Selenomethionine stimulates expression of glutathione peroxidase 1 and 3 and growth of bovine mammary epithelial cells in primary culture

S. G. Miranda,*† Y. J. Wang,† N. G. Purdie,† V. R. Osborne,† B. L. Coomber,‡ and J. P. Cant†¹

*Department of Animal Science, University of Zulia, Maracaibo, Venezuela 4005

†Department of Animal and Poultry Science, and

‡Department of Biomedical Sciences, University of Guelph, Ontario, N1G 2W1, Canada

ABSTRACT

This study examined the localization of cellular glutathione peroxidase (GPx1) and extracellular glutathione peroxidase (GPx3) in lactating mammary tissue and in primary cultures of bovine mammary epithelial cells (BMEC). The effect of selenium as selenomethionine (SeMet) on the growth and viability of BMEC and GPx protein expression and activity were also studied. Single mammary epithelial cells were recovered by serial collagenase/hyaluronidase digestion from lactating bovine mammary tissue and cultured in a low-serum collagen gel system enriched with lactogenic hormones and 0, 10, 20, or 50 nM SeMet. Positive immunostaining with anti-cytokeratin and bovine anti-casein confirmed the epithelial nature and differentiated state of BMEC. Addition of SeMet to media facilitated rapid confluence of BMEC and formation of dome structures. Immunohistochemical and immunocytochemical staining revealed that both GPx1 and GPx3 are synthesized by BMEC and localized in the cytoplasm and nucleus. Up to 50 nM SeMet linearly increased BMEC number and viability over 5 d of culture. Bovine mammary epithelial cells cultured in SeMet-supplemented medium also exhibited markedly elevated GPx activity and linear increases in abundance of GPx1 and GPx3 proteins. It is apparent that SeMet degradation to release Se for synthesis of selenoproteins is carried out by BMEC. Results indicate that bovine mammary epithelial cells express GPx1 and GPx3 *in vivo* and *in vitro*; SeMet enhances expression of these selenoproteins *in vitro* and the growth and viability of BMEC.

Key words: bovine, mammary epithelial cell, selenomethionine, glutathione peroxidase

INTRODUCTION

Selenium is an essential mineral for animal nutrition. Its biological functions are associated with the

Se-containing proteins, which possess selenocysteine residues at the active site. In mammals, there are 19 selenoproteins with known functions and all of them are enzymes (Behne and Kyriakopoulos, 2001). The glutathione peroxidase (GPx) family of selenoproteins catalyzes the reduction of lipid and hydrogen peroxides to lipid alcohols and water, respectively, with glutathione as a reductant. The GPx family is an essential component of the mammalian antioxidative system protecting membrane lipids and macromolecules from oxidative damage (Arthur, 2000). Both cellular GPx (GPx1) and extracellular GPx (GPx3) are expressed at the mRNA and protein level in liver, heart, placenta, gastrointestinal tract, thyroid, kidney, and red blood cells in many species including human, rat, and mouse (Chu et al., 1992; Arthur, 2000). Recently, GPx activity and mRNA for GPx1, GPx3, and GPx4 were detected in bovine mammary tissue (Bruzeliuss et al., 2007). In MAC-T cells cultured in 25 nM selenite, GPx1 was by far the predominant selenoprotein, whereas GPx3 could not be distinguished from other GPx (Bruzeliuss et al., 2008). The final Se-containing member of the family, GPx2, is primarily a gastrointestinal enzyme, and abundance of the protein in mammary tissue is very low (Naiki-Ito et al., 2007).

Oxidative damage of mammary epithelial cells has been proposed as one mechanism to explain the decline of milk production as lactation progresses (Hadsell et al., 2002). Reactive oxygen species (ROS) play an important role in signaling initiation of apoptosis in cells (Tang et al., 2008) and GPx removes ROS. Thus, we hypothesized that improvement of GPx status of mammary epithelial cells would improve their survival in culture. Selenium status can modulate GPx expression and activity (Saedi et al., 1988; Zeng et al., 2008) so that GPx activity is often used to assay Se availability from foods (Finley, 2006). In dairy cows, plasma GPx activity was correlated positively to Se intake (Juniper et al., 2008). A key factor in this effect is the synthesis of selenocysteine on its specific transfer RNA for incorporation into the polypeptide chain of GPx during translation (Allmann and Krol, 2006). It

Received November 14, 2008.

Accepted January 28, 2009.

¹Corresponding author: jcant@uoguelph.ca

has been proposed that a lack of Se blocks elongation of the polypeptide chain (Saedi et al., 1988), and the nonsense selenocysteine codon leads to degradation of the mRNA (Moriarty et al., 1998).

Dietary Se exists in both inorganic and organic forms. The preferred form of dietary Se for feeding dairy cows is organic, which is predominantly selenomethionine (**SeMet**), because it is more effective at enriching the Se content of milk (Ortman and Pehrson, 1999). In order for SeMet to stimulate synthesis of selenoproteins such as GPx, the Se must be released by enzymatic degradation and converted to selenophosphate, which is the substrate for cotranslational selenocysteine synthesis (Allmang and Krol, 2006). Different epithelial cell types do not share the same features of Se metabolism and GPx production (Rebsch et al., 2006). To our knowledge, there has been no study of GPx responses to Se status in primary cultures of bovine mammary epithelial cells (**BMEC**). Given that GPx1 and GPx3 are the main GPx forms expressed in mammary epithelial cells and milk of several species including the bovine (Chu et al., 1992; Lindmark-Månsson et al., 2001; Bruzelius et al., 2007, 2008), we hypothesized that BMEC can express both GPx1 and GPx3, and that an increase in the level of SeMet in culture media would increase absolute GPx activity and cell growth. To test the hypotheses, we localized GPx1 and GPx3 in lactating bovine mammary tissue and BMEC and evaluated the effect of SeMet on BMEC growth and GPx activity.

