microplates from Costar (Cambridge, MA). The anti-cytokeratin AE1/AE3 antibody was purchased from Dako (Mississauga, Ontario, Canada).

BMEC Isolation and Culture

Epithelial cells were isolated as described previously (Miranda et al., 2009) from healthy mammary glands

of 8 multiparous, lactating Holstein cows at 97 ± 12 DIM, producing 30.0 ± 5.0 kg milk/d, culled due to locomotor disorders and reproductive failure from the Elora Dairy Research Centre herd (Elora, Ontario, Canada), and slaughtered at the University of Guelph Meat Laboratory (Guelph, Ontario, Canada). Briefly, minced mammary tissue was digested with collagenase, hyaluronidase, and DNase, and cells were separated by serial filtration. Cells recovered on a Percoll density gradient were 98% immunopositive to anti-cytokeratin AE1/AE3 antibody (1:100), which verified their epithelial nature. Cells were cultured in media containing 45% RPMI-1640, 45% Dulbecco's modified Eagle's medium, 5% FBS, 2% antibiotic-antimycotic solution, 1 mM sodium pyruvate, 2 mM L-glutamine, 40 mM HEPES buffer, bovine insulin (5 $\mu\text{g}/\text{mL}$), hydrocortisone (1 $\mu\text{g}/\text{mL}$), sheep prolactin (1 $\mu\text{g}/\text{mL}$), murine epidermal growth factor (5 ng/mL), and bovine transferrin (10 $\mu\text{g}/\text{mL}$). Cell counting was performed in an improved Neubauer chamber (Fisher Scientific, Whitby, Ontario, Canada) and the proportion of viable cells was 95% by trypan blue dye (0.1% wt/vol) exclusion.

Oxidative Stress Model

To determine the concentration of H_2O_2 that would produce cell death rates reported in vivo (Wilde et al., 1997), BMEC were plated at $3 \times 10^4/\text{cm}^2$ in 24-well culture plates coated with collagen type I from rat tail tendon and grown to semiconfluent monolayers in a humidified environment (5% CO_2 at 37.5°C). Cells were exposed to 0, 50, 100, 200, 500, or 1,000 μM H_2O_2 for 24 h. Cells were harvested by trypsinization and counted in a hemocytometer chamber under an inverted light microscope (Leica Microsystems, Heidelberg, Germany) after diluting 1:2 with 0.1% trypan blue in PBS (Liu and Janeway, 1990). Stained cells were considered dead. The H_2O_2 dose of 100 μM produced 2.7% dead cells (Figure 1) and was selected for subsequent oxidative stress experiments.

Cell Morphological Analysis

Epithelial cells were seeded at an approximate density of 1.0×10^5 into 35-mm tissue culture dishes coated with collagen type I from rat tail tendon, and allowed to attach and spread into monolayers over 48 h of incubation in a humidified 37.5°C , 5% CO_2 atmosphere. Cells were then treated with 0 or 100 μM H_2O_2 with each of 4 levels of SeMet (0, 10, 20, or 50 nM) for 3 d. Every 24 h, 3 dishes on each treatment were carefully rinsed with ice-cold PBS (pH 7.5) and immediately observed under an inverted light microscope with phase contrast (Leica Microsystems) and photographed using a CoolSNAP

Figure 1. Induction of cell death in primary bovine mammary epithelial cells examined by trypan blue dye exclusion assay. Mammary cells were treated with 50 to 1,000 μM H_2O_2 for 24 h. Data are expressed as mean $\pm$ SEM of 3 independent experiments.

camera (RS Photometrics, Tucson, AZ). Subsequently, monolayers were harvested by trypsinization and collected by centrifugation at 100 g for 5 min. Cell pellets were washed twice in PBS supplemented with FBS and then fixed in 2% paraformaldehyde and 2.5% glutaraldehyde for 24 h at 4°C. Samples were dehydrated in a graded series of ethanol (50%, 70%, 90% and 100% vol/vol) and infiltrated in LR-White resin (Polysciences, Warrington, PA). Serial sections of polymerized blocks were cut with a Leica/Reichert-Jung Ultracut ultramicrotome (Vienna, Austria), and stained with uranyl acetate and lead citrate. The thin-sections were examined on a Philips CM10 transmission electron microscope (TEM; Philips Electron Optics, Eindhoven, Netherlands) at an accelerating voltage of 80 kV under standard operating conditions. All observations illustrated in the present paper were based on interpretations of at least 5 thin sections from each treatment group.

