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Regulation of selenoprotein mRNA expression by hormones and retinoic acid in bovine mammary cells

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Short title: Regulation of selenoprotein mRNA expression in bovine mammary cells

Summary

Selenium is essential for maintaining many body functions through the actions of selenoproteins. To find factors regulating selenoprotein biosynthesis in the bovine mammary cell line MAC-T, the effects of supplementation with selenite and also with retinoic acid, insulin, hydrocortisone and prolactin on the mRNA expression of a number of selenoproteins were investigated. It was found that MAC-T cells express glutathione peroxidase (GPx) 1 and 4, thioredoxin reductase 1 and selenoprotein P, but not GPx 3, which is interesting considering that GPx 3 is one of only few selenoproteins detected in milk so far. Addition of selenite to the cell culture resulted in a large increase in GPx 1 expression and an increase in selenoprotein P expression, which is similar to the findings made in other systems investigated. Increased mRNA levels of GPx 1 were also observed in cells treated with insulin and hydrocortisone or with retinoic acid. The expression of thioredoxin reductase 1 was increased in cells treated with retinoic acid, whereas that of selenoprotein P was decreased in cells exposed to insulin. The results indicate that several hormones, selenium, and retinoic acid regulate the biosynthesis of various selenoproteins differently in the bovine mammary cell. The possible implications of the findings for processes related to milk formation and mammary carcinogenesis will need additional investigation. Further study of the detailed mechanisms involved is also necessary.

Keywords: Selenoproteins, MAC-T, retinoic acid, insulin, hydrocortisone, prolactin

Introduction

The selenoproteins containing the amino acid selenocysteine are essential for many body functions. Several selenoproteins were discovered fairly recently [1], and the functions of many of them are so far not fully elucidated. The most extensively studied selenoproteins are the glutathione peroxidases (GPx), the thioredoxin reductases (TrxR), the iodothyronine deiodinases (DI) and selenoprotein P (SeP) [2-5].

Dairy products are an important source of selenium in the Swedish diet supplying on average 17% of the selenium intake [6]. Little is known about the formation of selenoproteins in the mammary gland and GPx 3 is the only selenoprotein thus far detected in milk [7, 8] together with an observation of SeP in mouse milk [9]. In a study of mouse mammary tissue in culture the combined addition of the lactogenic hormones prolactin, insulin and hydrocortisone was found to stimulate the production of the mouse selenocysteine transfer RNA gene transcription activating factor, mStaf, and the subsequent transcription of the selenocysteine transfer RNA, $tRNA^{Sec}$, which is required for selenoprotein synthesis [10]. Prolactin is one of the main hormones regulating mammogenesis and lactation and it stimulates the production of milk proteins, lactose and lipids [11, 12]. The combination of prolactin and the metabolic hormones insulin and hydrocortisone is thus commonly used *in vitro* for studies of mammary cell lines or mammary explants [13-16]. The effects of the combined hormonal treatment on the biosynthesis of individual selenoproteins and the corresponding effects of single hormones are not known.

Another substance found to affect both mammary cell biology and selenoprotein mRNA expression is all-*trans* retinoic acid (RA), which has profound effects on the expression of many genes [17]. Chu *et al*. found that the addition of RA increased the expression of GPx 2 mRNA in a human mammary cell line and other studies have shown that treatment by RA increased the activity of DI 1 and DI 3 in cell systems [18-20]. Other experiments have shown that RA inhibited the proliferation of both primary bovine mammary epithelial cells and mammary cell lines in culture [21-23]. One of the bovine mammary cell lines known to be affected by RA is MAC-T, which is an immortalized alveolar cell line. When cultured on collagen, the cells may form secretory dome structures similar to those found in bovine mammary alveoli *in vivo*, they may produce α- and β-caseins and are responsive to prolactin [16]. RA is also known to promote differentiation, cell cycle arrest and apoptosis in many

cancer cell lines and much research is currently being done regarding the inhibitory effect of RA and other retinoids on breast cancer [24-27].

