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Research Communications

Zinc-related metallothionein metabolism in bovine pulmonary artery endothelial cells

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Bovine pulmonary artery endothelial cells (BPAEC) were cultured in vitro under a variety of conditions to investigate how metallothionein (MT) might participate in zinc homeostasis. Experimental conditions included 10% serum to ensure that the in vitro environment would be a better reflection of the in vivo situation than with protein-free medium. MT was increased by acutely high zinc concentrations (100–200 μmol/L) in the extracellular environment. MT was relatively insensitive to moderate changes in zinc concentration (2–50 μmol/L), even after prolonged exposure for 7 to 12 days. BPAEC had reduced MT content when grown in medium containing serum that had been dialyzed to remove components with a molecular mass of less than 1,000, including zinc. Because the principal source of the major minerals in the experimental medium was not the serum, their concentrations in the final medium were not significantly influenced by serum dialysis. Restoring the zinc concentration in the medium containing the dialyzed serum did not restore MT content in BPAEC, suggesting that some small molecular weight molecule other than zinc established their basal MT content. This study did not identify these putative factors in serum, but hormones are likely candidates. Forty-eight–hour incubations of BPAEC with interleukin (IL-6) or dexamethasone increased cellular MT; however, 17β-estradiol decreased MT, and IL-1 and adenosine 3′,5′-cyclic phosphate (cAMP) had no discernible effect. We conclude that extracellular zinc concentrations have relatively little impact on the cellular concentrations of MT and zinc of BPAEC in vitro. Zinc homeostasis by BPAEC is not maintained by changing the MT concentration in response to changes in the extracellular zinc environment. (J. Nutr. Biochem. 10:00–00, 1999) (J. Nutr. Biochem. 10:139–145, 1999)

Keywords: metallothionein; zinc; endothelial cell; IL-6; dexamethasone; 17β-estradiol

Introduction

Metallothionein (MT) is a low molecular weight metal-binding protein with multiple functions, including protection against mineral toxicity.[1] MT is present in nearly all cells that have been examined, but its regulation is tissue specific.[2,3] The cellular concentration of MT is under multiple points of regulation,[4] and its synthesis is induced by steroid hormones, cytokines, and metals including lead, cadmium, copper, and zinc. MT seems to be integrally connected to intracellular zinc metabolism,[5] although the precise role of MT in zinc metabolism is uncertain.[6]

The endothelium of blood vessels constitutes a barrier to the transfer of substances between the blood plasma and the tissues. Nutrient exchange between the plasma and the

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0955-2863/99/$–see front matter
PII S0955-2863(98)00099-0

interstitial fluid occurs through endothelial cells; thus, endothelial cells can be regarded as a potential site for maintenance of homeostatic integrity of the adjacent tissues, as exemplified by the blood-brain barrier.

MT is believed to participate in zinc homeostasis in some tissues, and the tissues most responsive to zinc status in their production of MT are those primarily responsible for absorption and excretion.[6] MT also might contribute to tissue zinc homeostasis in endothelial cells. Therefore, we investigated the hypothesis that changing the extracellular zinc environment will significantly influence the intracellular zinc and MT content in cultured bovine pulmonary artery endothelial cells (BPAEC). We also tested several cytokines and steroids for their influences.

Methods and materials

Cell culture

BPAEC were obtained from the American Type Culture Collection (#CCL-209; Rockville, MD USA) at passage 16. Cells typically were grown in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum (FBS; Summit Biotechnology, Ft. Collins, CO USA). The growth medium typically contained 5 to 7 μmol zinc/L, by analysis. Cultures for the cytokine studies were grown in medium containing 20% FBS in MEM and contained 12 μmol zinc/L, by analysis. Some experiments (as indicated) involved media containing 10% FBS that had been dialyzed (described below) to remove endogenous zinc; these media contained 1 to 2 μmol zinc/L, by analysis. Cultures were incubated in a water-jacketed incubator (Forma Scientific, Marietta, OH USA) at 37°C, with 95% relative humidity, and 5% carbon dioxide. Experiments were conducted by using cells at passages 18 through 22 and were subcultured into 25 cm^2 plastic culture flasks (T25, Corning Costar Lab. Sci. Co., Park Ridge, IL USA) at a density of 15,000 cells/cm^2.

