Generation of Oxygen Free Radicals in Thyroid Cells and Inhibition of Thyroid Peroxidase

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We examined whether superoxide (O_2^-) is produced as a precursor of hydrogen peroxide (H_2O_2) in cultured thyroid cells using the cytochrome c method and the electron paramagnetic resonance (EPR) method. No O_2^- or its related radicals was detected in thyroid cells under the physiological condition. The presence of quinone, 2,3-dimethoxy-l-naphthoquinone (DMNQ), or 2-methyl-1, 4-naphthoquinone (menadione), in the medium produced O_2^- and hydroxyl radicals (OH·); the amount of H_2O_2 generation was also increased. Incubation of follicles with DMNQ or menadione inhibited iodine organification (a step of thyroid hormone formation) and its catalytic enzyme, thyroid peroxidase (TPO). This inhibition should be caused by reactive oxygen species because the two quinones, particularly DMNQ, exert their effect through the generation of reactive oxygen species. It is speculated that the site-specific inactivation of TPO might have occurred at the heme-linked histidine residue of the TPO molecule, a critical amino acid for enzyme activity because OH· (vicious free radicals) can be formed at the iron-linked amino acid. TPO mRNA level and electrophoretic mobility of TPO were not inhibited by quinones. Our study suggests that thyroid H_2O_2 is produced by divalent reduction of oxygen without O_2^- generation. If thyroid cells happen to be exposed to significant amount of reactive oxygen species, TPO and subsequent thyroid hormone formation are inhibited.

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The thyroid gland produces H_2O_2 by the NADPH oxidase system of the apical membrane (1–3) and it utilizes H_2O_2 as a substrate of thyroid peroxidase (TPO) for thyroid hormone formation. The thyroid gland contains superoxide dismutase (SOD) (4–6), which converts O_2^- to H_2O_2. However, whether the thyroid cell actually produces O_2^- has been controversial; conflicting results have been published (7–9). In a simplified cell-free experiment, incubation of lactperoxidase (an analog of TPO) with excess H_2O_2 in the presence of iodide has been shown to produce O_2^- and hydroxyl radicals (OH·) (10). Whether this phenomenon actually happens in the thyroid cell has not been tested. It is conceivable that the thyroid gland is exposed to oxygen free radicals during radiation therapy to the neck (ionized radiation) (11, 12), acute bacterial infection of the thyroid gland (respiratory burst from activated leukocytes) (13), and *ras*-related thyroid tumors (as an O_2^- signal from mutated *ras* oncogen) (14). However, the effect of oxygen free radicals on thyroid cell function, particularly on thyroid hormone formation, is still unknown. In this study, we first examined whether thyroid cells produce O_2^- under the physiological condition. Then, intracellular oxygen free radicals were generated by menadione or 2,3-dimethoxy-l-naphthoquinone (DMNQ) (15, 16), and the effects of oxygen free radicals on iodine organification and cellular TPO were examined.

Materials and Methods

Primary Culture of Porcine Thyroid Follicles. Fresh porcine thyroid tissue were procured from a local abattoir (Farmer John Co., Los Angeles, CA) and follicles were isolated by collagenase digestion as described (17). Cultured porcine follicles were used to examine generation of reactive oxygen species, iodide uptake, iodine organification, TPO activity, TPO immunoblot, and TPO mRNA levels. The culture conditions were described previously (17). For the demonstration of reactive oxygen species, cultured FRTL-5 rat thyroid cells were also used.

Biochemical Analysis *Reagents.* Reagents and culture medium were obtained from Sigma Chemical Co. (St. Louis, MO) unless specified. DMNQ was obtained from Oxis International (Portland, OR).

Measurement of H_2O_2 production. The content of H_2O_2 in the medium was measured by the homovanillic acid

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method (18). Porcine follicles or FRTL-5 rat thyroid cells cultured in 12-well plates up to 80% confluency were washed twice with Tyrode salt solution. The incubation medium for the H_2O_2 assay contained testing agents, 440 μM homovanillic acid, and 0.5 units/ml horseradish peroxidase in a total volume of 1.0 ml of Tyrode salt solution containing 1.8 mM $CaCl_2$, pH 7.4. Incubation was done for 1 hr at 37°C by floating plates in a water bath without a cover lid. The amount of H_2O_2 generated during incubation was measured in an Amicon fluorometer at the excitation and emission wavelengths of 315 nm and 425 nm, respectively (18). All reagents used for experiments were tested for the presence of nonspecific interference in the homovanillic acid method.

