EXPERIMENTAL STUDY

Selenium has a protective role in caspase-3-dependent apoptosis induced by H₂O₂ in primary cultured pig thyrocytes

Abeba Demelash, Jan-Olof Karlsson, Mikael Nilsson and Ulla Björkman

Institute of Anatomy and Cell Biology, Sahlgrenska Academy at Gothenburg University, Gothenburg, Sweden

(Correspondence should be addressed to A Demelash; Email: abeba.demelash@anatcell.gu.se)

Abstract

Objective: Hydrogen peroxide (H₂O₂), necessary for thyroid hormonogenesis, is produced at the apical surface of the thyroid follicular epithelium. Excess H₂O₂ is potentially cytotoxic and may contribute to the development of hypothyroidism, e.g. in severe selenium deficiency. Yet it is unclear how H₂O₂ contributes to thyroid cell death.

Design and methods: H₂O₂-induced apoptosis and necrosis were studied in primary cultured pig thyroid cells. Glutathione peroxidase (GPx) activity was altered by culture in low serum with or without selenite substitution. Apoptosis was evaluated by spectrofluorometric measurement of caspase-3-specific substrate cleavage, and by analysis of DNA fragmentation by agarose gel electrophoresis. Necrosis was detected by ⁵¹Cr release from prelabeled cells.

Results: Exogenous H₂O₂ dose-dependently (100–400 µmol/l) activated caspase-3 within 3–12 h, and DNA degradation was observed after 24 h. The potency of H₂O₂ to induce apoptosis was low compared with that of staurosporine, a strong proapoptotic agent. H₂O₂-treated cells with reduced GPx activity showed increased caspase-3 activation. Incubation of serum-starved cells with selenite (10–100 nmol/l) normalized the GPx activity and reduced the activation of caspase-3 by H₂O₂. High H₂O₂ concentrations (400–800 µmol/l) were required to obtain necrosis. The H₂O₂-induced necrosis was exaggerated by both low GPx activity and catalase inhibition.

Conclusions: Cytotoxic effects of H₂O₂ on thyroid cells include caspase-3-dependent apoptosis that occurs at H₂O₂ concentrations insufficient to induce necrosis. Selenium deficiency aggravates the apoptotic response, probably due to impaired capacity of GPx to degrade H₂O₂.

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Introduction

Cytotoxic effects of oxygen is mediated by its reduced metabolites superoxide (O₂^{•−}), hydrogen peroxide (H₂O₂), hydroxyl radicals and singlet oxygen. At high concentrations these reactive oxygen species (ROS) have been reported to induce oxidative damage to membrane lipids, proteins and DNA that may result in cell death by necrosis or apoptosis (1, 2). The most important sources of superoxide in eukaryotic cells are the electron transport chains of mitochondria and the endoplasmic reticulum, but superoxide is also generated by several enzymes e.g. NADPH oxidase, xanthine oxidase and lipoxygenase. H₂O₂ is formed by dismutation of superoxide and can further react to form hydroxyl radicals (3). For their protection against ROS all cells possess several defense systems comprising antioxidant molecules, e.g. α-tocopherol, ascorbic acid and glutathione, and antioxidant enzymes, i.e. superoxide dismutases, catalase and glutathione peroxidases (GPxs). These enzymes have different substrate

specificity and subcellular localization. Superoxide dismutases that convert superoxide into O₂ and H₂O₂ are located in both the cytosol and mitochondria. Catalase mainly located in the peroxisomes degrades H₂O₂ into H₂O and O₂ (4). The five selenocysteine-containing GPxs that convert organic peroxides and H₂O₂ into the corresponding alcohols, using reduced glutathione as substrate, are either cytosolic or membrane-bound or both.

In the thyroid gland the follicular epithelial cells have, in addition to the above described mechanisms of ROS formation, a physiologically important production of H₂O₂ that is essential for thyroid hormonogenesis (5). H₂O₂ acts as an electron acceptor in the thyroperoxidase-catalyzed iodination of thyroglobulin that takes place in the follicle lumen at the external side of the apical plasma membrane. At the same membrane surface H₂O₂ is produced by a Ca²⁺-dependent NADPH oxidase (6, 7). The main component of the thyroid NADPH oxidase is a flavoprotein (8), and some of the elements show great similarity to the

H₂O₂-generating system in leukocytes (9). The regulation of H₂O₂ generation and its role in thyroid hormone formation have been extensively studied, but less is known of defense mechanisms that protect the thyrocytes from the potentially harmful effects of excess H₂O₂.