Cells were seeded into experimental flasks in control medium, which was composed of 10% FBS in MEM. In the longitudinal studies, this medium was replaced with the experimental media on day 1 and changed every 3 days thereafter. In the acute studies, the cells were grown to confluency in the control medium, applying fresh control medium on day 3 and replacing this with the treatment media on day 6.

The influence of cytokines and steroids was examined by using medium supplemented at the high end of the effective concentration range as determined by the supplier (Sigma, St. Louis, MO USA) for use in cell culture: 20 pg interleukin-1β (IL-1)/mL, 13.3 ng interleukin-6 (IL-6)/mL, 10 ng 17β-estradiol/mL, 0.33 μg adenosine 3′,5′-cyclic phosphate (cAMP)/mL, and 40 ng dexamethasone/mL. Cells were grown to confluency in 20% FBS in MEM. On day 6, the medium was replaced with treatment media containing the hormones and subsequently incubated for 48 hours, followed by MT analysis.

Serum dialysis

FBS was dialyzed at 4°C by methods previously described.[7,8] Briefly, the serum was placed inside a 1000-MW cutoff dialysis bag (Spectra/Por 6, Spectrum Medical Industries, Los Angeles, CA USA) and dialyzed for 3 days each (1:40, changed daily) against 10 mM EDTA and 100 mM NaBr, and then dialyzed for 5 days against 150 mM NaCl. The EDTA was included to remove protein-bound zinc, NaBr to facilitate removal of the EDTA, and NaCl to reestablish nearly physiologic conditions to the serum.

MT analysis

Cellular MT was analyzed using the cadmium/hemoglobin affinity assay of Eaton and Toal.[9] The medium was removed and the cells were washed twice with phosphate buffered saline (PBS; 8.00 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KCl, 0.2 g KH_2PO_4 per liter), then briefly bathed with PBS containing 0.2% EDTA, and released from the culture flask surface by exposure to 0.05% trypsin and 0.1% EDTA in PBS for 3 to 4 minutes. After the endothelial cells had rounded up, they were collected in 10% FBS in MEM. Cells were centrifuged at 500 × g, washed with 5 mL PBS, centrifuged again, and resuspended in 0.70 mL of 10 mM Tris buffer. Cells were lysed with a sonicator (Sonic Dismembrator 50, Fisher Scientific) and centrifuged at 500 × g to separate soluble and insoluble components. The soluble fraction was collected in duplicate (0.20 mL) and mixed with an equal volume of ^{109}Cd solution (3.7 kBq/mL in 2.0 μg Cd/mL in 10 mM Tris buffer). After equilibration, 0.10 mL of a 2% hemoglobin solution in 10 mM Tris buffer was added. Tubes were heated at 100°C for 2 minutes and then centrifuged twice at 13,000 × g for 5 minutes. The supernatant was analyzed for ^{109}Cd gamma emissions of the sample using a #1282 Compugamma detector (LKB Nuclear, Gaithersburg, MD USA), and the values converted to pmol MT per milligram cellular protein or microgram DNA.[9] An internal MT standard was prepared and frozen in liquid nitrogen for analysis along with the samples to correct for any day-to-day variation in the assay procedure.

Zinc analyses

Cellular zinc concentrations were determined by first washing the cell monolayer thoroughly with zinc-free HEPES buffer containing 10 mmol HEPES, 140 mmol NaCl, 7 mmol KCl, and 5.6 mmol glucose, pH 7.4, per liter of buffer. The cells were then dissolved in 2.0 mL of 0.2 N NaOH and 0.2% SDS. First, an aliquot was removed for protein analysis and the remaining cell suspension was transferred to an acid washed Teflon tube and heated to dryness at 90°C. The residue was dissolved in nitric acid and heated to dryness. The residue was then suspended in hydrogen peroxide and heated to dryness, and subsequently dissolved in nitric acid and heated to dryness. Finally, the cell residue was dissolved in 2% nitric acid and analyzed by flame atomic absorption spectrophotometry (Smith Hieftje model 12, Jarrell Ash Co., Franklin, MA USA). Blanks and liver standards (#1577b; National Institute of Standards and Technology, Gaithersburg, MD USA) were prepared in 2% nitric acid and analyzed simultaneously. Media for growth and treatment of the cells were analyzed directly and compared with standards prepared in 1% nitric acid.

Additional analyses

Protein was measured by using the bicinchoninic acid method of Smith et al.[10] DNA was measured by the method of Labarca and Paigen.[11] Unless otherwise stated, all reagents were obtained from Sigma.