Measurement of O_2^- by the reduction of acetylated cytochrome c. The amount of O_2^- generated during incubation was measured by the degree of the reduction of acetylated cytochrome c (19). The advantage of using acetylated cytochrome c over nonacetylated cytochrome c is to eliminate interference by cytochrome c reductase as previously described (8, 19). Porcine thyroid follicles or FRTL-5 cells cultured in 12-well plates were washed twice with Tyrode salt solution. Then, 1 ml of Tyrode salt solution containing agents to be tested was added to each well with or without 30 μg of SOD. The reaction for the O_2^- assay was initiated by adding acetylated cytochrome c with a final concentration of 80 μM, and incubation was carried out for 1 hr at 37°C in a water bath without a cover lid. At the end of incubation, the medium was removed and centrifuged at 10,000*g* for 2 min. The absorbance of the supernatant was measured at the wavelength of 550 nm (20) in a 160 U Shimadzu double beam spectrophotometer (Shimadzu, Kyoto, Japan). Nonspecific reduction of cytochrome c by testing agents and SOD without cells was examined and subtracted in this assay system. The amount of O_2^- produced was calculated based on the extinction coefficient of $21 \times 10^3\ M^{-1}\ cm^{-1}$ (20).

Electron paramagnetic resonance (EPR) spectroscopy. To confirm superoxide production in the thyroid cell, cultured FRTL-5 rat thyroid cells were used for EPR experiment because these cells were convenient for transporting to the experimental site. Oxygen free radicals were detected by EPR in conjunction with the spin trapping technique. The spin trap used was 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) because it diffuses through the plasma membrane (21). DMPO was purified by repeated charcoal filtration until the EPR spectrum of the spin trap alone was signal-free. The reaction mixture contained FRTL-5 cells (1×10^6 cells), 20 μmol/L menadione, and 200 mM DMPO in 1 ml of phosphate-buffered saline (PBS). In some experiments, 1 μM ionomycin was used without menadione. The reaction mixtures, after 6–60 min of incubation, were transferred into sealed capillary ends of Pasteur pipettes. EPR spectra were recorded at 9.81 GHz on an ECS spectrometer (Burker, Billerica, MA). Measurement was carried out with a 100 kHz field modulation at room temperature. Instrument settings were as follows: 20 mW microwave power, 0.5 G modulation amplitude, 1.3 s time constant, 18 G/min scan rate, and 100 G sweep width.

Effects of menadione and DMNQ on iodide uptake and iodine organification. Porcine follicles (8000 follicles/well) were plated in 12-well culture plates and cultured in the presence of 1 mU/ml bovine thyroid stimulating hormone (TSH) and 0.1 μM KI for 1 week. After washing, follicles were incubated with 0–25 μM menadione or DMNQ, 10 pmol NaI, and 0.1 μCi of $Na^{125}I$ for 1 hr in the presence of 1 ml of Tyrode salt solution. To determine iodide uptake, follicles were washed with Tyrode salt solution, and the radioactivity of follicles was counted. Iodine organification, which represents intrafollicular protein iodination, was measured by precipitating thyroid protein with 10% trichloloacetic acid as previously described (22). The results of iodide uptake and iodine organification were expressed as percentages of ^{125}I-iodine taken by the follicles and thyroid protein, respectively, from the medium.