In an earlier study (10), we demonstrated in cultured thyrocytes that both catalase and GPx have a high capacity to degrade exogenous H₂O₂. Specifically, the observations indicate that GPx is involved in the degradation of fairly low H₂O₂ levels (100 µmol/l) whereas catalase is required to degrade H₂O₂ at mmol/l concentrations. In thyroid tissue, two GPxs, the cytosolic GPx (GPx1) and the phospholipid hydroperoxide GPx (GPx4), have been identified (11, 12). As both GPx1 and GPx4 are active in the cytosol they conceivably degrade H₂O₂ produced at the apical plasma membrane as soon as it enters the cell. Catalase, on the other hand, is mainly enclosed in the peroxisomes and therefore not directly accessible at the site of H₂O₂ production. It is thus possible that a reduced GPx activity may increase the susceptibility of thyroid cells to H₂O₂-induced damage related to cytosolic oxidative stress despite a normal catalase level. Indeed, *in vivo* experiments in the rat have demonstrated that selenium deficiency in combination with moderate iodide excess results in necrosis and subsequent fibrosis of the thyroid tissue (13, 14).

Previous *in vitro* studies (15, 16) indicate that excess H₂O₂ also is able to induce apoptosis, or programmed cell death, in the thyroid. The H₂O₂-mediated cytotoxicity appears to be dose-dependent, requiring only low concentrations to result in thyroid cell apoptosis rather than necrosis. However, recent findings in the rat thyroid suggest that the predominating cytotoxic response to oxidative stress may differ depending on the functional state of the gland (17). Moreover, antioxidants like vitamin E preferably protect against necrosis accompanying experimental goiter and involution (17). The mechanism by which H₂O₂ causes apoptosis in thyrocytes is not well defined. Studies on other cell types, mainly various tumor cell lines, indicate that apoptosis induced by exogenous H₂O₂ involve activation of caspase-3 and subsequent DNA cleavage (2, 18, 19). The purpose of this study was to determine if H₂O₂-induced apoptosis of primary cultured pig thyrocytes is caspase-dependent and if GPxs have a protective role in the process.

Materials and methods

Primary culture of thyroid cells

Thyroid follicle segments were isolated from pig thyroid glands obtained at the local abattoir, as earlier described (20). Briefly, the glands were disintegrated by repeated collagenase incubations and pipetting, after which the digest was filtered through nylon

mesh and washed by centrifugation several times to yield a purified follicle preparation. The follicle segments were seeded in 96-well plates or in 35- or 100-mm culture dishes and grown to nearly confluence at 37°C in an incubator with a humidified atmosphere of 5% CO₂. The culture medium was 6H medium consisting of, according to the original description (21), Coon's modified Ham's F12, 5% donor calf serum (DCS), 1 mmol/l non-essential amino acids, crude bovine thyrotropin (10 mU/ml), insulin, hydrocortisone, transferrin, glycyl-L-histidyl-L-lysine acetate, and somatostatin (medium and all additives were from Sigma-Aldrich Corp., St Louis, MO, USA). The medium was in addition supplemented with penicillin (200 U/ml), streptomycin (200 U/ml) and Fungizone (2.5 µg/ml). In order to change the cellular GPx activity levels, subconfluent cultures were grown for a period of 5 days in 6H medium with 5 or 0.5% DCS supplemented or not with varying concentrations (3–100 nmol/l) of sodium selenite (Sigma-Aldrich).

Experimental procedures

All experiments with exogenous H₂O₂ (Merck, Darmstadt, Germany) were carried out in Minimal Essential Medium (MEM) (Invitrogen) in the presence of 2% DCS and devoid of phenol red, as this compound was found to rapidly reduce H₂O₂ once it was added. Staurosporine (Sigma-Aldrich) kept in a 10 mmol/l stock solution in dimethylsulfoxide (DMSO) was added to a final concentration of 5 µmol/l. The pan-caspase inhibitor zVAD-fmk (Enzyme System Products, Livermore, CA, USA) was prepared as a 50 mmol/l stock solution in DMSO and was added to a final concentration of 50 µmol/l. Cells grown in ordinary 6H medium or in low serum medium with varying selenite concentrations were incubated with H₂O₂ or staurosporine in the presence or absence of zVAD-fmk for 2 h. Agents were then washed out by replacing the medium with 6H medium containing 5 or 0.5% DCS and the cells were further incubated and analyzed as described.