MATERIALS AND METHODS

Animals and Mammary Tissue Sample Collection

Mammary tissue was collected from 8 multiparous lactating Holstein cows at 172 ± 16 DIM producing an average of 31.0 ± 2.1 kg milk/d culled from the Elora Dairy Research Centre herd (Elora, Ontario, Canada) and sent for slaughter at the University of Guelph Meat Laboratory (Guelph, Ontario, Canada). All cows were clinically healthy and free of mammary infection (SCC <150,000 cells/mL milk). After stunning, cows were bled out and their mammary glands were excised, washed with Betadine (Perdue Frederick Co., Norwalk, CT), and rinsed with 70% ethanol. Samples of mammary parenchyma (four 30-g pieces) were aseptically removed from the interior of the gland within 30 min of death and transferred to the laboratory at 37°C in a 500-mL bottle containing sterile Hanks' balanced salt solution (Sigma Chemical Co., Oakville, Ontario, Canada) supplemented (**HBSSs**) with antibiotic-antimycotic solution (10,000 U/mL penicillin G, 10 mg/mL

streptomycin, and 25 µg/mL amphotericin B; Sigma Chemical Co.), 1.5 mL of 1 M HEPES buffer (Sigma Chemical Co.), and 15 mL of fetal bovine serum (**FBS**; Invitrogen, Burlington, Ontario, Canada).

Isolation of Bovine Primary Mammary Epithelial Cells and Cell Cultures

Primary mammary epithelial cells were isolated by adaptations and modifications of the methods of Baumrucker et al. (1988) and Cifrian et al. (1994) to decrease contamination with erythrocytes. Both methods serve to reduce contamination with fibroblasts and myoepithelial cells. In a laminar flow hood, six 10-g pieces of mammary tissue were washed 3 times in HBSSs, trimmed of all visible nonepithelial parenchyma, and thoroughly minced in 50-mL beakers with scissors. Minced tissues were washed 4 times with HBSSs to remove milk and blood, and transferred to a 100-mL bottle containing 50 mL of HBSSs supplemented with collagenase type III from *Clostridium histolyticum* (400 U/mL; Worthington Biochemicals, Freehold, NJ), hyaluronidase (100 U/mL; Sigma Chemical Co.), and DNase (2 U/mL; Sigma Chemical Co.) for 2 h at 37°C in a shaking water bath at ~110 oscillations/min. Every 60 min, the digestion suspension was passed sequentially through nylon mesh of 200- (Spectrum, Los Angeles, CA), 100-, and 40-µm mesh (BD Biosciences, Mississauga, Ontario, Canada), and dispersed cells were collected by centrifugation at $100 \times g$ for 5 min after addition of FBS (about 1/10 vol). The supernatant was decanted and the cell pellet was washed twice in HBSSs to eliminate residual enzyme. The resulting preparation consisted mainly of single and clumped epithelial cells and single erythrocytes. To separate BMEC from erythrocytes, the cell suspension was incubated for 1 h at 37°C in a ThinCert tissue cell culture insert (452.4 mm² culture surface, 8.0 µm pore size; Greiner Bio-One, Monroe, NC) and washed every 20 min in HBSSs. Erythrocytes filtered through to the bottom of the plate, and BMEC were recovered by inversion and washing of the membrane. After centrifugation at $100 \times g$ for 5 min, HBSSs was decanted and the final cell pellet was resuspended in primary culture medium (**PCM**) containing 45% RPMI 1640 (Sigma Chemical Co.), 45% Dulbecco's modified Eagle's medium (Sigma Chemical Co.), 5% FBS, 2% antibiotic-antimycotic solution, 1 mM sodium pyruvate (Sigma Chemical Co.), 2 mM L-glutamine (Sigma Chemical Co.), 40 mM HEPES buffer, bovine insulin (5 µg/mL; Sigma Chemical Co.), hydrocortisone (1 µg/mL; Sigma Chemical Co.), sheep prolactin (1 µg/mL; Sigma Chemical Co.), murine epidermal growth factor (5 ng/mL; Sigma Chemical Co.),

and bovine transferrin (10 µg/mL; Sigma Chemical Co.); PCM was changed every 24 h. The BMEC were counted on a hemacytometer slide (improved Neubauer chamber; Fisher Scientific, Whitby, Ontario, Canada) and checked for viability by exclusion of 0.4% (wt/vol) trypan blue dye (Sigma Chemical Co.). More than 90% of the single cells were found to be viable and erythrocytes were absent.

Cell Number Estimates

To quantify the effect of SeMet on cell count and viability, BMEC were seeded at an approximate density of 3×10^4 cells/well, from 1 mL of culture medium, onto 24-well plates of BioCoat Cellware coated with collagen type I from rat tail tendon (BD Biosciences) and incubated in an atmosphere of 5% CO₂ in air at 37°C for 5 d. One plate and 3 parallel wells for each treatment were seeded from each cow. DL-Selenomethionine (C₅H₁₁NO₂Se; Fisher Scientific) was added to the medium at concentrations of 0, 10, 20, and 50 nM. Cells were harvested every 24 h for 5 d by trypsinization and were resuspended in Hanks' balanced salt solution containing 50% FBS. Cells were counted on a hemacytometer, and DNA was quantified in cell suspensions washed twice with ice-cold PBS and lysed in CellLytic MT Cell Lysis Reagent (Sigma Chemical Co.) containing protease inhibitor cocktail (Sigma Chemical Co.). Supernatants resulting from a 10-min centrifugation of the lysate at 4°C and $13,000 \times g$ were assayed for DNA by fluorescent Hoechst-33258 dye binding (DNAQF kit, Sigma Chemical Co.) in a Wallac Victor 1420 Multilabel Counter (Perkin-Elmer, Wellesley, MA) with excitation and emission wavelengths set at 355 and 460 nm, respectively (Labarca and Paigen, 1980). Relative fluorescence from cultures with unknown cell numbers was compared with standard curves to determine cell number (Rago et al., 1990). Morphology of cultured cells was evaluated under a Leica DM IL inverted microscope with phase contrast (Wetzlar, Germany).

GPx Enzymatic Activity Assay

To quantify effects of SeMet on GPx activity, 6 parallel wells of BMEC were plated for each SeMet concentration of 0, 10, 20, or 50 nM for each cow. Cells were harvested every 24 h for 5 d by mechanical scraping, and lysates were prepared as described previously. Glutathione peroxidase activity was assayed by an enzyme-coupled spectrophotometric procedure (CGP1 kit, Sigma Chemical Co.) according to Paglia and Valentine (1967). Protein concentrations of cell lysates were quantified using the bicinchoninic acid assay (Pierce, Rockford, IL) using BSA as a standard.