The aim of this study was to investigate the effects of selenium, RA and the lactogenic hormones prolactin, hydrocortisone and insulin on the mRNA expression of individual selenoproteins in the mammary gland. The bovine mammary cell line MAC-T was used as a model system.

Experimental

Chemicals

Ovine prolactin (P), bovine insulin (I), hydrocortisone (H), rat tail collagen, all-*trans* retinoic acid, selenite, NADPH, reduced glutathione, *tert*-butyl hydroperoxide, glutathione reductase, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and mercaptosuccinic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Gold thioglucose was purchased from Research Diagnostics (Flanders, NJ, USA). Other chemicals used were of analytical or molecular biology grade.

Cell culture and harvest

MAC-T cells (a kind gift from Dr. Stig Purup, Research Centre Foulum, University of Aarhus, Denmark) were cultured in Dulbecco's Modified Eagle Medium, containing 4.5 g/L glucose (Invitrogen, Carlsbad, CA, USA), 10% foetal bovine serum (FBS) (Biochrom AG, Berlin, Germany), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Biochrom AG). Two batches of FBS containing 0.48 (expt in Figure 1) and 0.71 µmol/l of Se (expt in Figure 2) were used. Cells received new medium every 2-3 days and were subcultivated twice a week. PBS and trypsin/EDTA used for the washing and subcultivation were also purchased from Invitrogen. The cells used in the present experiments were between passages 8 and 19. The cells were routinely maintained in plastic flasks, but before the different experiments the cells were cultured in FBS-containing medium until confluent in plastic dishes coated with a thin layer of rat tail collagen (9 µg/cm^2). Thereafter, cells were maintained in serum-free medium for one day before the treatments. At the end of the experiment the cells were harvested for RNA extraction by first removing the medium and washing the cells briefly in PBS and then scraping the cells in the lysis buffer from the RNeasy Mini Kit (Qiagen GmbH, Hilden,

Germany). For enzyme measurement, the cells were harvested by scraping in a buffer containing 80 mmol/L potassium phosphate and 5 mmol/L EDTA, pH 7.6.

MessengerRNA expression analysis

Total RNA was purified from the cells using the RNeasy Mini Kit (Qiagen). The cells were lysed directly in the culture dish and homogenisation was performed using QIAshredder columns (Qiagen). The total RNA concentration was determined by the absorbance at 260 nm in a Beckman Coulter DU 640 Spectrophotometer (Fullerton, CA, USA). Messenger RNA was extracted using the Oligotex mRNA kit (Qiagen) according to the manufacturer's instructions, starting with the same amount of total RNA for each sample in each particular experiment. The extracted mRNA was divided into two aliquots and run on two gels thus creating two membranes containing the same samples. In experiments using analysis of total RNA, samples were also run on duplicate gels and the same amount of RNA, usually 10 µg, was loaded for each sample within an experiment.

The electrophoresis, blotting, probe labelling and hybridisation were carried out essentially as described previously [28]. In short, the RNA samples were separated on a 1% agarose gel containing formaldehyde and MOPS buffer according to the protocol suggested in the RNeasy Mini handbook, but without the addition of ethidium bromide to the gel. The separated samples were then transferred to a neutral nylon membrane (Hybond-N, GE Healthcare, Chalfont St. Giles, UK) by upward capillary transfer in a 20X saline sodium citrate buffer (20X SSC, 3 mol/L NaCl, 0.3 mol/L sodium citrate, pH 7.0) and crosslinked to the membrane by exposure to UV light. The membranes containing samples of total RNA were stained in a methylene blue solution to visualise the 18S and 28S bands and then rinsed in 2X SSC and either hybridised directly, or dried and stored at 8 ^{o}C. The cDNA probes were labelled with ^{32}P using the Rediprime II Random Prime Labelling System (GE Healthcare) and purified by eliminating unbound ^{32}P using a Microspin S-200 column (GE Healthcare). The prehybridisation and hybridisation were performed in a hybridisation oven at 65 ^{o}C, using a phosphate hybridisation buffer (0.25 mol/L sodium phosphate, 1 mmol/L EDTA, 0.24 mol/L SDS, 10 g/l BSA, pH 6.8). After the prehybridisation, most of the solution was discarded and new hybridisation buffer containing labelled probe was added. Hybridisation was performed over night, after which the blots were washed repeatedly with wash buffers containing 3.5 mmol/L SDS and decreasing concentrations of SSC, while at 65 ^{o}C. The membranes were then placed against a storage phosphor screen for exposure. After detection, the bound probe