Cell Death Assay

Dead adherent cells were determined by terminal dUTP nick end-labeling (TUNEL) assays using the ApopTag kit. The BMEC were grown in 2-chamber, collagen-coated culture slides at an approximate density of 4×10^5 cells/chamber. Incubation times and conditions were as described above. After initiation of SeMet and H_2O_2 treatment, cells were fixed in 1% paraformaldehyde every 24 h for 3 d. New 3'-OH ends of double- or single-stranded DNA produced by fragmentation were detected using terminal deoxynucleotidyl transferase enzyme, which chemically labels DNA fragments with a fluorescein nucleotide. Nuclei were counterstained with 1 $\mu\text{g}/\text{mL}$ propidium iodide. Cells were visualized in a Leica TCS SP5 multiphoton laser-scanning confocal

system (Leica Microsystems) fitted with a Leica DM 6000B microscope and those that showed greenish yellow fluorescence in the nucleus were taken as TUNEL-positive. At least 1,000 nuclei were counted for each treatment in 3 independent experiments. Cell death was presented as the percentage of total cells identified as TUNEL-positive.

Cell Proliferation Assay

Synthesis of DNA was measured with a BrdU cell proliferation assay kit according to the manufacturer's instructions. The BMEC were plated at 4×10^5 cells/well in 2-chamber collagen-coated culture slides, cultured for 48 h, and treated with the indicated doses of SeMet and H_2O_2 for 72 h. Each 24 h posttreatment, epithelial cells were labeled with BrdU at 37.5°C and 5% CO_2 for 60 min and then reacted with mouse antibody against BrdU. Unbound antibody was removed by washing, and anti-mouse IgG-alkaline phosphatase was added. Antibody banding complexes were visualized by reacting nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate under an inverted light microscope with phase contrast (Leica Microsystems) using the 20 $\times$ objective. At least 1,000 cells in random fields of view were counted for each treatment in 3 independent experiments to quantify the percentage of cells exhibiting nuclear BrdU incorporation.

Cell Viability Assay

Cell viability under H_2O_2 stress was estimated using the CellTiter 96 commercial kit. This kit quantified the cellular conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt (MTT) into a purple formazan product. Forty-eight hours after seeding cells into 96-well plates at 2×10^4 cells/well in 100 μL media containing 0 μM H_2O_2 , or different levels of SeMet (0, 10, 20 and 50 nM) with 100 μM H_2O_2 , was added for continued incubation at 37.5°C in 5% CO_2 . At 24, 48, and 72 h posttreatment, MTT solution (0.5 mg/mL) was added to each well and incubated for 4 h at 37.5°C before adding solubilization/stop solution. The solubilized formazan product was measured by spectrophotometry (PowerWave X340, Bio-Tek Instruments Inc., Winooski, VT) at 570 nm. A standard curve of cell number against absorbance was used to identify the linear portion of the curve. All cell viability assays were carried out in triplicate and repeated 3 times.