TPO activity in thyroid follicles. Follicles were cultured in 6-well plates (16,000 follicles/well) in the presence of 1 μU/ml TSH and 0.1 μM KI for 8 days. The presence of KI greater than 5 μM tends to disturb the shape of follicles in our culture system. We chose eighth day of culture because TPO activity became relatively stable at this time (23). Follicles in two wells of 6-well culture plates were pooled, washed twice with PBS, pH 7.0, and sonicated. The pellet was then centrifuged at 100,000*g* for 60 min at 4°C;, reconstituted to 1 ml with PBS, sonicated, and used for measurement of TPO activity by the modified method of guaiacol oxidation (24). The reaction mixture for TPO assay contained PBS buffer, 100–200 μl of sonicated 100,000*g* pellet, 12 μmol guaiacol, and 880 nmol H_2O_2 in a total volume of 1 ml. The reaction was started by adding H_2O_2, and the increase in absorbance was measured at OD of 470 nm in a Shimadzu spectrophotometer. This crude TPO sample gave a linear increase in TPO activity up to 300 μl of the volume. The DNA content in the 100,000*g* pellet of the sonicate was measured by the mithramycin fluorescent method (25). TPO activity was expressed as guaiacol units per milligram of DNA; one guaiacol unit was arbitrarily defined as an increase in OD of 1.0 at 1 min.

TPO mRNA measurement. TPO mRNA levels were measured by competitive RT-PCR as we described previously (23).

Immunoblot analysis of TPO. Porcine polyclonal TPO antibody was kindly provided by Dr. Alvin Taurog (University of Texas). TPO samples were prepared from thyroid follicles in the presence of protease inhibitors. Electrophoresis of the sonicated 100,000*g* pellet of follicle (2 μg of protein) was performed under reducing condition using Novex apparatus (Novex, San Diego, CA). Immunoblot was done using Novex Western blot reagents.

Statistical Analysis. The significant differences of the mean values were analyzed by the Dunnett multiple comparison test when the experimental groups were compared with the corresponding control group. Also, the unpaired Student's *t* test was used when the mean values of two groups were compared.

Results

O_2^- Generation in Thyroid Cells. As shown in Table I, O_2^- was not detected in the control group of thyroid follicles (no quinone) by the cytochrome c method (Table I). To confirm the above results, we employed EPR with DMPO as a spin trapping agent. The medium in control cells (2×10^6), even after 60 min of incubation, did not show EPR signals (Fig. 1A). Intracellular EPR signal after washing cells was also absent (results not shown). Ionomycin, an agent known to increase H_2O_2 generation in dog thyroid cells (26), did not show any specific EPR signal (Fig. 1B). When quinone was added to the medium, O_2^- was detected in thyroid follicles (Table I) and in FRTL-5 cells (Fig. 1C). The medium from the cells incubated with 20 μM menadione exhibited EPR signals (Fig. 1C). The EPR signals consisted of a quartet with line intensities of 1:2:2:1. Based on these characteristics and hyperfine coupling constants ($a_H = a_N = 14.9G$), this spectrum was assigned to 5-hydroxy-2, 2-dimethyl-3-pyrrolidinyloxyl (DMPO-OH), the spin adduct from DMPO and hydroxyl radicals. The DMPO-OH adduct can be formed either by direct trapping of the hydroxyl radical or by rapid breakdown of DMPO-OOH, the adduct formed by O_2^- (27). The addition of 5 μM SOD to the medium completely quenched the signals, suggesting that the signals were mainly derived from O_2^-.

H_2O_2 Generation in Thyroid Cells. Both quinones (DMNQ and menadione) increased H_2O_2 production in a dose-response manner in cultured follicles (Table I). FRTL-5 cells also showed a similar response to DMNQ or menadione for H_2O_2 production (results not shown).

Effects of Quinones on Iodide Uptake and Iodine Organification. Figure 2 shows ^{125}I iodide uptake and ^{125}I iodine organification by thyroid follicles in the presence of 20 μM DMNQ or menadione. The presence of DMNQ caused a significant inhibition of iodine organification without affecting iodide uptake. Menadione showed inhibition of iodide uptake and iodine organification. To examine whether this inhibition was caused by H_2O_2 alone, we added H_2O_2 (10 and 20 nmol) directly to the medium every 10 min. The concentration of H_2O_2 was chosen based on the amount of H_2O_2 generated by DMNQ. The addition

Table I. Generation of O_2^- and H_2O_2 in Porcine Thyroid Follicles by DMNQ and Menadione

	O_2^- (nmol/well)	H_2O_2 (nmol/mg DNA)
Control	9	156 ± 4.6
DMNQ		
1 μM	ND	158 ± 8.7
10 μM	10.0 ± 0.3	476 ± 87
20 μM	ND	955 ± 103
Menadione		
1 μM	ND	144 ± 20.2
10 μM	5.7 ± 0.8	306 ± 21.5
20 μM	ND	876 ± 124

Note. The results are the means ± SD of triplicate samples. The detail method and the amount of superoxide and H_2O_2 produced for 1 hr are explained in the text. ND, not done.