Statistical analyses

An analysis of variance was performed with the Crunch Statistical Package (Version 4, Crunch Software Corp., Oakland, CA USA).

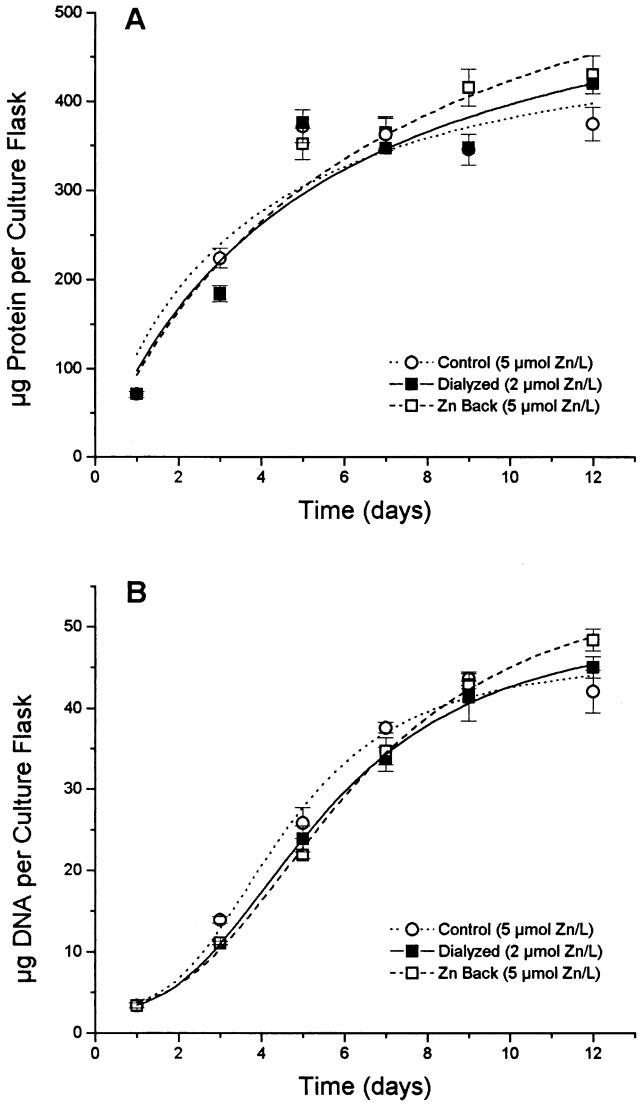

Figure 1 Growth curves of bovine pulmonary artery endothelial cells in three different media. *(A)* Growth rate as indicated by cellular protein in the culture. *(B)* Growth rate as indicated by number of cells (DNA) in the culture. Cells were seeded into T25 culture flasks at 15,000 cells/cm^2. Control medium was 10% fetal bovine serum (FBS) in minimum essential medium (MEM); dialyzed medium was 10% dialyzed FBS in MEM; Zn Back medium was 10% dialyzed FBS in MEM with zinc added back as $ZnCl_2$ to achieve the zinc concentration in the control medium. Results are presented as means ± SE (N = 5 flasks) on each day.

Fisher's protected LSD (least significant difference) test was used for pair-wise comparisons of multiple groups.[12] Differences were considered significant if the *P*-value was less than 0.05. Non-linear least-squares curve-fitting was performed on the growth curves with Origin (Version 4.0, Microcal Software, Inc., Northampton, MA USA).

Results

Figure 1 represents two approaches to evaluating the health of the BPAEC under three different environmental conditions that were employed to influence cellular zinc status. Growth patterns were similar in the three different media compositions. Growth of BPAEC is known to be density-inhibited, and in this study, cellular protein content of the flasks stabilized near day 5, indicating that the cells had occupied all of the available surface space in the flask. Cellular DNA content and the protein:DNA ratio stabilized soon thereafter, indicating that the endothelial cells were becoming a relatively stable monolayer near day 7. Daily visual observations by phase contrast microscopy did not find any significant differences in morphology caused by the different media compositions.