Measurement of Intracellular GPx Activity in BMEC

The BMEC were plated (2×10^6 cells) on 100-mm dishes culture coated with collagen type I from rat

tail tendon and grown to a semiconfluent monolayer in a humidified environment (5% CO₂ at 37.5°C) for 48 h. Cells were then treated with H₂O₂ and SeMet as described earlier. Every 24 h, cells were harvested by mechanical scraping and lysed in 500 µL of cold RIPA lysis buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1.0% Triton X-100, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 2 mM sodium vanadate, 2.1 µg/mL aprotinin) supplemented with 15 µL of protease inhibitor cocktail (104 µM 4-(2-aminoethyl) benzene-sulfonyl fluoride, 0.08 µM aprotinin, 2 µM leupeptin, 4 µM bestatin, 1.5 µM pepstatin A, and 1.4 µM E-64) on ice for 15 min. The remaining homogenates were centrifuged (12,000 × g, 4°C, 40 min) to remove cellular debris, after which absolute GPx activity recovered in the supernatant was estimated by GPx commercial kit, according to Paglia and Valentine (1967). Activity of GPx was normalized to total cell protein quantified by bicinchoninic acid assay.

Detection of Intracellular Hydrogen Peroxide

Intracellular H₂O₂ concentration was measured from the oxidation of 2'-7'-dihydrodichlorofluorescein diacetate to the fluorescent compound 2'-7'-dichlorofluorescein (DCF) in a spectrofluorometer as described previously (Grisham, 2008). The BMEC (2 × 10⁶) were cultured and treated with SeMet and H₂O₂ as indicated above for 72 h in 100-mm dishes coated with collagen type I from rat tail tendon. Every 24 h, monolayers were washed 3 times with PBS (pH 7.5). Epithelial cells were incubated with serum-free media containing 30 µM DCFH-DA for 90 min at 37°C in dark conditions. Postincubation, BMEC were washed 3 times with PBS and lysed in 0.5% (wt/vol) SDS solution. The 100 µL of cell lysate was transferred to flat-bottom, 96-well microplates and analyzed in a microplate reader (Wallac Victor 1420 Multilaber Counter, Perkin-Elmer, Wellesley, MA) with excitation and emission wavelengths set at 485 and 530 nm, respectively. After subtracting fluorescence of a blank cell suspension without DCFH-DA, fluorescence was expressed per milligram of protein.

Statistical Analysis

All experiments were carried out in triplicate and repeated independently 3 times, and numerical data are expressed as least squares means ± SEM. A mixed model in SAS (Windows version 9.1.3, SAS Institute Inc., Cary, NC) with repeated measures over time was used to analyze the effects of H₂O₂ (100 µM) and 4 levels of SeMet (0, 10, 20, or 50 nM) on BMEC apoptosis,

viability, proliferation, GPx activity and intracellular level of H₂O₂ during 3 d with the following model:

$$Y_{ijk} = \mu + M_i + T_j + MT_{ij} + \text{error}_{k(ij)},$$

where μ = overall mean, M_i = fixed effect of treatment ($i = 1$ to 5), T_j = repeated time effect ($j = 1$ to 3), MT_{ik} = treatment by time interaction effect, and $\text{error}_{k(ij)}$ = experimental error ($k = 1$ to 3). Covariance was fitted with a compound symmetry structure (Kaps and Lamberson, 2004). Differences among treatment means at each time point were evaluated using the pdiff option of SAS (SAS Institute Inc.). Significance was declared at $P < 0.05$.

RESULTS

Effect of SeMet and H₂O₂ on Intracellular H₂O₂ Levels in BMEC

The 100 µM H₂O₂ treatment without SeMet increased H₂O₂ concentrations inside BMEC to 2 and 3 times control concentrations ($P < 0.001$), according to DCFH-DA oxidation results (Figure 2). All concentrations of SeMet reduced intracellular H₂O₂ to control concentrations except at 72 h, when 10 nM SeMet only caused half of the H₂O₂ decrease ($P < 0.001$).

