RESEARCH COMMUNICATION
Thyroidal extracellular glutathione peroxidase: a potential regulator of thyroid-hormone synthesis

A. Forbes HOWIE,* Simon W. WALKER,* Björn ÅKESSON,† John R. ARTHUR‡ and Geoff J. BECKETT*§
*Cellular Endocrinology Unit, University Department of Clinical Biochemistry, Royal Infirmary, Edinburgh EH3 9YW, U.K.,
†Department of Applied Nutrition and Food Chemistry, Chemical Centre, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden,
and ‡Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, U.K.

Human thyrocytes were found to synthesize and secrete the selenoenzyme extracellular glutathione peroxidase (E-GPX), a process which was controlled by the Ca^{2+}/phosphoinositol second-messenger cascade. The potential involvement of thyroidal E-GPX in the regulation of thyroid-hormone synthesis and in the protection of the thyrocyte from peroxidative damage is discussed.

INTRODUCTION

The human thyroid gland contains higher concentrations of selenium (Se) than the liver and other human organs [1]. In conditions of Se deficiency induced either *in vivo* by dietary manipulation [2] or *in vitro* by using Se-deficient culture medium, thyrocytes are able to retain the trace element by an energy-dependent process [3,4]. These observations suggest that Se has a number of crucial roles within the thyroid gland.

Many of the functions of Se are exerted through the expression of specific selenoenzymes. Labelling of thyroid tissue both *in vivo* [2] and *in vitro* [5] with [^{75}Se]selenite has demonstrated that at least ten selenoproteins are expressed by the gland. Whilst most of these selenoproteins remain to be characterized, two seleno-enzymes, type I iodothyronine deiodinase (IDI) and cytoplasmic glutathione peroxidase (Cy-GPX), are considered to have important roles within the thyroid. IDI catalyses 5′-mono-deiodination of the prohormone thyroxine (T_4) to produce the metabolically active tri-iodothyronine (T_3) and in some species, including man, the enzyme may provide an important source of thyroidal T_3 [6]. It has also been suggested that intracellular glutathione peroxidase (GPX) protects the thyroid from H_2O_2-induced peroxidative damage [7].

The thyroid synthesizes H_2O_2 at the apical membrane of the thyrocyte, and the H_2O_2 is then utilized by thyroperoxidase (a non-selenoenzyme) for the oxidation of iodide and subsequent incorporation of iodine into the tyrosine residues on thyro-globulin situated in the colloid of the follicular lumen. In the presence of an adequate iodine supply, H_2O_2 production is thought to be the rate-limiting step in thyroid-hormone synthesis [7–11]. Control of H_2O_2 generation is exerted by both the cyclic AMP and Ca^{2+}/phosphoinositol signalling cascades, with the latter appearing to be the most important stimulator [7–11]. Excessive concentrations of H_2O_2 are harmful to the thyrocyte, and the GPX and catalase enzyme systems may act within the gland to prevent the accumulation of toxic levels. It has been

suggested that the thyroid atrophy found in myxoedematous cretinism may arise from prolonged combined Se and iodine deficiency since, in this situation, H_2O_2 production is increased, and protection from oxidative damage is lost as a consequence of impaired GPX synthesis brought about by lack of Se [7].

Four Se-dependent GPXs have been identified. Each has a single selenocysteine residue encoded by a TGA triplet, but each is expressed by a different gene and is immunochemically distinct. Three of these GPXs are intracellular and one is extracellular. The three intracellular enzymes are the ubiquitous cytoplasmic GPX (Cy-GPX), a membrane-associated GPX which can metabolize phospholipid hydroperoxides (PH-GPX) and gas-trointestinal GPX, an enzyme expressed only in liver and intestine (for reviews, see [12–14]).

A single extracellular GPX (E-GPX) has been identified and purified from plasma [12,15–17]. Plasma GPX comprises four identical subunits each having a molecular mass of approximately 24 kDa and is functionally, immunologically and structurally different from Cy-GPX and the other Se-dependent peroxidases [12]. Extracellular thioredoxin, glutaredoxin and thioredoxin reductase can act as reductants for plasma GPX in addition to GSH [18]. In the rat and man the major source of plasma GPX is the renal proximal tubules [19]. Although it has been suggested that plasma GPX may have an antioxidant role in plasma, another possibility is that the enzyme acts in the extracellular space of the tissues which secrete the enzyme; the enzyme has thus been named more appropriately as 'extracellular glutathione peroxidase' (E-GPX) [12]. The tissues identified as secretors of E-GPX are kidney, placenta and bronchio-epithelial tissue [12,19–23]. These tissues are under high degrees of oxidative stress and thus may require protection from injury caused by peroxide and oxygen-radical formation. Certain cultured trans-formed cells of kidney and lung origin also secrete the enzyme [21–23].