Measurement of GPx activity

Cells grown in 100-mm dishes were harvested after trypsinization, washed in PBS, and suspended in 100 mmol/l Tris-HCl buffer, pH 7.4, containing 5 mmol/l EDTA. Cell suspensions were sonicated for 3 × 15 s in an ice bath and aliquots were taken for determination of DNA content. The remaining homogenates were cleared by centrifugation at 20 000 *g* for 2 h, after which GPx activity recovered in the supernatant was determined by a modification of the method described by Paglia & Valentine (22). Samples were incubated in a final volume of 1 ml 100 mmol/l Tris-HCl containing 5 mmol/l EDTA, 0.13 mmol/l NADPH, 2 mmol/l reduced glutathione, 1 U/ml glutathione reductase and 0.16 mmol/l butylhydroperoxide

(all reagents from Sigma-Aldrich). GPx activity was expressed as nmol NADPH oxidized per min and mg DNA, which is equivalent to the same amount of oxidized H_2O_2 .

Measurement of caspase-3 activity

Cells grown in 96-well plates were frozen and thawed, and further disrupted by incubation under slow agitation for 20 min in room-temperature CHAPS buffer consisting of 50 mmol/l Tris-HCl, 100 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l EGTA, 3 mmol/l $NaNO_3$, and 0.2% CHAPS, pH 7.3 (100 μ l per well). The buffer was supplemented with trypsin inhibitor (5 μ g/ml), pepstatin (0.5 μ g/ml), leupeptin (1.25 μ g/ml), and phenylmethylsulfonyl fluoride (0.5 mmol/l) to minimize inappropriate proteolysis (all reagents from Sigma-Aldrich). Aliquots (20 μ l) were withdrawn for protein determination and the remaining samples (80 μ l) were incubated with the synthetic caspase-3 substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) (Calbiochem, Darmstadt, Germany) diluted from a 40 mmol/l stock solution in DMSO to 50 μ mol/l in 100 μ l of a buffer consisting of 50 mmol/l Tris-HCl, 100 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l EGTA and 3 mmol/l $NaNO_3$, pH 7.3. After addition of dithiothreitol to a final concentration of 2 mmol/l, the fluorescent signal of the cleavage products AMC was measured over time at 37°C in a SpectraMax Gemini microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 380 nm and an emission wavelength of 440 nm. Proteolysis was recorded for 3–5 h and V_{max} determined by computing the SOFT max PRO version 2.6 software (Molecular Devices).

DNA fragmentation assay

DNA from cells grown in 35-mm culture dishes was extracted with the Wizard genomic purification kit (Promega Corp., Madison, WI, USA). To ensure that all cells were analyzed, also the medium containing variable amounts of detached cells depending on treatment was collected. DNA was analyzed by electrophoresis on 1.5% or 4% agarose gels. Molecular mass standards were run in adjacent lanes and DNA was stained by ethidium bromide and visualized by fluorescence under UV light.

Analysis of nuclear morphology

Cells were grown on 8-well Lab-Tek chamber slides (Nunc, Naperville, IL, USA) and treated with H_2O_2 (200 μ mol/l) or staurosporine (5 μ mol/l) for 8 h. After fixation of cells with 4% paraformaldehyde for 20 min at room temperature followed by washing three times with PBS, pH 7.0, the slides were mounted with Vectashield mounting media containing 4,6-diamidino-2-phenylindole (DAPI) nuclear stain (Vector Laboratories,

Burlingame, CA, USA). Evaluation of nuclear morphology was performed in a Nikon Microphot FXA epifluorescence microscope equipped with a digital camera (Visa Tech International, Sunderland, UK). Digital images were obtained using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