Effect of SeMet on GPx Activity in BMEC Under H₂O₂ Treatment

At 24, 48, and 72 h, absolute GPx activity was 3 times higher ($P < 0.001$) in secretory cells treated simultaneously with H₂O₂ and SeMet than in cells treated only with 0 or 100 µM H₂O₂ (Figure 3). Although no effect of incubation time on GPx activity was observed during the first 48 h, secretory cells exposed to H₂O₂ with 10 nM SeMet exhibited a decrease in the activity of this selenoprotein by 72 h of incubation. Absolute GPx activity was 1.5-fold and 1.6-fold higher ($P < 0.05$) in cells cultured with 20 and 50 nM SeMet, respectively, than in cells cultured with 10 nM SeMet at 72 h. No effect of H₂O₂ treatment on GPx activity was observed at 24 and 72 h of incubation ($P > 0.41$); however, GPx activity was reduced 33% by H₂O₂ at 48 h ($P < 0.05$).

Influence of SeMet on Cell Morphology in BMEC Exposed to Subcytotoxic Level of H₂O₂

Before treatment, BMEC were flat and homogeneous in size and appearance, with morphology in culture typical of epithelial cells (cobblestone conformation). These characteristics changed after 48 h of treatment.

Figure 2. Selenomethionine (SeMet) decreased intracellular levels of H_2O_2 in primary bovine mammary epithelial cells during oxidative stress. Results are means $\pm$ SEM of 3 independent experiments. Means within each period with different letters are different ($P < 0.05$).

Secretory cells treated with a sublethal level of H_2O_2 without SeMet gradually became enlarged and elongated compared with untreated cells (data not shown). These changes were more marked after 72 h, where cells lost their cuboidal morphology and exhibited large vacuoles in the cytoplasm (Figure 4A). Additionally, zones devoid of cells were observed, which suggests disruption of the integrity of cell-to-cell contacts (Figure 4A). In contrast, when BMEC were exposed to SeMet in addition to H_2O_2 , no morphologic changes were detected at 72 h (Figure 4B). The control group showed few unhealthy cells with cell elongation (Figure 4C).

Ultrastructural features were examined by transmission electron microscopy at 72 h of treatment. Normal cells were observed in all treatments, displaying lipid droplets in their cytoplasm, a clear nucleus and intact specialized surface structures such as microvilli (Figure 5A). Apoptotic cells were detected across treatments,

Figure 3. Effect of selenomethionine (SeMet) on glutathione peroxidase activity (GPx) of primary bovine mammary epithelial cells under H_2O_2 stress. Data are expressed as means $\pm$ SEM of 3 independent experiments. Means within each period with different letters are different ($P < 0.05$).

showing margination and condensation of nuclear chromatin into more densely packed material along the nuclear membrane, intact plasma membrane, with prominent cytoplasmic vacuoles and loss of microvilli (Figure 5B).

Effect of SeMet on DNA Fragmentation in BMEC Under H_2O_2 Treatment

Fragmentation of DNA is a well-characterized biochemical marker of cell death. Exposure of BMEC to 100 μM H_2O_2 without SeMet resulted in an increase ($P < 0.001$) in the proportion of TUNEL-positive cells at all time points (Figure 6). Over 72 h of incubation, TUNEL-positive cell percentage increased in a time-dependent manner ($P < 0.001$) in all treatments, but the increase was greatest at 129% with 0 nM SeMet + 100 μM H_2O_2 compared with 39% on the control. When SeMet-containing medium was added, a reduction in TUNEL-positive cells was detected at each time point ($P < 0.001$). Maximal protection was achieved at 24 h. The decrease in TUNEL-positive cells was 76% for 20 and 50 nM SeMet, and 41% for 10 nM SeMet compared with 0 nM SeMet + 100 μM H_2O_2 .

Effect of SeMet on Cell Proliferation in BMEC Under H_2O_2 Treatment

According to the BrdU incorporation assay (Figure 7), no effect of H_2O_2 stress on numbers of cells with new nuclear DNA synthesis was found compared with control ($P > 0.38$). In contrast, SeMet at all doses increased proliferating cell number compared with 0 and 100 μM H_2O_2 treatments ($P < 0.001$), and to a greater extent as incubation time increased ($P < 0.001$). At 72 h, proliferating cell number was 4.2-fold higher on all SeMet treatments.