In the present paper we report that human thyrocytes grown in primary culture synthesize and secrete E-GPX into the culture

medium. Results are also presented to show that this process is under hormonal control, being negatively regulated by the Ca^{2+}/phosphoinositol signalling pathway.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM), Earle's balanced salt solution (EBS), penicillin, streptomycin, amphotericin B and glutamine were all obtained from ICN Flow (Costa Mesa, CA, U.S.A.). Collagenase was purchased from Worthington Biochemicals Corporation via Lorne Laboratories (Twyford, Berks., U.K.), and dispase supplied by Boehringer Mannheim U.K. (Lewes, East Sussex, U.K.). [75Se]selenite was obtained from the University of Missouri (Columbia, MO, U.S.A.). All other reagents, including control processed serum replacement 1 (CPSR-1), were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.).

Isolation and culture of human thyrocytes

Human thyrocytes were isolated from thyroid tissue (surplus to routine histopathological examination) from patients with functional nodules undergoing thyroid surgery. Cells were isolated using a modified version of a dog thyroid cell culture method [24] as previously described [25]. Briefly, the tissue was finely minced with scissors and the resulting fragments washed four times with EBS before digestion for 2 h in 50 ml of an enzyme cocktail containing dispase (0.5%, w/v), trypsin (0.25%, w/v), collagenase (0.1%, w/v) and BSA (2%, w/v) in EBS. Following digestion, an equal volume of EBS was added and the mixture filtered through a 100 μm-mesh gauze to remove undigested tissue. The resulting filtrate, containing released thyroid cells, was centrifuged at 450 g for 15 min to pellet the thyrocytes. The pellet was washed twice with EBS and the cells finally resuspended in 50 ml of DMEM containing 10% (v/v) CPSR-1 (fetal-calf serum treated to remove immunoglobulins and endotoxins), penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml) and glutamine (2 mM). The cell suspension was then filtered through a 30 μm-mesh gauze and the cell yield measured with a modified Neubauer haemocytometer. Finally the thyrocytes were plated out in DMEM/10% CPSR-1 into 75 cm^2 flasks in 25 ml of medium at a density of 10^7 cells/flask and incubated at 37 °C in an atmosphere of 5% CO_2.

After an overnight incubation, the culture medium was removed, the cells washed twice with EBS and fresh culture medium added. The thyrocytes were then grown for a further 2 days, after which time they had achieved confluence. The cells were then washed with EBS and cultured for a further 72 h with DMEM (with no CPSR-1) in the presence of 0.02 MBq/ml [75Se]selenite. Mimics of the cyclic AMP and Ca^{2+}/phosphoinositol second-messenger cascades and the isoprenoid fungal metabolite Brefeldin A (a compound which blocks intracellular transport of secretory proteins and thus prevents their export) were also added to the culture medium for the final 72 h incubation at the specified concentrations. After this 72 h period the culture medium was removed, centrifuged at 3000 g for 20 min and dialysed over 2 days against 2 litres of 60 mmol/l Tris buffer, pH 7.8, containing 1 mM dithiothreitol and 1 mM EDTA, with four changes of buffer being used over the dialysis period. This dialysis step was included to remove the majority of the unbound [75Se]selenite. The dialysed medium was then concentrated to 2.5 ml by placing the dialysis sack in a saturated poly(ethylene glycol) solution prepared in the dialysis buffer. Also after this 72 h period the cultured thyrocytes were washed

twice with EBS, harvested into 25 ml of EBS by scraping, followed by centrifugation at 2000 g for 10 min. The thyrocytes were resuspended in 1 ml of dialysis buffer and sonicated.

Identification of extracellular and intracellular [75Se]selenoproteins

After dilution to a common final protein concentration, the samples of concentrated culture medium and sonicated thyrocytes were diluted 2:1 with 'boiling mix' (SDS, 35 mM; glycerol, 1.4 mM; 2-mercaptoethanol, 0.3 mM; Bromophenol Blue, 15 mM) and heat-treated at 90 °C for 10 min. The [75Se]selenoproteins were separated by SDS/PAGE on a 12% gel; the resulting gel was dried and the selenoproteins revealed by autoradiography using Kodak X-OMAT XAR-5 film.