Gel Electrophoresis and Western Blotting of GPx1 and GPx3

To examine the effect of SeMet on GPx1 and GPx3 protein abundances in BMEC, secretory cells were plated into 60-mm culture dishes of BioCoat Cellware (BD Biosciences) at a density of 2×10^6 cells/dish and treated with 0, 10, 20, or 50 nM SeMet for 5 d. Cell lysates containing 35 µg of protein were boiled in an equal volume of 2× sample loading buffer [4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.125 M Tris HCl (pH 6.8), and 0.004% bromophenol blue; Sigma Chemical Co.] at 95°C for 5 min and separated on 12.5% SDS-PAGE with a 5% stacking gel in SDS-Tris-glycine running buffer. Proteins were electrotransferred (Mini Trans-Blot Cell, Bio-Rad Laboratories Inc., Mississauga, Ontario, Canada) to a polyvinylidene fluoride membrane (Millipore, Mississauga, Canada) in Tris-glycine-methanol buffer at 100 V for 60 min. The membranes were then dried for 30 min at room temperature and cut into 2 pieces, horizontally, along the 35 kDa size marker to allow primary detection of GPx1 (22 kDa) or GPx3 (25 kDa) and β-actin (42 kDa) from the same blot. Membranes were blocked with blocking buffer [5% (wt/vol) nonfat dry milk in 0.36% (wt/vol) Tris base (pH 7.5), 0.1% (vol/vol) Tween 20, and 1.16% (wt/vol) NaCl] overnight at 4°C. The upper molecular weight portions were incubated with primary mouse monoclonal anti-β actin (42 kDa; 1:5,000; Abcam, Cambridge, MA), and the lower portions were incubated with polyclonal rabbit anti-GPx1 (1 µg/mL; Abcam) or monoclonal mouse anti-GPx3 (1:1,000; Abcam) overnight at 4°C. After washing 6 times with blocking buffer, membranes were incubated with goat anti-rabbit IgG (1:4,000; Abcam) or rabbit anti-mouse IgG (1:4,000; Abcam) labeled with horseradish peroxidase for 45 min at room temperature. After washing 6 times with 0.36% (wt/vol) Tris base (pH 7.5), 0.1% (vol/vol) Tween 20, and 1.16% (wt/vol) NaCl, proteins were developed by autoradiography utilizing Enhancer ChemiLuminescence (Amersham, Arlington Heights, IL). Finally, images from radiographic film were scanned and the integrated density was determined by ImageJ software (<http://rbs.info.nih.gov/ij>). Relative density was quantified by normalization of the integrated density of each blot to that of the corresponding β-actin.

Immunohistochemical Staining and Microscopy Procedure

Immunohistochemical localization of GPx1 and GPx3 in normal lactating bovine mammary tissue was performed similarly to Komatsu et al. (2001). Samples collected at slaughter were fixed in 10% (vol/vol) neu-

tral buffered formalin for 24 h at room temperature, embedded in paraffin wax, and sectioned into 5.0- μ m sections. After deparaffinization, sections were heated at 400 W in a microwave oven for 7.5 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. Nonspecific binding sites were blocked by incubation in 3% (vol/vol) normal goat serum (Sigma Chemical Co.) in PBS for 30 min at room temperature. Sections were incubated with polyclonal rabbit anti-GPx1 (1:200; Abcam) and monoclonal mouse anti-GPx3 (1:200; Abcam) overnight in a humidified chamber at 4°C. For negative controls, PBS was used instead of primary antibody. Staining of GPx1 and GPx3 was revealed by incubation with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit or goat anti-mouse for 1 h. Vectashield mounting medium with 0.15% (wt/vol) 4',6 diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, Canada) was used to counterstain the nucleus and to preserve fluorescence. Images were obtained on a Leica DMR microscope equipped with epifluorescence illumination and a CoolSNAP camera (RS Photometrics, Tucson, AZ) interfaced with digital imaging analysis software (OpenLab 2.2; Improvision, Boston, MA). Filter sets utilized were 360/440 nm for DAPI and 450/515 nm for FITC.

Immunocytochemical Staining and Microscopy Procedure

Immunocytochemical localization of GPx1, GPx3, cytokeratin, and caseins in BMEC was performed by a modification of the method of Schmid et al. (1991). Bovine mammary epithelial cells (1×10^5) were grown to near confluency or confluency on collagen type I-coated 22-mm diameter glass coverslips (BD Biosciences) that fit into a 6-well plate. Medium was supplemented with 4 levels of SeMet (0, 10, 20, and 50 nM) and replaced every 48 h. By d 5, monolayers were fixed in 2.0% (wt/vol) paraformaldehyde (Sigma Chemical Co.) in PBS (pH 7.2) for 1 h at 4°C and washed in washing buffer (PBS, 0.1% wt/vol sodium azide, 2% vol/vol FBS, pH 7.2). Cell membranes were permeabilized with 0.2% Tween 20 solution in PBS for 15 min at 37°C in the water bath, washed in washing buffer, and blocked in washing buffer containing 10% (vol/vol) nonimmune goat serum for 30 min. Coverslips were incubated at 4°C overnight in a humid chamber with either monoclonal mouse antibody against cytokeratin AE1/AE3 (1:100; Dako, Mississauga, Ontario, Canada), polyclonal rabbit anti-GPx1 (1:200; Abcam), monoclonal mouse anti-GPx3 (1:200; Abcam), or polyclonal rabbit antibody against bovine milk caseins (1:300; Immunology Consultants Laboratory Inc., Newberg, OR). Cells were washed twice in washing buffer for 10 min

each and subsequently incubated for 1 h in the dark with goat FITC-labeled anti-rabbit (Abcam), goat FITC-labeled anti-mouse (Abcam), or goat Texas Red-labeled anti-rabbit (Abcam). After 2 more washes, cells were counterstained and fluorescence preserved with Vectashield mounting medium containing 0.15% (wt/vol) DAPI (Vector Laboratories). Images were obtained as described above with the addition of a 560/675 nm filter set for Texas Red.