Effect of SeMet on Viable Cell Number in BMEC Under H_2O_2 Treatment

After 72 h of incubation, 100 μM H_2O_2 reduced the number of viable BMEC by 25% ($P < 0.001$) compared with the control (Figure 8). In contrast, addition of SeMet to media increased ($P < 0.001$) viable cells throughout the experimental period in a time- and dose-dependent manner. Secretory cells cultured with both 20 and 50 nM SeMet exhibited higher viable numbers ($P < 0.001$) than those grown with 10 nM SeMet.

DISCUSSION

H_2O_2 Stress Changes Morphology, Increases Apoptosis, and Has No Effect on Proliferation of BMEC

To study the antioxidant role of SeMet in BMEC dynamics, an oxidative stress model was used where

Figure 4. Representative phase-contrast photomicrographs of living bovine mammary epithelial cells illustrating the effect of selenomethionine (SeMet) on morphological changes under H_2O_2 stress. A) Mammary cells exposed to $100 \mu M H_2O_2$ after 72 h became enlarged, elongated, lost their cuboidal shape, with enlarged cytoplasmic vacuoles (arrows) and disrupted cell-cell contacts (arrowheads); B) cells under H_2O_2 stress treated with $50 nM$ SeMet for 72 h without morphological changes; C) cells grown without H_2O_2 or SeMet after 72 h, revealing few unhealthy cells with elongated shape (arrows). Bar = $50 \mu m$. Color version available in the online PDF.

Figure 5. Transmission electron micrographs of cultured bovine mammary epithelial cells. A) A healthy cell displaying lipid droplets (LD), a clear nucleus (Nu) and intact plasma membrane (PM) with microvilli (Mi) were seen in the 5 treatments, and B) an apoptotic cell exhibiting marginal condensation of nuclear chromatin (arrows), intact PM, and a loss of Mi and LD were detected in all treatments.

$100 \mu M H_2O_2$ was added to BMEC culture media. The H_2O_2 -induced enlargement and elongation of cells that were observed over 3 d was reported previously in mouse mammary epithelial cells (Mori et al., 2004), as was the loosening of gap junctions and reorientation of the cytoskeleton (Alexandrova et al., 2006). Prolonged exposure to H_2O_2 may result in malignant transformation of epithelial cells (Mori et al., 2004), but the BMEC under oxidative stress for 72 h retained their epithelial phenotype as evidenced by detection of cytokeratins.

The MTT assay indicated the number of viable cells in a sample (Denizot and Lang, 1986). The decrease in MTT index with H_2O_2 stress appears to be caused by increased apoptosis alone, because the proportion of TUNEL-positive cells increased and proliferating cell number did not change. Electron microscopy revealed morphological features in unhealthy cells typi-

Figure 6. Inhibitory effect of selenomethionine (SeMet) on apoptosis in primary bovine mammary epithelial cells under H_2O_2 stress. Results are means $\pm$ SEM from 1,000 nuclei for each treatment in 3 independent experiments. Means within each period with different letters are different ($P < 0.05$). TUNEL = terminal dUTP nick end-labeling.

cal of apoptosis, suggesting that DNA fragmentation detected in the TUNEL assay was due to apoptosis, as opposed to other types of cell death (such as necrosis) in which DNA fragmentation also occurs. Apoptotic features observed included margination and condensation of chromatin, as well as loss of microvilli and plasma membranes remained intact (Krysko et al., 2008). There was no evidence of necrosis such as swollen mitochondria and loss of plasma membrane integrity in unhealthy cells. In several cell types extracellular H_2O_2 concentrations up to $200 \mu\text{M}$ induce apoptosis, but concentrations greater than 1 mM induce death by necrosis (Creagh et al., 2000).

Figure 7. Stimulatory effect of selenomethionine (SeMet) on proliferation of primary bovine mammary epithelial cells (BMEC) under H_2O_2 stress. The number of BMEC with BrdU incorporation were counted and related to the total BMEC number by percentage. Data are expressed as means $\pm$ SEM of 3 independent experiments. Means within each period with different letters are different ($P < 0.05$). BrdU = 5-bromo-2'-deoxyuridine.