was removed from the membranes by pouring boiling 3.5 mmol/L SDS on the membranes and letting them cool. Each membrane was generally hybridised with two of the selenoprotein probes and two housekeeping genes; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. Both mRNA and total RNA were used because the transcript for TrxR 1 migrated with the 28S ribosomal RNA, which appeared to inhibit the hybridisation of the probe to the target, resulting in a sharper and stronger band of TrxR 1 on mRNA membranes. Total RNA membranes were also used to assess the stability of the housekeeping genes.

The probes for the selenoproteins GPx 1, 3 and 4, SeP and TrxR 1 and the housekeeping gene β-actin were prepared as described previously [28]. The GAPDH probe, was created with a reverse transcriptase PCR reaction (Titan One Tube RT-PCR System and Nucleotide mix from Roche Diagnostics GmbH, Mannheim, Germany) using the forward primer TGA CCC CTT CAT TGA CCT TC, the reverse primer GGT CAT AAG TCC CTC CAC GA and an annealing temperature of 55 ^{o}C, resulting in a 427 bp cDNA probe.

Enzyme activity

Cell samples were lysed prior to the enzyme assays by sonication in ice (3 times for 5 s at 20% pulsar, power output 3 using a Branson sonicator) followed by centrifugation (3500 rpm, 10 min, 4 ^{o}C) in order to eliminate cell debris. The protein concentration was measured using a modified Lowry procedure (Kit no TP0300 from Sigma-Aldrich) in a 96-well plate format at 620 nm. The GPx activity assay used was adapted from a previously described method [29]. The sample was added to the reaction mixture and incubated for four minutes at 37 ^{o}C after which *tert*-butyl hydroperoxide was added, resulting in the following final concentrations in the cuvette: 50 mM potassium phosphate buffer (pH 7.6), 0.63 mmol/L reduced glutathione, 0.25 mmol/L NADPH, 5 mmol/L EDTA, 5 μg/ml glutathione reductase and 0.1 mmol/L *tert*-butyl hydroperoxide. Starting one minute after the last addition, the absorbance at 340 nm was measured for four minutes at 37 ^{o}C in an Ultrospec 3000 (Amersham Biosciences, Uppsala, Sweden). Duplicate measurements were performed for most samples. To evaluate the non-GPx dependent NADPH oxidation, mercaptosuccinic acid (4 mmol/L) was added to a sample to create a non-GPx blank, and it was found that this oxidation was negligible. One enzyme activity unit (U) was defined as one μmol of NADPH oxidised per min.

The assay of TrxR activity was based on a method in which the reduction of DTNB was monitored at 412 nm [30]. The TrxR activity was defined as the activity in the sample minus the non-TrxR dependent activity determined by addition of the TrxR inhibitor gold thioglucose to a sample blank. The reaction mixture and the sample were equilibrated separately at 37 ^{o}C for three min and then mixed. Gold thioglucose or water was added to parallel samples and absorption measurement was started at 37 ^{o}C in an Ultrospec 3000. After five min, NADPH was added to the cuvettes resulting in the following final concentrations: DTNB 5 mmol/L, EDTA 12 mmol/L, BSA 0.2 g/l, NADPH 0.2 mmol/L, potassium phosphate buffer 130 mmol/L pH 7 and in sample blanks, 20 μmol/L gold thioglucose. The reaction was monitored for 30 min. One enzyme activity unit (U) was defined as one μmol of DTNB reduced per min. Due to the large amount of sample required for this assay, only one sample could be measured.