Immunoprecipitation of GPX in growth medium

Rabbit antisera (20 μl) raised to E-GPX [26] or Cy-GPX were added to concentrates of 75Se-labelled dialysed culture medium (200 μl) and were incubated overnight at 4 °C; non-immune rabbit serum was also used as a control. Pre-precipitated donkey anti-rabbit antiserum (DARS) was then added (50 μl) to each incubation and shaken at room temperature for 4 h. The DARS was then sedimented by centrifugation at 3000 g for 15 min and the pellet washed with EBS and then taken up in boiling mix. The immunoprecipitated selenoproteins were identified as described above.

Effect of Brefeldin A, thyrotropin and different second-messenger systems on extra-cellular and intra-cellular [75Se]selenoprotein expression

The effects of the various test compounds on the [75Se]selenoprotein content of the culture medium were investigated by adding these compounds at specified concentrations for the last 72 h of culture. These compounds were: Brefeldin A (5 μg/l), thyrotropin (TSH, 1.0 units/litre), 8-bromoadenosine 3':5'-cyclic monophosphate (8-bromo cAMP, 10^{-4} M), the phorbol ester, phorbol 12-myristate 13-acetate (PMA, 10^{-6} M) and the calcium ionophore A23187 (10^{-6} M).

GPX activity in culture media

For these experiments thyrocytes were cultured as described above but in the absence of [75Se]selenite. Three control flasks plus three flasks containing A23187 (10^{-6} M) were used. After the final 72 h period the culture media were concentrated as described above and the activity of GPX in the concentrates was determined as described previously using H_2O_2 as substrate [27]. Results were expressed as units/litre ± S.D. in the concentrated culture medium. GPX activity was also determined in the concentrated medium after the addition of iodoacetic acid 2×10^{-3} M and 100×10^{-6} M gold thioglucose, both known inhibitors of Se-dependent GPX.

Determination of protein

The protein content of sonicated thyrocytes and culture medium was determined using the Bradford dye-binding method [28], adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, U.K.).

RESULTS

Expression of [75Se]selenoproteins in thyrocytes and culture medium

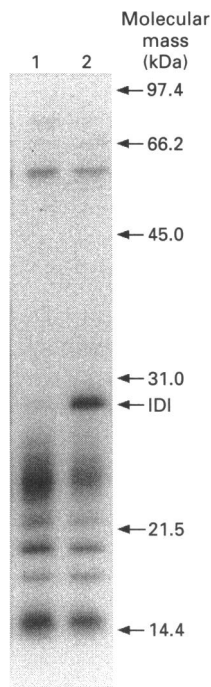At least 11 intracellular proteins were labelled in thyrocytes

Figure 1   Autoradiograph of an SDS/PAGE gel of sonicated thyrocytes

Thyrocytes were obtained as follows: lane 1, thyrocytes grown in the presence of 0.02 MBq/ml [^{75}Se]selenite; lane 2, thyrocytes grown in the presence of 0.02 MBq/ml [^{75}Se]selenite with 1 units/ml TSH. Note the induction by TSH of the 28.1 kDa IDI band (25 μg of protein was loaded on each lane)

grown in the presence of [^{75}Se]selenite, as revealed from the autoradiograph of the SDS/PAGE gels (Figure 1). Within the thyrocytes, labelled selenoproteins with a molecular mass of approx. 24 kDa comprised less than 10 % of the total seleno-proteins expressed by the cells.

In the culture medium two poorly separated selenoprotein bands were found, the band with the lower molecular mass being the most intense and having a mean molecular mass, calculated from four experiments, of 24 ± 1.6 kDa. These two selenoproteins
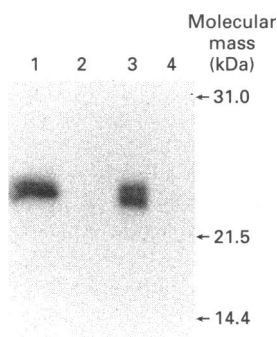

Figure 2   Autoradiograph of an SDS/PAGE gel of concentrated culture media obtained from two different thyrocyte cultures (a and b)

Lane 1, medium from thyrocyte culture (a) grown in the presence of 0.02 MBq/ml [^{75}Se]selenite.; lane 2, medium from thyrocyte culture (a) treated with Brefeldin A (5 μg/ml); lane 3, medium from thyrocyte culture (b); lane 4 medium from thyrocyte culture (b) treated with Brefeldin A (5 μg/ml) (10 μg of protein was loaded on each lane).
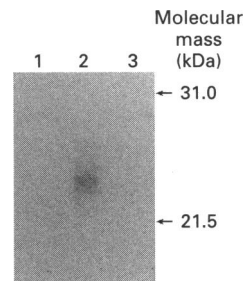

