EXPERIMENTAL STUDY

Selenium has a protective role in caspase-3-dependent apoptosis induced by \( \text{H}_2\text{O}_2 \) 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 (\( \text{H}_2\text{O}_2 \)), necessary for thyroid hormonogenesis, is produced at the apical surface of the thyroid follicular epithelium. Excess \( \text{H}_2\text{O}_2 \) is potentially cytotoxic and may contribute to the development of hypothyroidism, e.g. in severe selenium deficiency. Yet it is unclear how \( \text{H}_2\text{O}_2 \) contributes to thyroid cell death.

Design and methods: \( \text{H}_2\text{O}_2 \)-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 \( ^{51}\text{Cr} \) release from prelabeled cells.

Results: Exogenous \( \text{H}_2\text{O}_2 \) dose-dependently (100–400 \( \mu \text{mol/l} \)) activated caspase-3 within 3–12 h, and DNA degradation was observed after 24 h. The potency of \( \text{H}_2\text{O}_2 \) to induce apoptosis was low compared with that of staurosporine, a strong proapoptotic agent. \( \text{H}_2\text{O}_2 \)-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 \( \text{H}_2\text{O}_2 \). High \( \text{H}_2\text{O}_2 \) concentrations (400–800 \( \mu \text{mol/l} \)) were required to obtain necrosis. The \( \text{H}_2\text{O}_2 \)-induced necrosis was exaggerated by both low GPx activity and catalase inhibition.

Conclusions: Cytotoxic effects of \( \text{H}_2\text{O}_2 \) on thyroid cells include caspase-3-dependent apoptosis that occurs at \( \text{H}_2\text{O}_2 \) concentrations insufficient to induce necrosis. Selenium deficiency aggravates the apoptotic response, probably due to impaired capacity of GPx to degrade \( \text{H}_2\text{O}_2 \).

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Introduction

Cytotoxic effects of oxygen is mediated by its reduced metabolites superoxide (\( \text{O}_2^- \)), hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), 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. \( \text{H}_2\text{O}_2 \) 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. \( \alpha \)-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 \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) are located in both the cytosol and mitochondria. Catalase mainly located in the peroxisomes degrades \( \text{H}_2\text{O}_2 \) into \( \text{H}_2\text{O} \) and \( \text{O}_2 \) (4). The five selenocysteine-containing GPxs that convert organic peroxides and \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) that is essential for thyroid hormonogenesis (5). \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) is produced by a \( \text{Ca}^{2+} \)-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 GPxs 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 (DSC), 1 mmol/l non-essential amino acids, crude bovine thyrotropin (10 mIU/ml), insulin, hydrocortisone, transferrin, glycyrl-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$_2$O$_2$.

**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 NaN$_3$, and 0.2% CHAPS, pH 7.3 (100 µl per well). The buffer was supplemented with trypsin inhibitor (5 µg/ml), pepstatin (0.5 µg/ml), leupeptin (1.25 µg/ml), and phenylmethylsulfonyl fluoride (0.5 mmol/l) to minimize inappropriate proteolysis (all reagents from Sigma-Aldrich). Aliquots (20 µl) were withdrawn for protein determination and the remaining samples (80 µl) 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 µmol/l in 100 µl 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 NaN$_3$, 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 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 380 nm and an emission wavelength of 440 nm. Proteolysis was recorded for an excitation wavelength of 380 nm and an emission wavelength of 450 nm in a Perkin-Elmer spectrophotometer. Fluorescence was measured by Labarca & Paigen (24). Fluorescence was measured at the excitation wavelength at 360 nm emission wavelength of 450 nm in a Perkin-Elmer spectrophotometer. DNA fragments were analyzed by agarose gel electrophoresis with PBS, pH 7.0, and visualized by ethidium bromide and visualized by fluorescence under UV light.

**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$_2$O$_2$ (200 µmol/l) or staurosporine (5 µmol/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 $4 \mu$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$_2$O$_2$ 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$_2$O$_2$. $^{51}$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 bisbenzimide (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.

**Results**

**Effect of H$_2$O$_2$ and staurosporine on caspase-3 activity in pig thyrocytes**

The effect of externally added H$_2$O$_2$ on caspase-3 activity in primary cultured pig thyrocytes was studied by fluorimetric analysis of the cleavage of Ac-DEVD-AMC, a caspase-3-specific synthetic substrate. From earlier studies (10), we know that most exogenous H$_2$O$_2$ is already degraded 30 min after addition to the cells and that the degradation rate increases proportionally with increasing H$_2$O$_2$ 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$_2$O$_2$ during an equal limited time period. Incubation with a single dose of 100–400 µmol/l H$_2$O$_2$ caused a concentration-dependent
increase in the caspase-3 activity (Fig. 1), whereas higher H$_2$O$_2$ concentrations did not further stimulate substrate degradation (data not shown). The H$_2$O$_2$-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 H$_2$O$_2$ a nearly complete inhibition of Ac-DEVD-AMC hydrolysis was obtained (Fig. 2B). In order to estimate the potency of H$_2$O$_2$ 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 H$_2$O$_2$. However, caspase-3-dependent proteolysis induced by staurosporine was nearly 10 times higher than that maximally obtained by H$_2$O$_2$.