Experimental setup

Selenium supplementation. Cells were grown on collagen until they were confluent in medium containing only 5% FBS to reduce its selenium level. Serum-free medium was added for one day followed by incubation in 0, 5, 10, 50, 100 or 500 nmol/L sodium selenite for two days in serum-free medium. To investigate the response time of MAC-T cells to selenium, cells were cultured to confluence and washed with serum-free medium as above and then treated with serum free-medium, containing 100 nmol/L sodium selenite, for 6-48 h and compared to non-supplemented cells. Duplicate samples were used for each time point, and all the cells were harvested at the same time, i.e. all cells were cultivated for the same length of time and the selenite supplement was added at different times.

Lactogenic hormones. To investigate the effect of lactogenic hormones on the production of selenoproteins, MAC-T cells were grown until confluent in 10% FBS on collagen-coated dishes and then treated with serum-free medium containing prolactin (5 μg/ml), insulin (5 μg/ml) and hydrocortisone (1 μg/ml) in different combinations for three days. In one set of experiments cells were treated with prolactin (P), or insulin and hydrocortisone (IH), or prolactin, insulin and hydrocortisone (PIH) and compared to untreated cells. In the second set of experiments cells were treated with insulin (I) or hydrocortisone (H) or insulin and hydrocortisone (IH) and compared to untreated cells. All cells received selenium supplementation in the form of 100 nmol/L sodium selenite in the medium and each treatment was made in triplicate.

Retinoic acid. MAC-T cells were grown on collagen with 10% FBS added to the medium, until confluency and then they received serum-free medium. One day later, the cells received either serum-free medium supplemented with 1 μmol/L RA and 25 nmol/L selenite or medium supplemented only with 25 nmol/L selenite (in triplicate). The cells were harvested after three days of treatment.

Statistical calculations

To determine any significant differences in mRNA expression and enzyme activity between treatments, the nonparametric Kruskal-Wallis H and Mann-Whitney U tests were used. For a difference to be considered significant, $p \leq 0.05$ and a mean difference from the control of at least 30% were required. The SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used to perform the calculations.

Results

Effects of sodium selenite

All the five selenoprotein genes assayed in this study except for the extracellular GPx 3 were found to be expressed in the MAC-T cells. After supplementing the cells with sodium selenite, the mRNA expression of GPx 4 and TrxR 1 was unchanged, while the expression of SeP and GPx 1 was found to increase when increasing amounts of selenium were added (Figure 1). The expression of both SeP and GPx 1 appeared to reach a plateau at 50 nmol/L selenite and the increase in expression was more than twofold for SeP and more than fourfold for GPx 1, compared to that of the untreated cells. Also when the MAC-T cells were supplemented with 100 nmol/L of selenite for different lengths of time, it was found that the mRNA expression of TrxR 1 and GPx 4 was not affected by selenium. The expression of GPx 1 and SeP were also in this experimental setup increased by selenite supplementation, that of GPx 1 reaching a plateau after 24 h and that of SeP slightly later (Figure 2). The extent of the changes observed in this experiment was slightly higher than in the experiment investigating different selenium concentrations but this was not related to the selenium content of the FBS used in the initial phase. Selenium supplementation did not appear to have any effect on the transcription level of β-actin or GAPDH mRNA. The latter was used as the reference gene for the selenium supplementation studies.