Se Content of Culture Media and FBS

Samples of PCM and FBS were analyzed for Se content by inductively coupled plasma mass spectrometry (Anderson, 1999). The media and FBS used in these experiments contained 4.2 ± 2.2 and 56 ± 8.6 nM Se, respectively ($n = 6$; mean $\pm$ SD).

Statistical Analysis

Results are presented as means $\pm$ SEM. The effects of 4 SeMet levels (0, 10, 20, or 50 nM) on BMEC growth, viability, and GPx activity in BMEC over 5 d of incubation were analyzed by a mixed model in SAS (SAS Institute Inc., Cary, NC) with repeated measures over time according to

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

where μ = overall mean, S_i = fixed effect of SeMet level ($i = 1$ to 4), T_j = repeated time effect ($j = 1$ to 5), ST_{ij} = SeMet $\times$ time interaction effect, and $\text{error}_{k(ij)}$ = experimental error ($k = 1$ to 3). Covariance in repeated measures on the same cow was accommodated with a compound symmetry structure. Linear and quadratic orthogonal contrasts of SeMet level were determined with coefficients calculated in PROC IML of SAS (SAS Institute Inc.) to match the SeMet level.

Western blotting data were analyzed with PROC GLM of SAS (SAS Institute Inc.). Linear and quadratic contrasts of SeMet level were calculated. Significance was declared at $P < 0.05$.

RESULTS

Immunohistochemical Localization of GPx1 and GPx3 in Bovine Mammary Tissue

Polyclonal anti-GPx1 and monoclonal anti-GPx3 primary antibodies were used to detect GPx1 and GPx3 proteins, respectively. These primary antibodies were demonstrated to be non-cross-reactive by Western blotting analysis of bovine plasma and erythrocyte hemolysate. The anti-GPx1 specifically detected the 22-kDa

Figure 1. Immunohistochemical detection of cellular glutathione peroxidase (GPx1) and extracellular glutathione peroxidase (GPx3) in normal lactating bovine mammary tissue. Cross-reactivity of anti-GPx1 and anti-GPx3 in bovine plasma (2 μ L) and bovine erythrocyte lysate (10 μ g) were resolved by Western blotting analysis. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded lactating bovine mammary tissue and immunostained with anti-GPx1 or anti-GPx3 by fluorescein isothiocyanate-labeled method. A) In the lactating bovine mammary gland, GPx1 localization is clearly seen in the cytoplasm of mammary epithelial cells (arrow) and red blood cells (arrowhead), with no staining in the extracellular space; B) GPx3 is immunolocalized in the cytoplasm of mammary epithelial cells (arrow) and extracellular fluid (arrowhead); C) 4',6 diamidino-2-phenylindole (DAPI) was used as a nuclear marker (arrow); and D) PBS was used instead of primary antibody as control.

GPx1 protein in erythrocyte hemolysate (Figure 1A) but did not detect the 25-kDa GPx3 protein that was recognized in plasma by anti-GPx3 (Figure 1B). Bovine mammary epithelial cells showed positive staining with both primary antibodies. In epithelial and red blood cells, anti-GPx1 produced prominent cytoplasmic staining with low nuclear staining (Figure 1A). Immunoreactivity to GPx3 was found in the cytoplasm of epithelial cells with reduced or absent nuclear staining (Figure 1B). A strong staining pattern from anti-GPx3 was seen in the interstitial space, consistent with its localization in the extracellular fluid. Cell nucleus was stained with DAPI, which revealed mononucleate cells (Figure 1C). Conversely, when PBS was used instead of primary antibody as a negative control, staining was not detected (Figure 1D).

Effect of SeMet on BMEC Culture

Cells collected after 2 h of digestion with collagenase/hyaluronidase were predominantly viable single BMEC and a few epithelial cell clumps. The major cellular contaminants were erythrocytes, which were removed using tissue cell culture inserts. Phase-contrast microscopy of BMEC revealed a prominent nucleus and cytoplasmic vacuoles (Figure 2A), which have been observed in normal mammary secretory epithelial cells (Wooding, 1973). The BMEC and epithelial cell clumps attached to the collagen gel within 12 to 24 h and spread out to monolayers with a typical cobblestone organization in all treatments (Figure 2B). By d 2, cells had flattened with a prominent nucleus, but cytoplasmic vacuoles were reduced in size and numbers. The BMEC cultured

in 10, 20, or 50 nM SeMet reached 90% confluency with dome-like structures by d 5 (Figure 2C). In contrast, BMEC with 0 nM SeMet remained subconfluent without dome-like structures and failed to become confluent even if kept for up to 12 d (Figure 2D).

Functional differentiation of BMEC was confirmed in the different treatments by positive staining for bovine caseins in coverslip monolayers prepared from BMEC (Figure 2E). The epithelial nature of BMEC was confirmed with a monoclonal antibody against cytokeratin AE1/AE3. After 7 d of culture, cells were identified as more than 95% epithelial in each treatment by positive staining of cytoplasm and characteristic tonofilament connections between cells, which are important to intracellular communication and cell polarity (Figure 2F).

Effect of SeMet on BMEC Growth and Viability

The BMEC were cultured for 5 d to evaluate treatment effects on cell growth and viability. Both cell number (Figure 3A) and DNA (Figure 3B) increased linearly ($P < 0.001$) and quadratically ($P < 0.001$) with the level of SeMet added to media. Numbers of BMEC showed as much as a 5-fold increase in 5 d with SeMet added to the media, compared with 3-fold for cells without SeMet.

Viability of BMEC cultured in media without added SeMet decreased ($P < 0.05$) from 90 to 70% during the first 24 h of incubation and remained at 70% thereafter (Figure 4). The addition of SeMet caused linear ($P < 0.001$) and quadratic ($P < 0.001$) increases in the capacity of BMEC to maintain viability during 5 d of culture.

Effect of SeMet on BMEC GPx Activity

There was a linear ($P < 0.001$) and quadratic ($P = 0.003$) increase of GPx activity in BMEC cultured with increasing levels of SeMet as early as 24 h after the start of incubation (Figure 5). This effect of SeMet persisted until 120 h of incubation. There was no effect of time on GPx activity.