^{51}Cr release

Plasma membrane leakage designating necrotic cell death was assessed by the release of ^{51}Cr (23). Cells grown in 24-well plates were incubated with 4 μ Ci/ml $Na_2^{51}CrO_4$ at 37°C for 18 h. ^{51}Cr -labeled cells were washed three times in radioactivity-free MEM and immediately used in H_2O_2 experiments, as detailed in the results. To reduce catalase activity the cells were incubated with 10 mmol/l aminotriazole (ATZ) (Sigma-Aldrich) for 1.5 h before the addition of H_2O_2 . ^{51}Cr release experiments were stopped by collecting the medium, after which the cells were washed twice with MEM and lysed by freezing and thawing in 0.05 mol/l sodium phosphate buffer, pH 7.4, containing 2 mol/l NaCl and 2 mmol/l EDTA. Radioactivity present in cell lysates and in culture media was measured in a gamma-counter and the amount of released ^{51}Cr was calculated as percent of total ^{51}Cr .

Quantification of protein and DNA

Total cellular protein content was determined with the Micro BCA protein assay kit (Pierce, Rockford, IL, USA) using BSA as standard. Absorbance was measured in a microplate reader at 570 nm. DNA was quantified by enhanced fluorescence after binding of bisbenzimidazole (H33258; Sigma-Aldrich) to DNA, as described by Labarca & Paigen (24). Fluorescence was measured at the excitation wavelength at 360 nm emission wavelength of 450 nm in a Perkin-Elmer spectrophotometer Becton Dickinson.

Results

Effect of H_2O_2 and staurosporine on caspase-3 activity in pig thyrocytes

The effect of externally added H_2O_2 on caspase-3 activity in primary cultured pig thyrocytes was studied by fluorometric analysis of the cleavage of Ac-DEVD-AMC, a caspase-3-specific synthetic substrate. From earlier studies (10), we know that most exogenous H_2O_2 is already degraded 30 min after addition to the cells and that the degradation rate increases proportionally with increasing H_2O_2 concentrations ranging from 0.01 to 1 mmol/l. This implies that independently of the amounts added and the subsequent incubation time the cells have been exposed to H_2O_2 during an equal limited time period. Incubation with a single dose of 100–400 μ mol/l H_2O_2 caused a concentration-dependent

Figure 1 Activation of caspase-3 by H₂O₂ in pig thyroid cells. The cells were exposed to 100–400 μmol/l H₂O₂ for 8 h, after which cell lysates were prepared and caspase-3 activity determined using the fluorogenic substrate Ac-DEVD-AMC. The amounts of cleaved substrate (AMC) were quantified by spectrofluorometry. Means ± s.d. (*n* = 4).

increase in the caspase-3 activity (Fig. 1), whereas higher H₂O₂ concentrations did not further stimulate substrate degradation (data not shown). The H₂O₂-induced activation of caspase-3 increased with prolonged incubation and reached a maximum after 8 h, after which the activity declined towards the baseline level (Fig. 2A). When the pan-caspase peptide inhibitor zVAD-fmk was added together with H₂O₂ a nearly complete inhibition of Ac-DEVD-AMC hydrolysis was obtained (Fig. 2B). In order to estimate the potency of H₂O₂ to induce thyroid cell apoptosis, we compared the effect on caspase-3 with that of staurosporine, a *Streptomyces* metabolite known to be a strong inducer of apoptosis in a variety of cell types (25, 26). The time dependency of staurosporine-induced caspase-3 activation (Fig. 3A) and the inhibitory effect of zVAD-fmk (Fig. 3B) were similar to the responses to H₂O₂. However, caspase-3-dependent proteolysis induced by staurosporine was nearly 10 times higher than that maximally obtained by H₂O₂.

Effect of H₂O₂ and staurosporine on DNA fragmentation

The cytotoxic effect of H₂O₂ was further characterized by analysis of DNA integrity. We found that H₂O₂ dose-dependently caused DNA degradation that was detectable by electrophoresis 24 h after addition of H₂O₂ (Fig. 4A). The degraded DNA was resolved as a rather diffused smear (Fig. 4A), which contrasted

Figure 2 H₂O₂-induced caspase-3 activity of thyroid cells. (A) Time course of caspase-3 activation. After addition of 200 μmol/l H₂O₂ the cells were incubated for 4–24 h. Cell lysates were incubated in the presence of Ac-DEVD-AMC and the amounts of cleaved substrate analyzed. Means ± s.d. (*n* = 4). (B) Inhibition of caspase-3 activity. The cells were exposed to 200 μmol/l H₂O₂ in the absence or presence of 50 μmol/l of the caspase inhibitor zVAD-fmk for 8 h, after which cell lysates were prepared and assayed for caspase-3 activity. Means ± s.d. (*n* = 4).