Effects of lactogenic hormones

The cells treated with insulin generally gave a higher yield of total RNA and protein than the control cells or the cells treated with only prolactin. The total RNA obtained from the latter cell samples was on average 63% of the amount of total RNA extracted from cells treated with insulin. In the experiments with lactogenic hormones, the expression of β-actin mRNA appeared to be unaffected while that of GAPDH was increased by the addition of insulin and consequently β-actin was used as the reference gene in these experiments. In cells treated with insulin only or in combination with other hormones, an approximate reduction of 45% in the mRNA level of SeP compared with that in control cells (normalised to β-actin expression) was observed (I, $p=0.001$; IH, $p<0.001$; PIH, $p=0.001$). There was no apparent difference in the decrease in SeP expression for cells receiving the combinations of hormones compared to the ones receiving insulin alone (Figure 3). In contrast, there was an increase of about 55% in the mRNA levels of GPx 1 in cells treated with insulin and hydrocortisone compared with the control cells, although it was difficult to determine the contribution of each hormone to this effect (Figure 3). On the other hand, there was no significant difference in GPx activity expressed as U per gram of total protein between samples in this experiment, but an increase of about 95% in enzyme activity per millilitre of sample was seen in samples receiving the IH and PIH treatments ($p=0.01$ and $p=0.004$, respectively), with a corresponding increase in total protein (Table 1). The mean GPx activity in MAC-T cells was 16 U/g of protein. No apparent differences in the mRNA expression of TrxR 1 and GPx 4 or in TrxR activity per gram of protein were found after the addition of lactogenic hormones. The same twofold increase in TrxR activity per millilitre in the samples receiving the IH and PIH treatments, as seen for the GPx activity, was seen here as well ($p=0.046$). The mean TrxR activity was about 0.5 U/g of protein.

Effects of retinoic acid

In these experiments it appeared that RA increased the expression of β-actin mRNA in the cells, while that of GAPDH was less affected. The level of GAPDH mRNA did however appear to be slightly elevated in cells treated with RA, so both the expression in relation to GAPDH and the absolute values of expression from the membranes were used to evaluate the results. A mean increase of about 40% in mRNA expression of GPx 1 ($p<0.001$) and a mean increase in TrxR 1 of about 75% ($p=0.001$), were found when using GAPDH as the reference gene. Using the absolute values, the changes were even larger (Figure 4). The mean GPx activity was 13 U/g of protein and as in the experiments with the lactogenic hormones, no

significant difference in GPx activity per gram of total protein was found after the addition of RA. The TrxR activity in these samples was slightly lower than in the experiments using added hormones, and no significant differences in enzyme activity per gram of protein could be verified. Addition of RA caused slight increases in total RNA and total protein.

Discussion

Effects of selenite

The experiments with sodium selenite supplementation were performed partly to characterise the experimental system, and to investigate the usefulness of MAC-T cells for this type of study. The results showed that GPx 1 mRNA expression was strongly affected by selenite supplementation. This is consistent with prior studies showing that selenium deficiency leads to a reduction of GPx 1 expression down to 9-35% of the selenium-sufficient expression in different systems [31-34] compared to about 20% found in the present study. The effect of the selenium supply on GPx 1 mRNA levels has also been observed in whole bovine mammary tissue [28]. Selenite supplementation also increased the expression of SeP mRNA in MAC-T cells 2-3 fold. This is a slightly greater change, but still in the same range as has been previously found in arterial cells and kidney [31, 34]. A tendency towards a difference in SeP mRNA expression due to selenium status was also found in bovine mammary tissue [28]. The expression of SeP in the liver has, however, been found to be more stable [31, 33]. The mRNA level of GPx 4 appeared to be unaffected by selenium supplementation in MAC-T cells which is consistent with other studies. The expression of TrxR 1 mRNA has been found to be slightly reduced by selenium deficiency in some systems but increased in others [33-35]. The TrxR 1 mRNA level in MAC-T cells remained virtually unaltered by selenium supplementation in the present study. GAPDH, the reference gene used here, has also been found to be unaffected by selenium status in other studies [33].