Figure 3   Autoradiograph of an SDS/PAGE gel of ^{75}Se-labelled proteins immunoprecipitated from culture medium by: non-immune rabbit serum (lane 1), E-GPX antibody (lane 2) and Cy-GPX antibody (lane 3)
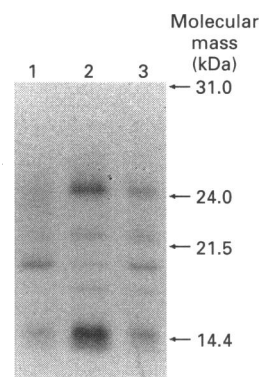

Figure 4   Autoradiograph of an SDS/PAGE gel of sonicated thyrocytes that had been treated with various agents in the presence of 0.02 MBq/ml [^{75}Se]selenite

Lane 1, no additions; lane 2, Brefeldin A (5 μg/ml); lane 3, Calcium ionophore A23187 (10^{-6} M) (25 μg of protein was loaded on each lane).

accounted for more than 95 % of the total ^{75}Se-labelled proteins (Figure 2, lanes 1 and 3). Antisera to E-GPX, but not Cy-GPX or non-immune rabbit serum, immunoprecipitated the 24 kDa selenoprotein band (Figure 3).

Effect of Brefeldin A, thyrotropin and second messenger systems on the secretion and intracellular accumulation of E-GPX

Inclusion of Brefeldin A into the culture medium prevented the appearance of E-GPX in the culture medium (Figure 2, lanes 2 and 4), but the amount of enzyme within the thyrocytes (as judged from the intensity of the 24 kDa band) appeared to be increased (Figure 4, lane 2). Treatment with Brefeldin A also produced an increase in the amount of a 14.4 kDa selenoprotein within the thyrocytes (Figure 4, lane 2), but no similar seleno-protein was identified in the culture medium; no further charac-terization of this intracellular selenoprotein was performed.

The calcium ionophore A23187 diminished the amount of E-GPX secreted into the culture medium to levels which were less that 10 % of control values (Figure 5, lane 3). Whilst PMA had little or no effect on the amount of the E-GPX in the medium when added alone (Figure 5, lane 2), it augmented the effect of A23187 when added in conjunction with this compound (Figure 5, lane 4). The addition of PMA with A23187 produced an increase in the intensity of the intracellular 24 kDa band similar to the effect seen with Brefeldin A (Figure 4, lanes 2 and 3).
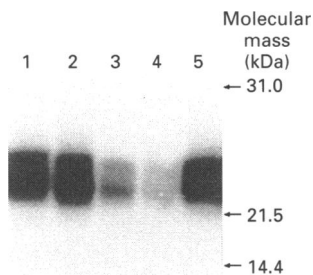
Figure 5 Autoradiograph of an SDS/PAGE gel of concentrated culture media obtained from thyrocytes treated with various agents in the presence of 0.02 MBq/ml [^{75}Se]selenite

Lane 1, no additions; lane 2, PMA (10^{-6} M); lane 3, calcium ionophore A23187 (10^{-6} M); lane 4, PMA (10^{-6} M) with calcium ionophore A23187 (10^{-6} M); lane 5, 8-bromo cAMP (10^{-4} M) (25 μg of protein was loaded on each lane).

Addition of 8-bromo cAMP had no significant effect on the extracellular (Figure 5, lane 5) or intracellular accumulation of E-GPX. Similarly TSH, when added at 1 U/L, had no effect on the intracellular or extracellular amounts of E-GPX (results not shown).

Effect of calcium ionophore with phorbol ester on GPX enzymic activity in culture medium

The addition of A23187 to the culture medium resulted in a significant decrease ($P < 0.05$) in GPX activity from 41 ± 7 units/litre in culture media obtained from untreated cells to 25 ± 4 units/litre in cells treated with A23187, as determined from triplicate incubations. Iodoacetic acid and gold thioglucose added to the assay buffer completely inhibited GPX activity.