Figure 3 Staurosporine-induced apoptosis of thyroid cells. (A) Time course of caspase-3 activation. Cells were treated with staurosporine (5 μmol/l) for 2 h and then further incubated until cell lysates were prepared at the indicated times for measurement of Ac-DEVD-AMC cleavage. Means ± s.d. (*n* = 4). (B) Inhibition of caspase-3 activity. Cells were exposed to 5 μmol/l staurosporine for 2 h in the absence or presence of 50 μmol/l zVAD-fmk. After a total incubation time of 8 h cell lysates were analyzed as in (A). Means ± s.d. (*n* = 3).

Figure 4 H_2O_2 - and staurosporine-induced DNA fragmentation. (A) Effect of single dose H_2O_2 . Cells were exposed to 200–800 μM /l H_2O_2 for 24 h, after which total DNA was extracted and resolved by electrophoresis on a 1.5% agarose gel. (B) Effect of staurosporine. Cells were exposed to 5 μM /l staurosporine for 2 h and then taken for electrophoretic DNA analysis at the indicated times following the addition of the drug. (C) Effect of repetitively added H_2O_2 . Cells were exposed to single (at 0 h), double (at 0 and 3 h) or triple (at 0, 3 and 6 h) doses of 100 μM /l H_2O_2 . After 24 h DNA was extracted and analyzed using a 4% gel. 'bp' indicates size of standard DNA fragments.

with the distinct domain-sized DNA cleavage products obtained after treatment with staurosporine (Fig. 4B). Moreover, after repeated exposure to H_2O_2 , previously known to increase the number of cells committed

to apoptosis (16), the diffuse DNA degradation pattern did not change (Fig. 4C). The qualitative difference between the effects of H_2O_2 and staurosporine on DNA was also evident after nuclear staining. As shown in

Fig. 5, changes obtained after treatment with moderately low H_2O_2 concentrations (i.e. amounts of H_2O_2 that did not cause thyroid cell necrosis) were largely confined to shrinkage and condensation of the affected nuclei (Fig. 5B), whereas fragmentation into numerous, small nuclear bodies typically observed in staurosporine-treated cells (Fig. 5C) was rare or absent.

Effect of selenite on H_2O_2 - and staurosporine-induced caspase-3 activation

The influence of GPx activity on apoptosis was studied in thyroid cells cultured for several days in medium with high (5%) or low (0.5%) serum content that was supplemented or not with various amounts of sodium selenite. In agreement with previous findings (19), low serum conditions substantially decreased the GPx activity, and addition of 10–100 nmol/l selenite was sufficient to recover and further increase the GPx activity above the level recorded in control cultures grown in 5% serum (Table 1). The cells were then exposed to 100–400 $\mu\text{mol/l}$ H_2O_2 for 8 h after which the caspase-3 activity was measured. In most experiments, low serum did not change the basal caspase-3 level, but the response to H_2O_2 was considerably higher than that observed in cells cultured in 5% serum (Fig. 6A). Importantly, selenite-substituted cultures were much less sensitive to H_2O_2 -induced activation of caspase-3 (Fig. 6A). In contrast, low serum and hence low GPx levels did not further increase the

Table 1 Effect of serum and selenite on GPx activity estimated by analysis of NADPH oxidation in primary cultured pig thyrocytes. Values are mean $\pm$ s.d. ($n = 3$).

Culture conditions*		NADPH oxidation
Serum (%)	Se ($\mu\text{mol/l}$)	(nmol/min per mg DNA $\pm$ s.d.)
5	0	342 $\pm$ 17
0.5	0	100 $\pm$ 33
0.5	0.01	177 $\pm$ 40
0.5	0.03	825 $\pm$ 75
0.5	0.1	3440 $\pm$ 180

*Subconfluent cells grown for 5 days in medium with the indicated modifications.

ability of staurosporine to activate caspase-3, and no corresponding reduction in proteolysis was found after selenite substitution (Fig. 6B). Altogether, this suggests that GPx exerts a protective effect specifically on H_2O_2 -induced apoptosis.