GPx 3 is one of only few selenoproteins so far detected in milk, the other one being SeP [9], but it was not found to be expressed in MAC-T cells. The validity of the observation is supported by the fact that a strong signal of GPx 3 was observed in kidney using the same method [28]. This could indicate that the GPx 3 found in milk is transported to milk and not produced in the alveolar cells. A similar finding has been made in three human breast epithelial cell lines [36]. However, the mRNA of GPx 3 has been detected in some other breast carcinoma cell lines [37] indicating that it has a variable expression in different

mammary cell lines. GPx 3 expression has been detected in bovine, human and mouse mammary tissue [28, 37], but further investigation is required to determine which cells in the mammary gland that are responsible for the production of this enzyme.

These results show that selenoproteins in MAC-T cells respond to selenite supplementation and that the changes in their mRNA levels are similar to the ones found in bovine mammary tissue and other systems. The MAC-T cell is therefore a useful model system for studying selenoprotein biosynthesis. One difficulty is that these cells will not proliferate in the absence of FBS, which may contribute considerable amounts of selenium in the cell culture medium. It is therefore difficult to deplete the cells of selenium extensively.

Effects of lactogenic hormones

The increase in total RNA observed in insulin-treated cells has also been documented in rat primary hepatocytes, in which it was found that insulin deprivation reduced the total RNA levels to 63% of that of cells treated with insulin [38], which is the same value as found in the present study. Hsu *et al.* also observed that the mRNA/total RNA ratio was not affected by the insulin treatment and the finding of a constant β-actin mRNA expression after the different treatments suggests that this might be the case here as well. The increase in GAPDH expression after the addition of insulin has also been found in other studies and insulin-responsive elements have been found in the human GAPDH gene [39, 40]. The approximate 55% increase in GPx 1 mRNA expression found in samples treated with insulin and hydrocortisone was not matched by an increase of GPx activity, which is probably mainly derived from GPx 1, when normalising against total protein. Instead there was a total increase in GPx activity of about 95% in these samples. It is hard to judge whether the increased protein content after the addition of hormones was due to increased cell proliferation or increased protein synthesis. Cell counts were not performed but previous information indicated that the MAC-T cells would not be expected to proliferate in serum-free medium, and thus an increase due to protein metabolism is most probable. There was no apparent effect of prolactin on selenoproteins in these cells. The profound effect of the hormones on total RNA and total protein in the MAC-T cells thus makes it difficult to determine the effects of mixtures of hormones on selenoprotein mRNA expression. Still it can be concluded that the mRNA expressions of SeP, GPx 1 and 4, and TrxR 1 in MAC-T cells are affected in different ways by insulin and hydrocortisone supplementation.

Previously the addition of insulin to adipocytes was found to inhibit GPx activity [41] which is in contrast to our findings in mammary cells. Inhibition of GPx activity by other methods in that system was linked to increased insulin resistance possibly mediated by increased GSH levels and decreased phosphorylation of the insulin receptor [41]. In animals and cells overexpressing GPx several contrasting changes in glycaemic control and insulin function have also been observed [42]. In mice overexpressing GPx 1 in pancreatic islets the administration of streptozotocin led to a much higher content of insulin in islets of overexpressing animals than in control animals [43]. Other studies showed that mice overexpressing GPx 1 developed insulin resistance and obesity [41]. Regarding SeP, insulin was found to inhibit SeP expression and secretion in hepatoma cells which was mediated by a concerted action of several factors, whereas dexamethasone stimulated both processes [44]. We also found a decreased SeP expression after the addition of insulin but no effect of hydrocortisone. In hepatoma cells insulin was also found to ameliorate the activity of the SeP promoter activity which probably was mediated by an inhibition of FoxO [45].