DISCUSSION

Human thyrocytes grown in primary culture secrete predominantly a selenoprotein which has a molecular mass on SDS/PAGE of 24 kDa. Secretion of this protein was prevented by Brefeldin A, and the selenoprotein profile in the growth media was quite different to the profile observed in the cell lysates, indicating that the 24 kDa protein did not arise from non-specific leakage of protein from thyrocytes. GPX activity was found in the culture medium of control cells and could be inhibited by the addition of iodoacetic acid and gold thioglucose, known inhibitors of Se-dependent GPX activity. GPX activity was also diminished in the culture medium of thyrocytes exposed to A23187, a treatment which also decreased the expression of the 24 kDa selenoprotein. The 24 kDa selenoprotein could be immunoprecipitated with antiserum raised to E-GPX, but not with antiserum raised to Cy-GPX or with non-immune serum. Cy-GPX and E-GPX are both tetrameric with a subunit molecular mass of approx. 24 kDa, but these enzymes are functionally, chemically and immunochemically distinct, the latter being exported from the cell after synthesis. Our observations are thus consistent with thyrocytes synthesizing and secreting E-GPX into the growth media. In many experiments a double selenoprotein band on SDS/PAGE was observed. This phenomenon may be the result of proteolysis of E-GPX after release into the culture medium or differences in disulphide bridges. This phenomenon of multiple bands has been described previously for Cy-GPX [19,29].
Previously E-GPX production from 'normal' human tissue

has been reported only in renal, lung and placental tissue [19,20,23], and current evidence would indicate that the E-GPX circulating in human plasma arises mainly from kidney [19]. In anephric patients the activity of E-GPX in plasma is decreased to 23 % of the values found in plasma from matched controls and, similarly, in nephrectomized rats the E-GPX in plasma is reduced to 30 % of control values [19]. These observations suggest that non-renal tissue may provide up to 30 % of circulating E-GPX, and the possibility arises that the thyroid may provide a significant contribution to this non-renal source of plasma E-GPX.

What is the function of E-GPX in the thyroid? Our data suggest that E-GPX may provide an important mechanism for the regulation of thyroid-hormone synthesis. Availability of thyroidal H_2O_2 in the follicular lumen represents the rate-limiting step in thyroid-hormone synthesis [8–11]. Production of H_2O_2 is stimulated through the Ca^{2+}/phosphoinositol cascade [8–11] and, in vitro, the addition of Ca^{2+} ionophores such as A23187 produces a marked stimulation in H_2O_2 production [9–11]. Phorbol ester (e.g. PMA) has a similar, though smaller, stimulatory effect on H_2O_2 production [10,11], but phorbol esters augment the stimulatory effect of calcium ionophore [10]. In the present study we have shown that E-GPX secretion by the thyrocyte is diminished considerably by the second-messenger systems which promote H_2O_2 synthesis, and this is also accompanied by a loss in GPX activity against H_2O_2 in the culture medium. Thus when increased thyroid-hormone synthesis is signalled by the Ca^{2+}/phosphoinositol pathway, this would lead to both an increase in H_2O_2 generation on the luminal side of the apical membrane of the thyrocyte and a reduction in H_2O_2 degradation within the follicular lumen. These concurrent changes would amplify the increase in H_2O_2 concentration within the follicular lumen during stimulation of hormone synthesis and thus potentiate the availability of this rate-limiting compound for thyroid hormone synthesis. When thyroid-hormone synthesis is not signalled, hormone production would be prevented both by diminished H_2O_2 production and by E-GPX secretion, which would degrade any H_2O_2 released into the lumen in the basal state.

E-GPX may have a role in protecting the colloid from peroxidative damage in the basal state but, as a protective agent, its site of action is more likely to be intracellular. We have shown that activation of the Ca^{2+}/phosphoinositol signalling cascade leads to an accumulation of E-GPX within the thyrocyte, where it could act, along with Cy-GPX, PH-GPX and catalase, to prevent intracellular peroxidative damage from any H_2O_2 which may diffuse into the cell from the follicular lumen, particularly during stimulation of thyroid-hormone synthesis when H_2O_2 production will be greatest. Similarly, intracellular GPX may also protect the thyrocyte from the accumulation of harmful lipid hydroperoxides.

In conclusion, human thyrocytes synthesize and secrete E-GPX, a process which can be controlled by the Ca^{2+}/phosphoinositol signalling cascade. Evidence suggests that the primary role of the enzyme may be in modulating a supply of H_2O_2 for thyroid-hormone synthesis rather than exerting a protective effect on the colloid against possible peroxidative cellular damage. However, translocation of E-GPX from the extracellular to the intracellular space may provide an important mechanism to allow a rapid adaptation to prevent peroxidative damage from diffusion of extracellular H_2O_2 during stimulation of thyroid-hormone synthesis.

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