Effect of H_2O_2 on ^{51}Cr release

It is well established that H_2O_2 , depending on the concentration, may trigger either apoptosis or necrosis in different cell types in culture. In order to characterize H_2O_2 -induced necrosis in the present experimental conditions, thyroid cells grown in 0.5% serum with or without 10 mmol/l selenite were prelabeled with ^{51}Cr , after which the effect of H_2O_2 on ^{51}Cr release was analyzed. As shown in Fig. 7, increasing amounts of ^{51}Cr were released from cells exposed to 400–800 $\mu\text{mol/l}$ H_2O_2 , whereas lower H_2O_2 concentrations had no effect. It was also found that selenite substitution prevented the H_2O_2 -induced necrosis (Fig. 7). Of note, the cytolytic response to these H_2O_2 concentrations was submaximal, as indicated by the finding that preincubation with the catalase inhibitor ATZ greatly enhanced the H_2O_2 -induced release of ^{51}Cr (Fig. 7). Nevertheless, this shows that high concentrations of H_2O_2 are required to induce necrosis in primary cultures of pig thyrocytes. Moreover, both GPx and catalase protect against necrosis resulting from H_2O_2 overload.

Discussion

Apoptosis and necrosis define distinct forms of cell death, but there is increasing evidence that they represent by their classical definitions the extremes of a spectrum of killing mechanisms with different biochemical and morphological features (27). Unlike necrosis, the apoptotic process is a regulated series of events that after initiation may follow different intracellular pathways. The execution of apoptosis is either caspase-dependent, involving the proteolytic activity of caspase-3 as the major effector mechanism (28), or caspase-independent (29). Both necrosis and apoptosis may be induced by H_2O_2 , but the sensitivity and type of response differ between cells of type of

Figure 5 Nuclear morphology of H_2O_2 - and staurosporine-treated thyroid cells. Nuclear DNA was stained with DAPI after paraformaldehyde fixation. (A) Untreated cells. (B) Cells exposed to H_2O_2 (200 $\mu\text{mol/l}$) for 8 h. (C) Cells treated with staurosporine (5 $\mu\text{mol/l}$) for 8 h.

Figure 6 Effect of low serum and selenium substitution on caspase-3 activation by H_2O_2 (A) and staurosporine (B). Cells cultured in media with different serum (FCS) and selenite concentrations for 5 days were exposed to H_2O_2 (100–400 μM /l) or staurosporine (5 μM /l) for 8 h. Cell lysates were prepared and analyzed for caspase-3 activity as outlined in preceding figures. Means $\pm$ S.D. ($n = 4$).

different origin. The present results collectively show that the cytotoxic effect of H_2O_2 on primary cultured pig thyrocytes switches from proapoptotic, involving caspase-3 proteolytic activity, to necrotic at increasing concentrations. Moreover, selenium deficiency increases the sensitivity to both H_2O_2 -induced apoptosis and necrosis.

The thyroid cells underwent caspase-3-dependent apoptosis in response to moderately low concentrations

Figure 7 H_2O_2 -induced necrosis of thyroid cells. Thyroid cells cultured in media with 0.5% serum with or without 10 nmol/l selenite (Se) to obtain low or high of GPx levels (see Table 1) were pre-labeled with ^{51}Cr and then exposed to different concentrations of H_2O_2 (100–800 μM /l). For comparison, H_2O_2 -treated cells were also preincubated with 10 mmol/l ATZ to inhibit catalase. After 4 h the amount of ^{51}Cr released into the medium was measured and calculated as percent of the total activity. Means $\pm$ S.D. ($n = 3$).