Effects of retinoic acid

In MAC-T cells treated with RA, elevated mRNA levels were found for both GPx 1 and TrxR 1. In the human breast cancer cell line MCF-7, addition of RA resulted in an increase in mRNA levels of GPx 2 and also a non-significant increase in GPx 1 of about 50% [19], which is in the same range as found in the present study. These authors used β-actin as the reference gene, which was not appropriate in the present study. Chu *et al.* [19] also found that the GPx activity increased in RA-treated cells after six days in culture, the increase becoming evident on the fourth day. In the present study, no significant increase in activity was found, but three days in culture may not have been sufficient to detect any possible increase in activity. It is not known if MAC-T cells express GPx 2. An increase in TrxR 1 mRNA was also found in the present study, although the absolute numbers are difficult to evaluate due to the possible slight increase in mRNA levels of the reference gene, GAPDH (Figure 4). As concluded above regarding hormones, also RA affects the regulation of mRNA levels differently for the various selenoproteins studied. Recently we studied the effect of RA on selenoprotein formation in MAC-T cells using a isotope-proteomic approach which also indicated that RA affected the pattern of selenoproteins formed [46]. Due to the low abundance of most selenoproteins and their co-migration there was an ambiguity in identifying in some of them. Further experiments are necessary to optimise this methodological approach.

The GPx activity was found to be around 15 U/g of protein in the experiments using RA and lactogenic hormones, which was somewhat lower than the mean activity found in bovine mammary tissue (mean 33 U/g, range 4-62 U/g) [28]. The TrxR activity in the present samples, 0.4-0.5 U/g of protein, was in the low range of that found in bovine mammary tissue (mean 1.5, range 0.4-2.4 U/g) and considerably lower than the activity found in the liver of selenium-sufficient rats (7 U/g of protein) [28, 30].

Further studies are necessary to unravel the mechanisms of action and the physiological importance of the changes observed. The novel findings indicate that several hormones, selenium, and RA regulate selenoprotein biosynthesis in the bovine mammary cell line studied. These findings might be of clinical importance especially for lactation under pathophysiological conditions, e.g., for diabetic mothers, mothers with dysregulated prolactin levels, mothers with severely low selenium status or mothers to very preterm children. Respective studies need to be performed to test these hypotheses. The possible implications of the findings for processes related to mammary carcinogenesis are a second interesting issue which should be addressed in future studies

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Table 1. Glutathione peroxidase activity in MAC-T cells.

The glutathione peroxidase activity expressed per gram of total protein and per mL of sample in MAC-T cells treated with prolactin, insulin and hydrocortisone for three days is shown (n=6).

	U/mL	U/g protein
Control	0.0212 (0.0033)	13.74 (2.51)
Prolactin ,	0,0212 (0.0031)	20.59 (6.92)
Insulin + hydrocortisone	0.0406 (0.0090)[a]	16.41 (4.69)
Prolactin + Insulin + hydrocortisone	0.0418 (0.0069) [b]	15.19 (2.64)

[a]P=0.010 vs. control

[b]P=0.004 vs control

Legends to Figures

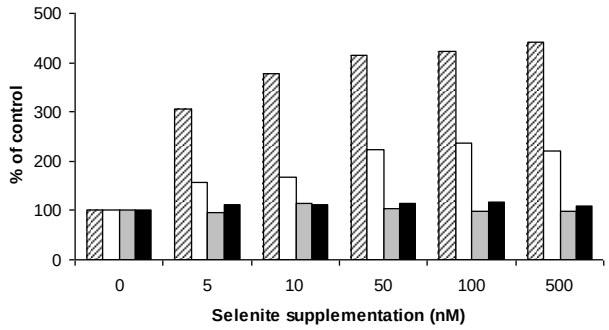
Figure 1. Effects of selenium concentration on the mRNA expression of selenoproteins.
The mean expression (n=2) of glutathione peroxidase 1 (hatched), selenoprotein P (white), thioredoxin reductase 1 (gray) and glutathione peroxidase 4 (black) in MAC-T cells supplemented with different concentrations of selenite for 48 h is shown. The expression was normalised to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Figure 2. Expression of selenoprotein mRNA:s in cells supplemented with selenium for different time intervals.
The mean expression (n=2) of glutathione peroxidase 1 (hatched), selenoprotein P (white), thioredoxin reductase 1 (gray) and glutathione peroxidase 4 (black) in MAC-T cells supplemented with 100 nmol/L of selenite for different time intervals is shown. The expression was normalised to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Figure 3. Changes in selenoprotein mRNA expression in hormone-treated cells.
A. The expression of glutathione peroxidase 1 (hatched) and selenoprotein P (white) in MAC-T cells treated with prolactin (P, 5µg/ml), insulin (I, 5µg/ml), hydrocortisone (H, 1 µg/ml) and combinations thereof for 3 days, compared to untreated control (C) is shown. All cells received 100 nmol/L selenite throughout the experiment. The expression was normalised against that of β–actin. Significant differences from the control are illustrated by *** $p \leq 0.001$. C: n=12; P: n=6; I: n=6; H: n=6; IH: n=12; PIH: n=6.
B. Corresponding Northern blot images illustrating the changes in selenoprotein expression in hormone-treated and control cells.