**Effect of H$_2$O$_2$ and staurosporine on DNA fragmentation**

The cytotoxic effect of H$_2$O$_2$ was further characterized by analysis of DNA integrity. We found that H$_2$O$_2$ dose-dependently caused DNA degradation that was detectable by electrophoresis 24 h after addition of H$_2$O$_2$ (Fig. 4A). The degraded DNA was resolved as a rather diffused smear (Fig. 4A), which contrasted

**Figure 1** Activation of caspase-3 by H$_2$O$_2$ in pig thyroid cells. The cells were exposed to 100–400 μmol/l H$_2$O$_2$ 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).

**Figure 2** H$_2$O$_2$-induced caspase-3 activity of thyroid cells. (A) Time course of caspase-3 activation. After addition of 200 μmol/l H$_2$O$_2$ 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 H$_2$O$_2$ 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).
with the distinct domain-sized DNA cleavage products obtained after treatment with staurosporine (Fig. 4B). Moreover, after repeated exposure to H₂O₂, 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 H₂O₂ and staurosporine on DNA was also evident after nuclear staining. As shown in

Figure 4 H₂O₂- and staurosporine-induced DNA fragmentation. (A) Effect of single dose H₂O₂. Cells were exposed to 200–800 μmol/l H₂O₂ 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 μmol/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 H₂O₂. 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 μmol/l H₂O₂. After 24 h DNA was extracted and analyzed using a 4% gel. ‘bp’ indicates size of standard DNA fragments.
Fig. 5, changes obtained after treatment with moderately low H$_2$O$_2$ concentrations (i.e. amounts of H$_2$O$_2$ 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$_2$O$_2$- 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 μmol/l H$_2$O$_2$ 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$_2$O$_2$ was considerably higher than that observed in cells cultured in 5% serum (Fig. 6A). Importantly, selenite-substituted cultures were much less sensitive to H$_2$O$_2$-induced activation of caspase-3 (Fig. 6A). In contrast, low serum and hence low GPx levels did not further increase the 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$_2$O$_2$-induced apoptosis.

**Effect of H$_2$O$_2$ on $^{51}$Cr release**

It is well established that H$_2$O$_2$, depending on the concentration, may trigger either apoptosis or necrosis in different cell types in culture. In order to characterize H$_2$O$_2$-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$_2$O$_2$ on $^{51}$Cr release was analyzed. As shown in Fig. 7, increasing amounts of $^{51}$Cr were released from cells exposed to 400–800 μmol/l H$_2$O$_2$, whereas lower H$_2$O$_2$ concentrations had no effect. It was also found that selenite substitution prevented the H$_2$O$_2$-induced necrosis (Fig. 7). Of note, the cytolytic response to these H$_2$O$_2$ concentrations was submaximal, as indicated by the finding that preincubation with the catalase inhibitor ATZ greatly enhanced the H$_2$O$_2$-induced release of $^{51}$Cr (Fig. 7). Nevertheless, this shows that high concentrations of H$_2$O$_2$ are required to induce necrosis in primary cultures of pig thyrocytes. Moreover, both GPx and catalase protect against necrosis resulting from H$_2$O$_2$ 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$_2$O$_2$, but the sensitivity and type of response differ between cells of type of

![Figure 5](https://www.eje.org)
different origin. The present results collectively show that the cytotoxic effect of \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \)-induced apoptosis and necrosis.

The thyroid cells underwent caspase-3-dependent apoptosis in response to moderately low concentrations (100–200 \( \mu \text{mol/l} \)) of \( \text{H}_2\text{O}_2 \), whereas considerably higher doses (400–800 \( \mu \text{mol/l} \)) were required to induce necrosis designated by \( ^{51}\text{Cr} \) 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 \( \text{H}_2\text{O}_2 \) exposure. Instead, \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) and staurosporine. It was also evident that \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ once it enters the cytosol and thereby effectively protects the thyrocytes from proapoptotic signal(s) that otherwise is likely to be provoked by H₂O₂. Interestingly, similar effects of selenium substitution were observed when necrosis was induced by high H₂O₂ concentrations. Further proof demonstrating the importance of sufficient GPx activity for protecting thyrocytes specifically against H₂O₂-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 H₂O₂-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 selenium 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 selenium 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 H₂O₂-induced apoptosis of pig thyroid epithelial cells. Moreover, both programmed thyroid cell death and necrosis elicited by H₂O₂, 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 H₂O₂ 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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