(100–200 μM /l) of H_2O_2 , whereas considerably higher doses (400–800 μM /l) were required to induce necrosis designated by ^{51}Cr release. When apoptotic death was studied by electrophoretic analysis of DNA, a characteristic fragmentation pattern commonly referred to as DNA laddering was observed in cells treated with staurosporine but not after H_2O_2 exposure. Instead, H_2O_2 at doses that activated caspase-3 caused a more diffuse or random degradation of DNA. It has been previously discussed (30) that lack of DNA fragmentation in multiples of about 200 bp is not reliable to exclusively distinguish necrotic from apoptotic cells. In fact, morphologically defined apoptosis may also occur in the absence of internucleosomal DNA cleavage. In line with this, we observed distinct changes in the nuclear morphology of apoptotic cells after treatment with respectively H_2O_2 and staurosporine. It was also evident that H_2O_2 only weakly activated caspase-3, the level of which was far from that maximally generated by staurosporine. Whether the observed biochemical and morphological DNA changes are due to a direct DNA-damaging effect of H_2O_2 or reflect the activation of an apoptosis-related but possibly caspase-3-independent mechanism of DNA degradation remains to be established.

Both GPx and catalase are major defense mechanisms against harmful side effects of ROS in most cell types. However, addition of selenite to cultured cells has been demonstrated to increase only the GPx activities, whereas catalase and other antioxidative enzymes are essentially unaffected (31, 32). It was therefore of interest to investigate if the ability of H_2O_2 to induce apoptosis was influenced by the cellular content of selenium-dependent GPxs. In accord with previous observations (10), thyroid cells grown in 0.5% serum had a very low GPx activity, but after exposure to 100 nmol/l selenite for several days the GPx activity was restored and further increased 5–10 times over the level recorded in control cells cultured in 5% serum. Whereas the basal caspase-3 activity was unchanged in serum-starved cells with low GPx, we found that H_2O_2 had a considerably higher capacity to activate caspase-3 than in cells with normal or supranormal GPx levels. This strongly suggests that GPx rapidly degrades H_2O_2 once it enters the cytosol and thereby effectively protects the thyrocytes from proapoptotic signal(s) that otherwise is likely to be provoked by H_2O_2 . Interestingly, similar effects of selenium substitution were observed when necrosis was induced by high H_2O_2 concentrations. Further proof demonstrating the importance of sufficient GPx activity for protecting thyrocytes specifically against H_2O_2 -induced cytotoxicity was obtained in experiments with staurosporine; the ability of this proapoptotic fungal toxin to strongly activate caspase-3 was not different in cells with low or high GPx content. A protective role of GPx in H_2O_2 -induced DNA damage has previously been recognized in different mammalian cell lines (30, 31).

Although selenium is an essential trace element for normal cell functions of all mammalian species, toxic effects of high selenite concentrations have been reported in both *in vivo* and *in vitro* studies. It has been suggested that selenium cytotoxicity is related to the generation of free radicals that induces apoptosis (33). However, the selenite concentrations (10–100 nmol/l) used here to experimentally modulate the GPx activity were within physiological range, and no unwanted side effects of the selenium substitution were observed. Another possible confounding factor to be considered is that selenite may actually repress proteolytic activity, i.e. of a caspase-3-like protease through a redox mechanism (34), which potentially could mimic the effects observed in the present study. The possibility of such interference was, however, ruled out by addition of dithiothreitol to samples taken for caspase-3 activity measurements. It therefore seems safe to conclude that the observed effects of selenium substitution reflect the biological activity of selenoproteins, i.e. GPxs.

In summary, the present findings provide evidence of a caspase-3-dependent mechanism in H_2O_2 -induced apoptosis of pig thyroid epithelial cells. Moreover, both

programmed thyroid cell death and necrosis elicited by H_2O_2 , although at different concentration intervals, are highly sensitive to reduced selenite and GPx levels, reinforcing previous notions of selenium deficiency as a condition that is potentially harmful to the thyroid (13, 14). In *in vitro* studies, it is well-known that serum withdrawal from the culture medium promptly induces apoptosis in many cell types (35, 36) including thyrocytes (37–39). This effect is generally believed to depend on loss of trophic effects of serum growth factors required for the cells to adhere properly to each other and to the underlying extracellular matrix. Consequently, lack of these factors will promote cell detachment that in turn is a powerful signal for the desquamated cells to undergo apoptosis (40). However, as we found that selenite-substituted cells with a high GPx activity had a much better capacity to withstand H_2O_2 and prevent apoptosis than cells with low GPx activity, the selenite content of serum should be considered equally important for thyroid cell survival.

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