The influence of a low zinc environment on the MT concentration of BPAEC is presented in *Figure 2*. By day 3, cells cultured in medium with dialyzed serum had a lower ($P < 0.01$) MT concentration than control cells cultured in medium with undialyzed serum, and MT remained lower throughout the study. Adding zinc back (as $ZnCl_2$) to the medium containing dialyzed serum did not reestablish control concentrations of MT. The influence of a high zinc environment on the MT concentration of BPAEC is presented in *Figure 3*. MT was higher ($P < 0.05$) in cells grown in 25 and 50 µmol zinc/L than in the control medium on days 3 and 7, but not on day 5.

After the BPAEC were grown to confluence (day 6) in 10% FBS in MEM, they were exposed to a variety of zinc concentrations for 24 hours to examine the effect of this acute exposure on zinc (*Figure 4A*) and MT (*Figure 4B*) content. Significant increases in MT required greater than 50 µmol zinc/L in the medium, unless dexamethasone was added. By comparison to the 6 µmol zinc/L treatment with serum (most analogous to a control group), cellular zinc concentration was lower ($P < 0.05$) after 24 hours in 1 µmol zinc/L and higher ($P < 0.05$) in greater than 50 µmol zinc/L or with dexamethasone added. These results were confirmed in other studies not presented in this report for the sake of brevity.

In studies to examine the influence of factors other than zinc, an increase ($P < 0.05$) in MT concentration was induced by 48-hour incubation with dexamethasone (116% of control) and IL-6 (112% of control). However, a decrease ($P < 0.05$) in MT concentration was observed following a 48-hour incubation with 17β-estradiol (88% of control). IL-1β and cAMP did not significantly influence MT concentration in BPAEC.

Discussion

The primary objective of this study was to manipulate the extracellular zinc environment and to measure the consequences of these manipulations on intracellular zinc and MT concentrations. However, it was imperative that the in vitro environment be within a physiologic range of conditions for the cells. It is our experience that the growth and morphology of BPAEC suffers significantly without 5 to 10% serum in the growth medium, and serum is the principal source of zinc in the medium. We found that the best way to reduce

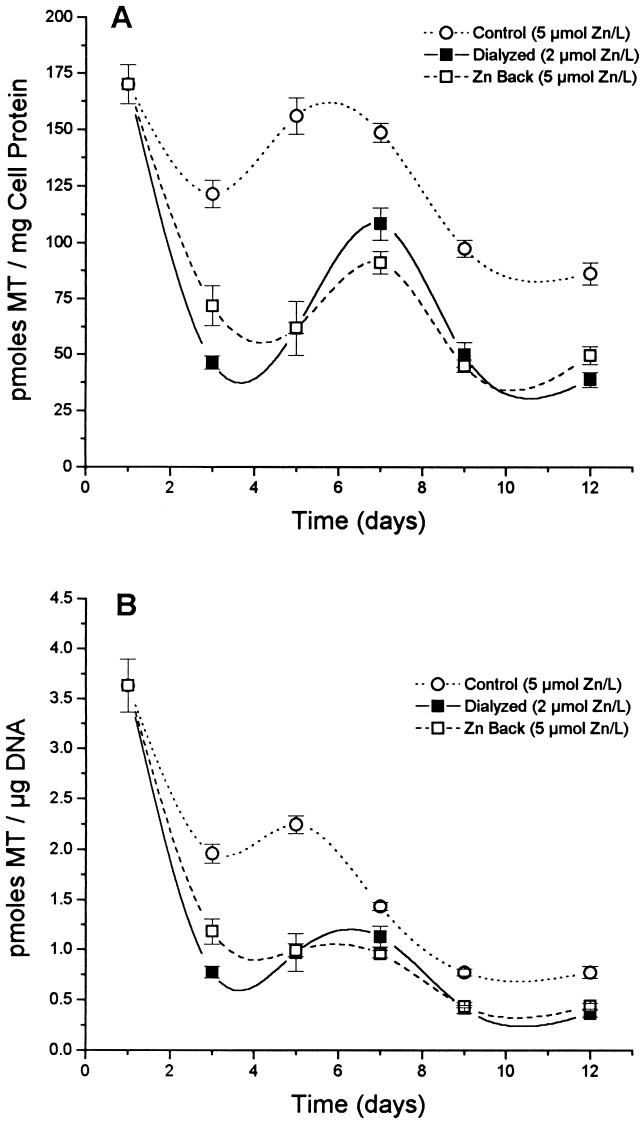

Figure 2 Longitudinal changes in metallothionein (MT) content of bovine pulmonary artery endothelial cells in three different media intended to evaluate low zinc conditions. *(A)* MT content relative to the total cellular protein in the culture. *(B)* MT content relative to the number of cells (DNA) in the culture. Cells were seeded into T25 culture flasks at 15,000 cells/cm^2. Control medium was 10% fetal bovine serum (FBS) in minimum essential medium (MEM); dialyzed medium was 10% dialyzed FBS in MEM; Zn Back medium was 10% dialyzed FBS in MEM with zinc added back as $ZnCl_2$ to achieve the zinc concentration in the control medium. Results are presented as means ± SE (N = 5 flasks) on each day.