GPx1 and GPx3 Protein Expression in BMEC

To determine whether GPx1 or GPx3 abundance in BMEC is regulated by SeMet, cell lysates from 5 d cultures were probed by Western blotting analysis (Figure 6A and Figure 7A). Both linear ($P < 0.001$) and quadratic ($P = 0.037$) increases in GPx1 abundance due to SeMet addition were apparent (Figure 6B). Although immunoblots for GPx3 were less intense than for GPx1, SeMet added to media linearly ($P < 0.001$)

and quadratically ($P = 0.002$) increased the protein expression of GPx3 in BMEC (Figure 7B). The highest expression of GPx1 and GPx3 proteins was found in BMEC cultured in 50 nM SeMet.

Immunocytochemistry of cells grown on coverslips revealed strong cytoplasmic staining, with weak or absent nuclear staining for GPx1 and GPx3, respectively (Figure 8A and Figure 8B). The control showed no staining (Figure 8C). These results confirm that BMEC express both selenoproteins after 5 d of culture.

DISCUSSION

GPx1 and GPx3 Are Both Synthesized by Bovine Mammary Epithelial Cells

This study demonstrates that both GPx1 and GPx3 are synthesized by lactating bovine mammary epithelial cells. First, GPx1 and GPx3 were localized cytoplasmically by immunofluorescent staining of mammary tissue collected at slaughter, extending the findings of Bruzelius et al. (2007) that both selenoproteins are expressed at the mRNA level. However, the origin of GPx3 was unclear. It is presumed that GPx3 is produced and secreted mainly by the liver and the kidney, from where it is transported through blood plasma to other tissues, including the mammary glands (Avissar et al., 1991; Whitten et al., 2002). Indeed, immunohistochemistry revealed the presence of GPx3 in the interstitial space and this observation does not preclude the possibility that GPx3 found inside mammary epithelial cells was of extracellular origin. Therefore, we isolated individual BMEC by enzymatic digestion of mammary parenchyma, taking care to remove erythrocytes that could be an extra-epithelial source of GPx1, and cultured them for 5 d on collagen type I. After 5 d of culture, by which time GPx in the original plated cells would have disappeared, GPx1 and GPx3 were detected inside cells by immunocytochemistry. The absence of nuclear GPx3 and the presence of cytoplasmic GPx1 were as expected from previous studies in the human glioma PU118-9 cell (Li et al., 2000). Furthermore, Western blotting showed that culture conditions (i.e., SeMet addition) could increase GPx1 and GPx3 abundances.

Glutathione peroxidase activity has been detected in human and bovine milk, where levels of GPx3 are 100 times higher than that of GPx1 (Avissar et al., 1991; Lindmark-Månsson et al., 2001). Our finding that GPx3 is expressed by cultured BMEC lends support to the hypothesis that GPx3 in bovine milk is synthesized and secreted by mammary epithelial cells. Although the biological functions of GPx3 in alveolar milk are unclear, the occurrence of GPx3 at this level may be associated with external protection of mammary secretory

Figure 2. Phase-contrast photomicrographs showing the effect of selenomethionine (SeMet) on bovine mammary epithelial cell (BMEC) culture. A) Minutes after plating, numerous cytoplasmic vacuoles (arrows) were detected in the 4 treatments; B) between 12 and 24 h after plating, cells formed monolayer aggregates with cobblestone morphology, which was found in all treatments; C) a representative micrograph of 7-d-old culture with 50 nM of SeMet added showing confluency and dome-like structures (arrow); D) 12-d-old culture without SeMet added, showing subconfluency and absence of dome-like structures; E) immunofluorescent staining of BMEC on collagen type I-coated glass coverslips with polyclonal antibody against bovine caseins (α , β , and κ) found in the different treatments; F) Immunofluorescent staining of BMEC on collagen type I-coated glass coverslips with monoclonal antibody against cytokeratin AE1/AE3 5 d postculture with evidence of tonofilament among cells found in all treatments.

Figure 3. Influence of selenomethionine (SeMet) on bovine mammary epithelial cell number: A) counted by hemacytometer; B) DNA assay. Data are expressed as mean $\pm$ SEM of 3 independent experiments. *Values are statistically different ($P < 0.01$) within each period.

cell apical membranes from lipid or hydrogen peroxides, which can be produced by at least 44 enzymes found in milk such as superoxide dismutase, xanthine oxidase, and sulphydryl oxidase (Avisar et al., 1991).

SeMet Helps to Maintain the Differentiated State of BMEC

Primary cultures of BMEC were used to observe cell behavior under conditions that mimic physiological conditions relevant to lactation. For example, the use of primary cultures allowed us to study the effect of SeMet on BMEC without the influence of other mammary cell types, tissues of the body, or the circulatory and immune systems. A good in vitro culture model should permit normal cell growth and maintenance of

cell differentiation, which underlies the biochemical and morphological characteristics typical of mammary epithelial cells in vivo. Two common strategies to induce mammary differentiation are incubation with the lactogenic hormones insulin, hydrocortisone, and prolactin, and seeding on extracellular collagen, both of which we used. Casein secretion in culture is a marker of the differentiated state (Talhouk et al., 1990). In our cultures, the positive staining of BMEC with anti-casein antibody suggests that cells grown with or without SeMet retained the ability to synthesize casein during the experimental period. Cytokeratin expression has also been used to distinguish between epithelial and nonepithelial cells in tissues and cultures (Cifrian et al., 1994), or to assay the malignant transformation of mammary epithelial cells (Dairkee and Heid, 1993). The epithe-

Figure 4. Influence of selenomethionine (SeMet) on the viability of bovine mammary epithelial cell measured by trypan blue exclusion. Data are expressed as mean $\pm$ SEM of 3 independent experiments. *Values are statistically different ($P < 0.05$) within each period.

lial nature of our BMEC was confirmed by positive staining with anti-cytokeratin cocktail antibody (AE1/AE3) after 7 d in culture. The immunostaining patterns against caseins and cytokeratins are consistent with previous studies (Talhouk et al., 1990; Cifrian et al., 1994). Additionally, the outgrowth pattern of attached BMEC, observed 48 h after seeding by phase contrast

microscopy, was notably affected by SeMet addition to the media. Cells cultured in SeMet-supplemented media grew into a confluent layer with dome formation by 5 d, whereas this outgrowth pattern was not seen without added SeMet. The in vivo arrangement of milk secretory cells is a hollow, spherical alveolus with tight contact between adjacent cells. Cell-cell contact is es-