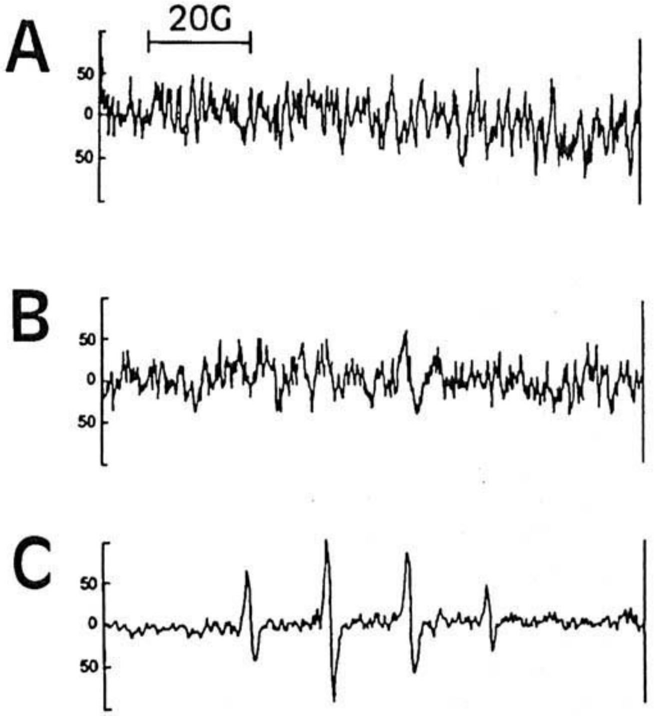

Figure 1. EPR spectra in FRTL-5 cells. (A) Control cells. (B) Cells treated with 0.1 μM ionomycin for 60 min. (C) Cells treated with 25 μM menadione for 60 min. An aliquot was taken from the medium for this experiment.

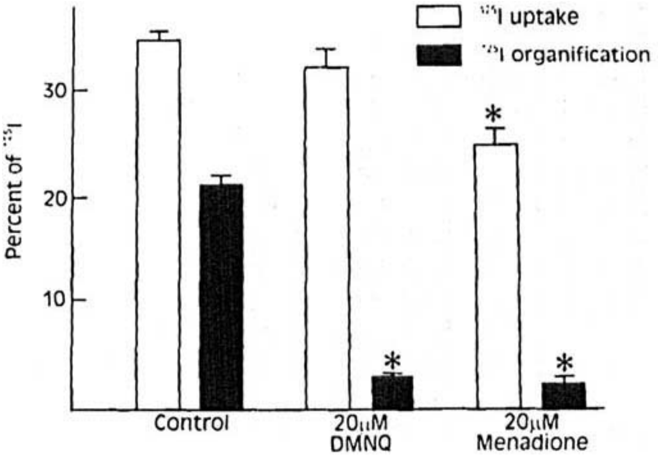

Figure 2. Effects of DMNQ and menadione on iodide uptake and iodine organification in porcine thyroid follicles. Follicular cells were exposed to 20 μM DMNQ or menadione for 60 min in the presence of Na^{125}I-KI mixture. The results were expressed as percentages of ^{125}I taken by follicles (iodide uptake) and bound thyroid protein (iodine organification). The results are the means ± SD of triplicate samples of one of representative experiments.

of 10 or 20 nmol H_2O_2 six times did not enhance or inhibit intrafollicular iodination.