Figure 4. Northern blot images illustrating the changes in selenoprotein mRNA expression in retinoic acid-treated cells.
The expression of glutathione peroxidase 1 (GPx 1) and thioredoxin reductase 1 (TrxR 1) in MAC-T cells treated with retinoic acid (RA) for three days compared with untreated control (C) is shown. The corresponding expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the same membrane is shown for comparison.

Figures



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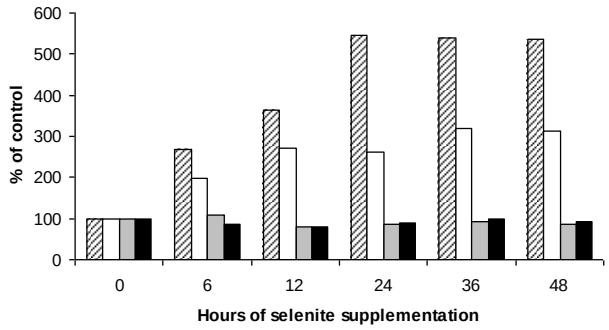
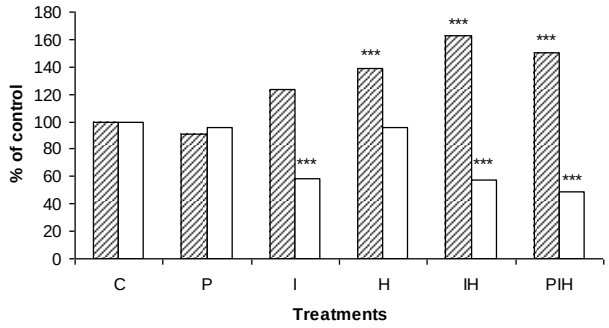


Figure 2. Expression of selenoprotein mRNA:s in cells supplemented with selenium for different time intervals.

The mean expression (n=2) of glutathione peroxidase 1 (hatched), selenoprotein P (white), thioredoxin reductase 1 (gray) and glutathione peroxidase 4 (black) in MAC-T cells supplemented with 100 nmol/L of selenite for different time intervals is shown. The expression was normalised to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

A



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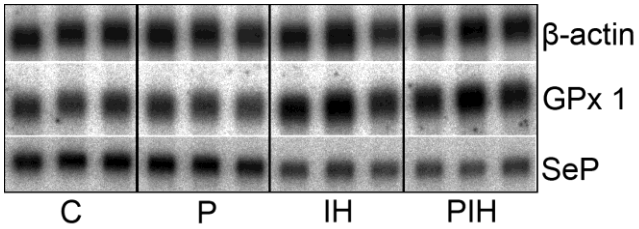

Figure 3. Changes in selenoprotein mRNA expression in hormone-treated cells.

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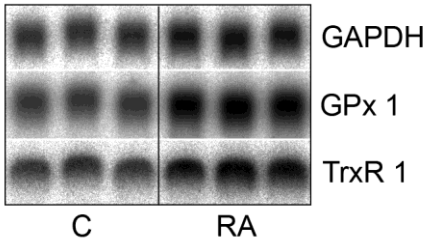

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