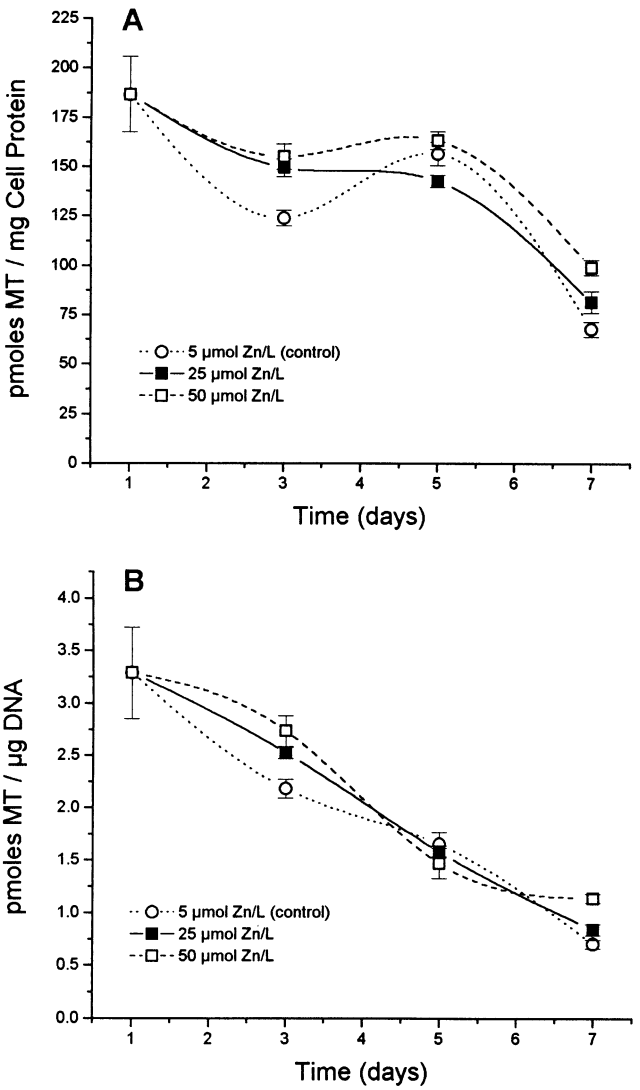

Figure 3 Longitudinal changes in metallothionein (MT) content of bovine pulmonary artery endothelial cells in three different media intended to evaluate high zinc conditions. *(A)* MT content relative to the total cellular protein in the culture. *(B)* MT content relative to the number of cells (DNA) in the culture. Cells were seeded into T25 culture flasks at 15,000 cells/cm^2. Control medium was 10% fetal bovine serum (FBS) in minimum essential medium (MEM); 25 and 50 µmol Zn/L treatments were prepared by adding $ZnCl_2$ to the control medium. Data are expressed as means ± SE (N = 5 flasks) on each day.

the zinc concentration of the medium was to dialyze the serum in an EDTA buffer prior to its incorporation into the culture medium. Dialysis of the serum in a 1,000-MW cutoff dialysis bag effectively removed zinc (based on analysis), but in addition, dialysis removed other small molecular weight serum components. Because the principal source of the major minerals and other nutrients in the experimental medium was not the serum, their concentrations in the final medium were not significantly influenced by serum dialysis. However, it is likely that many other small molecules were removed whose significance to endothelial cell health is unknown. We evaluated the influence of the dialyzed serum on cell growth and morphology, but could not detect any effect.

MT has been detected in most cells examined to date.[3] This report confirms that endothelial cells also express this protein. In addition, it is the first report that shows longitudinal changes in MT concentration in cells grown under various zinc environments and at different stages of growth.