Figure 5. Influence of 5 d of culture with selenomethionine (SeMet) on the glutathione peroxidase (GPx) activity of bovine mammary epithelial cells. Cells were harvested and lysed every 24 h up to 5 d. The GPx activity was measured in the cell lysis and the means $\pm$ SEM of 3 independent experiments are shown. One unit of GPx was defined as the amount of enzyme that caused the oxidation of 1.0 μ mol of NADPH to NADP⁺ per minute at pH 8.0 at 25°C in a coupled reaction with reduced glutathione, glutathione reductase, and *tert*-butyl hydroperoxides. Enzyme activity was expressed using a molar extinction coefficient of NADPH = 6.22 ϵ mM. * $P < 0.03$; ** $P < 0.05$; *** $P < 0.01$: values are statistically different within each period.

Figure 6. Effect of 5 d of culture with selenomethionine (SeMet) on cellular glutathione peroxidase (GPx1) protein expression in bovine mammary epithelial cells. A) A representative Western blot of cell lysates is shown; B) densitometric analysis of GPx1 protein expression relative to the corresponding β -actin control. Data are representative of 3 independent experiments. Asterisks above histograms show differences among treatments (* vs. **, *** $P < 0.01$; ** vs. *** $P < 0.05$).

essential for normal mammary tissue development, mediated by cadherins (Knudsen and Wheelock, 2005), and dome formation in vitro is recognized as a manifestation of the alveolus-forming behavior of milk secretory cells that facilitates polar secretion of milk components (Zucchi et al., 2002). The Se concentration of 4.2 ± 2.2 nM in complete media was apparently insufficient to support cell proliferation or survival necessary for rapid confluency and formation of epithelial domes, thus supplementation was required to maintain the differentiated state of these BMEC in vitro.

SeMet Improves Growth of BMEC

A linear relationship was detected between SeMet addition to culture media and growth in numbers of BMEC over 5 d. These results are consistent with those reported for HL-60 cells by Zeng (2002) and represent the first demonstration that Se as SeMet is an essential trace nutrient for growth of BMEC. The mechanism

of enhancement of cell growth induced by SeMet, however, remains unclear. Proliferation and death of cells are the 2 processes that will affect cell number. A Se-induced decrease in apoptosis has been observed in several experimental situations. For example, cardiac cell death due to oxidative stress during recovery from a heart attack is mitigated with Se (Venardos and Kaye, 2007), Se reduces apoptosis of neural progenitor cells following traumatic brain injury (Yeo and Kang, 2007), and lymphocyte apoptosis is reduced by antioxidant formulas containing Se (Mosca et al., 2002). Effects of Se on cell proliferation are less clear and have mostly been studied at supranutritional concentrations that inhibit cell proliferation (Golczewski and Frenkel, 1989) and stimulate apoptosis (Rikiishi, 2007), characteristics that instill Se with an anticarcinogenic property. However, growth of human leukemia HL-60 cells in serum-free media in vitro was stimulated by 250 nM SeMet, during which there was a stimulation of cell cycle progression to the M phase, mRNA expression

Figure 7. Effect of 5 d of culture with selenomethionine (SeMet) on extracellular glutathione peroxidase (GPx3) protein expression in bovine mammary epithelial cells. A) A representative Western blot of cell lysates is shown; B) densitometric analysis of GPx3 protein expression relative to the corresponding β -actin control. Data are representative of 3 independent experiments. Asterisks above histograms show differences among treatments (* vs. **, *** $P < 0.01$; ** vs. *** $P < 0.05$).

of proliferating cell nuclear antigen and several cyclins and cyclin kinases was elevated, and phosphorylation state of cellular protein was increased (Zeng, 2002). At 1,000-fold higher concentrations, Se is an insulin mimetic that can activate mitogen-activated protein kinase and S6 kinase by phosphorylation (Hei et al., 1998). These results suggest that Se status of cells may be detected and signaled to proliferation machinery by phosphorylation cascades.

SeMet Stimulates Expression of GPx1 and GPx3 in Bovine Mammary Epithelial Cells

It is well established that Se in many forms induces the expression and activity of the GPx enzyme family in the body of Se-deficient animals and in several cell lines in vitro (Finley, 2006; Zeng et al., 2008). However, there has been no previous demonstration of this effect

in BMEC. In order for SeMet to stimulate GPx expression, the Se must be released as H_2Se by the sequential actions of methionine β -lyase and a demethylase, or via the trans-sulfuration (trans-selenation) pathway to produce selenocysteine that is acted on by selenocysteine β -lyase (Suzuki et al., 2007). Although mammary catabolism of methionine is considered to be negligible so that the only use of methionine is for protein synthesis (Mephram, 1982), inclusion of [^{35}S]-methionine in the perfusate of lactating sheep mammary glands resulted in a small incorporation of label into cysteine residues of casein (Verbeke et al., 1967), indicating that trans-sulfuration is possible. In addition, Ip et al. (1999) found a low level, compared with liver, of β -lyase activity in extracts of rat mammary tissue that was able to release monomethylated Se from Se-methylselenocysteine. Our observation that GPx1 and GPx3 expression and GPx activity were progressively increased by nanomolar ad-

ditions of SeMet to cultures indicates that one or the other SeMet-degrading pathway is present in BMEC.

Glutathione peroxidases are just one of many selenoproteins that protect cells against oxidative damage by converting ROS to harmless molecules such as water. Increased expression of selenoproteins may have been responsible for the observed increase in growth of BMEC in vitro. Addition of 70 to 7,000 nM selenite to cultures of bovine corpora lutea decreased concentrations of lipid hydroperoxides and increased cell numbers over 8 to 14 d of culture (Kamada and Ikumo, 1997). Lipid-soluble antioxidants like α -tocopherol prevented both the increase in intracellular concentrations of ROS and death of leukemia cells induced in vitro by 3 d of Se deficiency (Saito et al., 2003). A ROS challenge with H_2O_2 causes apoptosis (Saito et al., 2003) and leakage of ROS from mitochondria is an integral component of both intrinsic and extrinsic pathways of apoptosis signaling (Tang et al., 2008). Thus, enzymatic activity of selenoproteins can account for the growth-stimulating effect of Se. If the decline in milk production of the dairy cow during mid and late lactation is due in part to apoptotic signaling from intracellular ROS (Hadsell et al., 2002), the GPx-promoting effects of Se supplementation of mammary epithelial cells may serve to improve the persistency of lactation. Indeed, dairy cows supplemented with Se have shown an increase in milk production (Wichtel et al., 1994; Silvestre et al., 2007).