Some cell types respond to $\text{H}_2\text{O}_2 < 200 \mu\text{M}$ with an increase in proliferation (Rao and Berk, 1992; Sigaud et al., 2005). Other cell types respond by decreasing proliferation rate (Rao and Berk, 1992). Hydrogen peroxide appears to act as a second messenger for some of the proliferative effects of growth factors (Burdon, 1995), whereas the mechanism of antiproliferative effects is not well characterized. No effect of $100 \mu\text{M}$ H_2O_2 was observed on BMEC proliferation, supporting the lack of effect seen on myofibroblast proliferation (Waghray et al., 2005).

SeMet Increases GPx Activity and Reverses ROS Effects

We have previously shown that 10 to 50 nM SeMet in the incubation medium increased GPx expression and activity in primary cultures of BMEC (Miranda et al., 2009). In this experiment, these increases in GPx activity (when extracellular H_2O_2 is $100 \mu\text{M}$) were associated with a return of intracellular H_2O_2 concentrations to the baseline level at $0 \mu\text{M}$ extracellular H_2O_2 . Apparently, GPx levels at 0 nM SeMet were not sufficient to handle the peroxide load imposed by $100 \mu\text{M}$ H_2O_2 . Further support for this conclusion comes from the 10 nM SeMet concentration at 72 h , where a decrease in GPx activity coincided with an increase in H_2O_2 fluorescence.

Reduction of intracellular H_2O_2 to baseline by GPx appeared to reverse the effect of extracellular H_2O_2 on BMEC morphology, viable cell number, and apoptosis, as all 3 of these variables returned at least to control levels. Similarly, Se reversed the increase in apoptosis and decrease in cell number induced by up to $250 \mu\text{M}$ H_2O_2 in normal and cancerous cells (Yoon et al., 2002;

Figure 8. Effect of selenomethionine (SeMet) on viable bovine mammary epithelial cell number under H_2O_2 stress. Data are expressed as means $\pm$ SEM of 3 independent experiments. Means within each period with different letters are different ($P < 0.05$).

Yeo and Kang, 2007). Therefore, the effects of Se on BMEC survival can be explained, in part, by its anti-oxidative functions.

SeMet Affects Apoptosis and Proliferation Independent of Oxidative Status

Selenomethionine caused intracellular H_2O_2 concentrations to return to the control level, but apoptotic cell number decreased to below control level. Likewise, proliferating cell number was not affected by H_2O_2 , but was increased by SeMet. Together these results indicated that SeMet affected apoptosis and proliferation independently of a GPx-mediated decrease in intracellular H_2O_2 . Yoon et al. (2002) found a stimulation of HT1080 cell population growth to higher than control levels when 2 μM Se was added to media containing 100 μM H_2O_2 . Even without exogenous H_2O_2 exposure, SeMet up to 50 nM accelerated BMEC population growth over 5 d of culture (Miranda et al., 2009). The anti-apoptotic effects of Se, independent of oxidative stress, have been attributed to activation of the insulin signaling kinase Akt (Yoon et al., 2002).

CONCLUSIONS

Oxidative stress in BMEC increased the number of apoptotic cells, decreased cell viability, and perturbed cell morphology, without affecting cell proliferation. Selenomethionine was able to reverse these negative effects of H_2O_2 by increasing absolute GPx activity and reducing intracellular levels of H_2O_2 . Furthermore, SeMet decreased the number of apoptotic BMEC and increased proliferating cell number independent of selenoprotein-mediated reductions in H_2O_2 . The turnover of mammary epithelial cells is a major determinant of the slope of the lactation curve after peak lactation. If the reduction in apoptosis and increase in proliferation brought about by SeMet in BMEC under oxidative stress in vitro can be recapitulated in vivo, it could enhance lactation persistency in dairy cows.

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