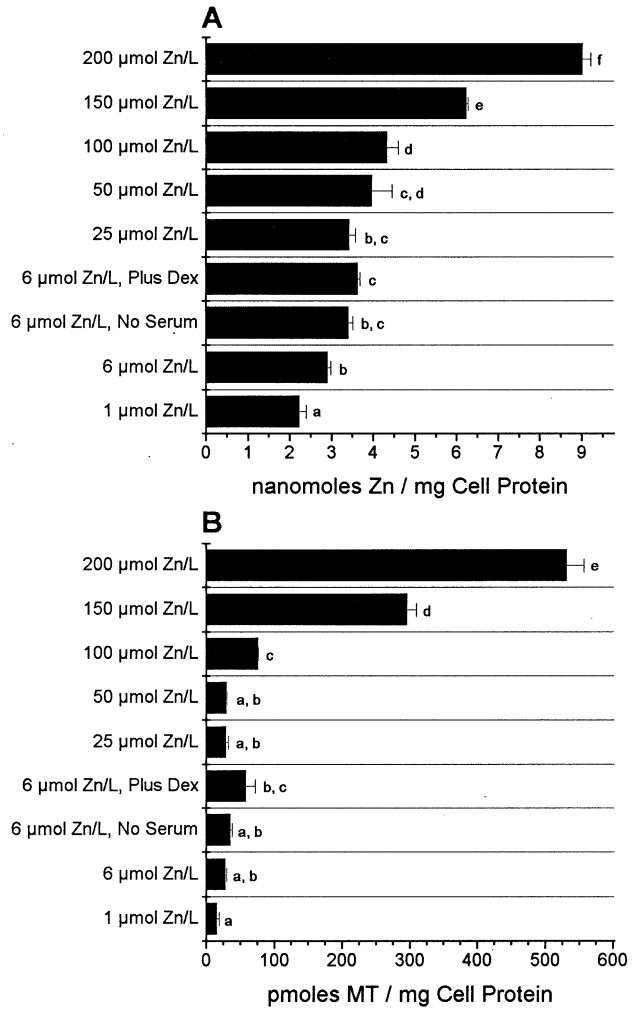

Figure 4 Concentrations of zinc *(A)* and metallothionein (MT) *(A)* in bovine pulmonary artery endothelial cells exposed to media with a variety of treatments for a 24-hour period beginning after growth to confluence (day 6) in medium containing 10% fetal bovine serum (FBS) in minimum essential medium (MEM; 6 µmol zinc/L). All treatment media had 10% dialyzed FBS in MEM, except the one treatment without serum. A low zinc concentration of 1 µmol/L zinc was achieved with 10% dialyzed FBS in MEM. The other media were made by adding $ZnCl_2$ to 10% dialyzed FBS in MEM. Dexamethasone was added at 0.1 µmole/L. Data are presented as means ± SE ($N = 4$ flasks). Bars with different letters have means that are different ($P < 0.05$) from each other.

Relative to total cellular protein, MT concentration was highest soon after the culture was initiated. This agrees with previous observations that MT is highest in rapidly dividing cells.[13] MT concentration in the control medium declined approximately 30% during the phase of rapid growth between days 1 and 3. When the control culture entered confluency, the MT concentration returned to values approximating those on day 1. Thereafter, the MT concentration declined to approximately one-half its zenith and stabilized as the culture became relatively nonmitotic, at least over the period we examined. Cells in the medium containing dialyzed serum had less MT, which remained below that of the control cells throughout the study. Restoring the zinc concentration to that of the control medium did not sustain control concentrations of MT, although it did slow the decline a small but significant amount.

Increasing the zinc concentration in the growth media from 5 to 25 or 50 µmol/L also had little effect on longitudinal changes in cellular MT concentration. The only difference was that the higher zinc concentrations reduced the decline in MT that occurred between days 1 and 3 in the control cells, but this difference was short lived. This delayed decrease in MT in the high zinc environments might reflect the longer half-life of zinc–MT compared with MT without zinc.[14] This evidence suggests that the extracellular concentration is relatively inconsequential in promoting the synthesis of MT in endothelial cells.