CONCLUSIONS

The results presented demonstrate expression of GPx1 and GPx3 proteins in mammary epithelial cells from the lactating dairy cow. Using primary cell cultures, we also show that addition of SeMet to cell culture media increases the protein expression of both selenoenzymes and absolute GPx activity in BMEC. In addition, SeMet at nanomolar concentrations promotes growth and viability of BMEC. Finally, our data confirm that SeMet as a principal source of Se represents an essential nutrient for BMEC culture.

ACKNOWLEDGMENTS

This study was supported by grants from NSERC Canada, the Ontario Ministry of Agriculture, Food and Rural Affairs, and University of Zulia, Venezuela. We would like to thank Helen Coates, Brian McDougall, Sam Leo, Qiumei You, and Linda Trouten-Radford (University of Guelph) for their skillful technical assistance. Special thanks are extended to Brian McBride (University of Guelph), who provided a critical review of this study.

Figure 8. Immunocytochemical detection of cellular glutathione peroxidase (GPx1) and extracellular glutathione peroxidase (GPx3) in bovine mammary epithelial cells grown on collagen type I-coated glass coverslips for 5 d to near confluency. Cells show strong cytoplasmic immunostaining with both A) GPx1 and B) GPx3, but moderate to weak immunostaining in nucleus; C) PBS was used instead of primary antibody as control.