Relative to the number of cells in the cultures, as indicated by the amount of DNA, longitudinal changes in the MT concentration were similar in some respects to comparisons made on a total cellular protein basis, but there were also differences. The differences in the MT concentrations presented in the two panels, whether reported relative to protein or DNA, probably reflect the changes that occur in the size and shape of endothelial cells as they grow into confluent monolayers in their culture flasks. Cellular MT concentration in the control medium declined approximately 40% between days 1 and 3. The MT concentration then stabilized for a few days as the culture grew into confluency, before subsequently declining to 21% of its zenith as the culture became relatively nonmitotic. The most rapid rates of growth coincided with the highest concentrations of MT, and presumably with high concentrations of zinc. This could reflect a greater requirement for zinc during periods of rapid growth.

Twenty-four–hour exposure of BPAEC to varying zinc concentrations did not significantly affect the cellular MT content unless the media zinc concentration was greater than 50 µmol/L. The MT concentration was not significantly different between 1 and 50 µmol zinc/L. Kaji et al.[15] also failed to observe an induction of MT with 10 µmol zinc/L in endothelial cells. On the other hand, cellular MT and zinc concentrations increased significantly when the extracellular zinc concentration was 100 µmol/L or higher. A similar response to high zinc exposure was observed with cultured hepatocytes.[16,17] The physiologic relevance of MT induction at this high concentration of zinc is questionable because these concentrations are very unlikely to occur in the extracellular environment of animals. Unlike some studies of previous investigators,[15] we used culture media that contained 10% serum (35 µmol albumin/L). Changes in the extracellular zinc concentration between 6 and 25 µmol/L did not affect the cellular zinc concentration enough to induce a change in MT concentration. This might be because other (unidentified) physiologic mechanisms were sufficient to adequately maintain cellular zinc homeostasis.

Zinc is not the most important media component responsible for regulation of the constitutive concentration of MT in endothelial cells, and serum dialysis effectively removed

other more important regulatory component(s). Possible candidates might include hormonal signals or other MT-binding minerals. Our primary goal in this study was to examine the influence of zinc status on MT concentration; however, we also conducted a few studies that tested the addition of steroids and cytokines to the growth media. MT concentrations were increased in cells incubated with dexamethasone in the medium. This response is similar to that observed by others in selected rat tissues[18–20] and cultured hepatocytes[17] when using glucocorticoids to induce MT production. We also saw an increase in MT with IL-6, but not with IL-1. This observation supports the hypothesis that IL-6 induces the MT response to stress at the cellular level, which is transmitted through the whole organism by IL-1.[21] 17β-Estradiol depressed the MT concentration in endothelial cells, which is similar to the effects recently observed in some human breast cancer cells.[22] The significance of this latter observation awaits further studies on the interaction between gender and zinc status and/or MT. It also should be noted that, although the different responses to these hormones were statistically significant, the quantitative differences were relatively small. Until further studies are performed, we encourage caution in concluding too much physiologic significance from these data.

Zinc is one of many minerals that bind MT, and serum dialysis also could have removed them effectively from the medium. Some of these minerals were returned to the medium in MEM, which contains supplemental calcium and magnesium, but not copper, cadmium, or lead. It is possible that one of these other minerals is preeminent in determining the cellular MT content, but this remains to be demonstrated.

Conclusion

The evidence from this study indicates that endothelial cells have a constitutive concentration of MT that is relatively unaffected by moderate changes in zinc status. It was only when the extracellular zinc concentration exceeded a relatively high threshold that endothelial cells increased their concentration of MT. If MT has a role in supporting tissue zinc homeostasis at the endothelium, it is not directly related to changes in intracellular concentration of MT in the endothelial cell itself. This is dissimilar to the role of MT in supporting zinc homeostasis by intestinal cells.[23] It is possible that the stable concentration of intracellular MT in endothelial cells might serve as a buffer against changes in zinc status, although this scenario is somewhat discounted by the absence of any significant changes in the intracellular zinc content during wide fluctuations of the zinc concentration in the extracellular environment, assuming that the zinc–MT complex remains inside the cell. A more likely possibility is that zinc homeostasis is sustained primarily by other non-MT mechanisms, including zinc transporter proteins in the plasma membrane[24] or vesicular membranes.[25]

Acknowledgments

The authors wish to thank James P. McClung and David J. Rowe for their contributions to serum dialysis and cell culturing. This is scientific contribution number 1990 of the New Hampshire Agricultural Experiment Station. This work was supported in part by National Institutes of Health grant R15 NS35285-01 and in part by University of New Hampshire Undergraduate Summer Fellowships and the UNH Undergraduate Research Opportunity Program.

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