REFERENCES

- Allmang, C., and A. Krol. 2006. Selenoprotein synthesis: UGA does not end the story. *Biochimie* 88:1561–1571.
- Anderson, K. A. 1999. Analytical Technique for Inorganic Contaminants. AOAC International, Gaithersburg, MD.
- Arthur, J. R. 2000. The glutathione peroxidase. *Cell. Mol. Life Sci.* 57:1825–1835.
- Avissar, N., J. R. Slemmon, I. S. Palmer, and J. H. Cohen. 1991. Partial sequence of human plasma glutathione peroxidase and immunologic identification of milk glutathione peroxidase as the plasma enzyme. *J. Nutr.* 121:1243–1249.
- Baumrucker, C. R., K. P. Deemer, R. Walsh, T. L. Riss, and R. M. Akers. 1988. Primary culture of bovine mammary acini on collagen matrix. *Tissue Cell* 20:541–554.
- Behne, D., and A. Kyriakopoulos. 2001. Mammalian selenium-containing proteins. *Annu. Rev. Nutr.* 21:453–473.
- Bruzellius, K., T. Hoac, R. Sundler, G. Önnings, and B. Åkesson. 2007. Occurrence of selenoprotein enzyme activities and mRNA in bovine mammary tissue. *J. Dairy Sci.* 90:918–927.
- Bruzellius, K., S. Purup, P. James, G. Önnings, and B. Åkesson. 2008. Biosynthesis of selenoproteins in cultured bovine mammary cells. *J. Trace Elem. Med. Biol.* 22:224–233.
- Chu, F. F., R. S. Esworthy, J. H. Doroshov, K. Doan, and X. F. Liu. 1992. Expression of plasma glutathione peroxidase in human liver in addition to kidney, heart, lung, and breast in humans and rodents. *Blood* 79:3233–3238.
- Cifrian, E., A. J. Guidry, C. N. O'Brien, S. C. Nickerson, and W. Marquardt. 1994. Adherence of *Staphylococcus aureus* to cultured bovine mammary epithelial cells. *J. Dairy Sci.* 77:970–983.
- Dairkee, S., and H. W. Heid. 1993. Cytokeratin profile of immunomagnetically separated epithelial subsets of the human mammary gland. *In Vitro Cell. Dev. Biol. Anim.* 29:427–432.
- Finley, J. W. 2006. Bioavailability of selenium from foods. *Nutr. Rev.* 64:146–151.
- Golczewski, J. A., and G. D. Frenkel. 1989. Cellular selenoproteins and the effects of selenite on cell proliferation. *Biol. Trace Elem. Res.* 20:115–126.
- Hadsell, D. L., S. G. Bonete, and A. V. Lee. 2002. Genetic manipulation of the IGF-I axis to regulate mammary gland development and function. *J. Dairy Sci.* 85:365–377.
- Hei, Y., S. Frahbakhshian, X. Chen, M. L. Battell, and J. H. McNeill. 1998. Stimulation of MAP kinase and S6 kinase by vanadium and selenium in rat adipocytes. *Mol. Cell. Biochem.* 178:367–375.
- Ip, C., Z. Zhu, H. J. Thompson, D. Lisk, and H. E. Ganther. 1999. Chemoprevention of mammary cancer with Se-allylselenocysteine and other selenoamino acids in the rat. *Anticancer Res.* 19:2875–2880.
- Juniper, D. T., R. H. Phipps, D. I. Givens, A. K. Jones, C. Green, and G. Bertin. 2008. Tolerance of ruminant animals to high dose in-feed administration of selenium enriched yeast. *J. Anim. Sci.* 86:197–204.
- Kamada, H., and H. Ikumo. 1997. Effect of selenium on cultured bovine luteal cells. *Anim. Reprod. Sci.* 46:203–211.
- Knudsen, K. A., and M. J. Wheelock. 2005. Cadherins and the mammary gland. *J. Cell. Biochem.* 95:488–496.
- Komatsu, H., I. Okayasu, H. Mitomi, H. Imai, Y. Nakagawa, and F. Obata. 2001. Immunohistochemical detection of human gastrointestinal glutathione peroxidase in normal tissue and cultured cells with novel mouse monoclonal antibodies. *J. Histochem. Cytochem.* 49:759–766.
- Labarca, C., and K. Paigen. 1980. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* 102:344–352.
- Li, S., T. Yan, J. Yang, T. D. Oberley, and L. W. Oberley. 2000. The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. *Cancer Res.* 60:3927–3939.
- Lindmark-Månsson, H., J. Chen, M. Paulsson, G. Alden, B. Ren, R. Ladenstein, and B. Åkesson. 2001. The effect of storage and heat treatment on glutathione peroxidase in bovine milk and whey. *Int. Dairy J.* 11:71–81.
- Mephram, T. B. 1982. Amino acid utilization by lactating mammary gland. *J. Dairy Sci.* 65:287–298.
- Moriarty, P. M., C. C. Reddy, and L. E. Maquat. 1998. Selenium deficiency reduces the abundance of mRNA for Se-dependent glutathione peroxidase 1 by a UGA-dependent mechanism likely to be nonsense codon-mediated decay of cytoplasmic RNA. *Mol. Cell. Biol.* 18:2932–2939.
- Mosca, L., S. Marcellini, M. Perluigig, P. Mastroiacovo, S. Moretti, G. Famularo, I. Peluso, G. Santini, and C. De Simone. 2002. Modulation of apoptosis and improved redox metabolism with the use of a new antioxidant formula. *Biochem. Pharmacol.* 63:1305–1314.
- Naiki-Ito, A., M. Asamoto, N. Hokaiwado, S. Takahashi, H. Yamashita, H. Tsuda, K. Ogawa, and T. Shirai. 2007. Gpx2 is an overexpressed gene in rat breast cancers induced by three different chemical carcinogens. *Cancer Res.* 67:11353–11358.
- Ortman, K., and B. Pehrson. 1999. Effect of selenate as a feed supplement to dairy cows in comparison to selenite and selenium yeast. *J. Anim. Sci.* 77:3365–3370.
- Paglia, D. E., and W. N. Valentine. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* 70:158–169.
- Rago, R., J. Mitchen, and G. Wilding. 1990. DNA fluorometric assay in 96-well tissue culture plates using Hoechst 33258 after cell lysis by freezing in distilled water. *Anal. Biochem.* 191:31–34.
- Rebsch, C. M., F. J. Penna III, and P. R. Copeland. 2006. Selenoprotein expression is regulated at multiple levels in prostate cells. *Cell Res.* 16:940–948.
- Rikiishi, H. 2007. Apoptotic cellular events for selenium compounds involved in cancer prevention. *J. Bioenerg. Biomembr.* 39:91–98.
- Saedi, M. S., C. G. Smith, J. Frampton, I. Chambers, P. R. Harrison, and R. A. Sunde. 1988. Effect of selenium status on mRNA levels for glutathione peroxidase in rat liver. *Biochem. Biophys. Res. Commun.* 153:855–861.
- Saito, Y., Y. Yoshida, T. Akazawa, K. Takahashi, and E. Niki. 2003. Cell death caused by selenium deficiency and protective effect of antioxidants. *J. Biol. Chem.* 278:39428–39434.
- Schmid, I., C. H. Uittenbogaart, and J. V. Giorgi. 1991. A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantification. *Cytometry* 12:279–285.
- Silvestre, F. T., H. M. Rutigliano, W. W. Thatcher, J. E.-P. Santos, and C. R. Staples. 2007. Effect of selenium source on production, reproduction, and immunity of lactating dairy cows. Pages 48–61 in Florida Ruminant Nutrition Symposium. Univ. Florida, Gainesville.
- Suzuki, K. T., K. Kurasaki, and N. Suzuki. 2007. Selenocysteine β -lyase and methylselenol demethylase in the metabolism of Se-methylated seleno compounds into selenide. *Biochim. Biophys. Acta* 1770:1053–1061.
- Talhok, R. S., R. L. Neiswander, and F. L. Schanbacher. 1990. In vitro culture of cryopreserved bovine mammary cells on collagen gels: synthesis and secretion of casein and lactoferrin. *Tissue Cell* 22:583–599.
- Tang, P. S., M. Mura, R. Seth, and M. Liu. 2008. Acute lung injury and cell death: How many ways can cells die? *Am. J. Physiol.* 294:L632–L641.
- Venardos, K. M., and D. M. Kaye. 2007. Myocardial ischemia-reperfusion injury, antioxidant enzyme systems, and selenium: A review. *Curr. Med. Chem.* 14:1539–1549.
- Verbeke, R., S. Simeonov, and G. Peeters. 1967. Incorporation of L-methionine-S35 into casein by the isolated mammary gland of the sheep. *Arch. Int. Physiol. Biochim.* 75:378–379.
- Whitin, J. C., S. Bhamre, D. M. Tham, and H. J. Cohen. 2002. Extracellular glutathione peroxidase is secreted basolaterally by human renal proximal tubule cells. *Am. J. Physiol.* 283:F20–F28.
- Wichtel, J. J., A. L. Craigie, H. Valera-Alvarez, and N. B. Williamson. 1994. The effect of intra-ruminal selenium pellets on growth rate, lactation and reproductive efficiency in dairy cattle. *N. Z. Vet. J.* 42:205–210.

- Wooding, F. P. 1973. Formation of the milk fat globule membrane without participation of the plasmalemma. *J. Cell Sci.* 13:221–235.
- Yeo, J. E., and S. K. Kang. 2007. Selenium effectively inhibits ROS-mediated apoptotic neural precursor cell death *in vitro* and *in vivo* in traumatic brain injury. *Biochim. Biophys. Acta* 1772:1199–1210.
- Zeng, H. 2002. Selenite and selenomethionine promote HL-60 cell cycle progression. *J. Nutr.* 132:674–679.
- Zeng, H., J. H. Botnen, and L. K. Johnson. 2008. A selenium-deficient Caco-2 cell model for assessing differential incorporation of chemical or food selenium into glutathione peroxidase. *Biol. Trace Elem. Res.* 123:98–108.
- Zucchi, I., L. Bini, D. Albani, R. Valaperta, S. Liberatori, R. Raggiaschi, C. Montagna, L. Susani, O. Barbieri, V. Pallini, P. Vezzoni, and R. Dulbecco. 2002. Dome formation in cell cultures as expression of an early stage of lactogenic differentiation of the mammary gland. *Proc. Natl. Acad. Sci. USA* 99:8660–8665.