Effects of Quinones on Cellular TPO. To further investigate the mechanism of decreased iodine organification, we measured cellular TPO activity after thyroid follicles were exposed to DMNQ or menadione for 2 hr. Figure 3 shows a profound inhibition of TPO activity in cultured thyroid follicles when concentrations of quinones were greater than 10 μM. We also measured TPO mRNA levels by competitive RT-PCR in thyroid follicles that were treated with DMNQ for 2 hr followed by washing and culturing for another 48 hr. TPO mRNA levels in the control and DMNQ-treated groups were 12.1 ng/μg poly(A)$^+$ RNA and 18.5 ng/μg poly(A)$^+$ RNA (mean of the two experiments), respectively. Electrophoretic mobility of TPO protein showed the same pattern and the same staining intensity between control cells and DMNQ-treated cells by immunoblotting (results not shown).

Discussion

The thyroid gland contains tissue specific NADPH oxidase (2, 3) and produces H_2O_2. It is theoretically possible that H_2O_2 can be derived from O_2^- because SOD, an enzyme to convert O_2^- to H_2O_2, is also present in the thyroid cell (4–6). Nakamura *et al.* (7, 8) used thyroid cell membrane fraction and demonstrated O_2^- production; however, there was no O_2^- detected in the medium of the intact thyroid cell (8). They proposed that O_2^- is produced inside of the plasma membrane where O_2^- is converted to H_2O_2 by SOD followed by diffusion of H_2O_2 through the plasma membrane (8). We have tried to demonstrate intracellular O_2^- by analyzing the EPR spectra from control cells (FRTL-5 cells) incubated with 200 μM DMPO for 15 min followed by washing. The EPR spectra from whole-cell suspension did not show O_2^- or O_2^--derived signals, suggesting no intracellular O_2^- formation in the physiological condition. The concept of O_2^- as the initial product for thyroid H_2O_2 has been questioned by Dupuy *et al.* (9). Jenzer *et al.* (10) showed production of O_2^- and OH· by incubating lactoperoxidase with excessive H_2O_2, which generates free radicals by the Fenton reaction (iron-mediated H_2O_2 breakdown). However, their finding cannot be extrapolated to the cellular level because the high concentrations of H_2O_2 used in their experiment should not prevail in the thyroid cell. Our present study could not demonstrate O_2^- formation under the physiological condition in the medium and cells. Even addition of ionomycin, a stimulator of thyroid H_2O_2 in dog thyroid cells (26), failed to show O_2^- in the thyroid cell (Fig. 1B). The question can be raised that the methodology is not sensitive enough to pick up O_2^- generation. However, if thyroid H_2O_2 production depends on solely from O_2^- or has stoichiometry relationship with O_2^-, EPR should have picked up O_2^- as the detection limit of free radicals by the EPR method is micromolar ranges (10). The thyroid gland has been shown to posses calcium and TSH dependent NADPH oxidase (2, 3). In contrast to leukocyte NADPH oxidase that can produce O_2^-, thyroid NADPH oxidase appears to produce H_2O_2 by divalent reduction of oxygen without O_2^- generation.

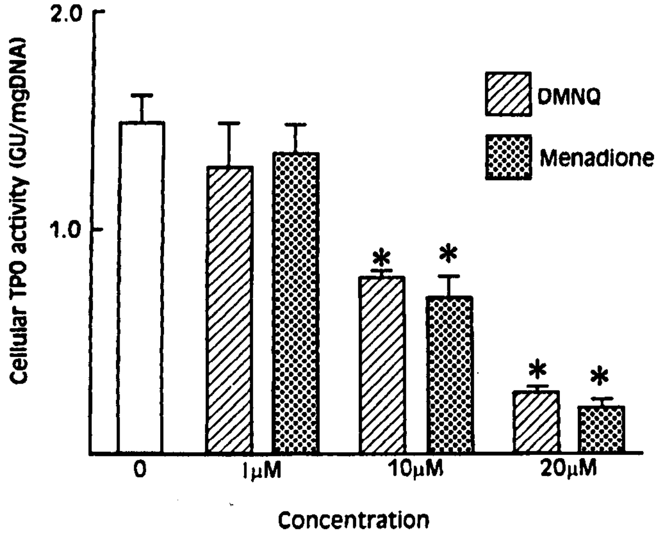

Figure 3. Cellular TPO activities in porcine thyroid follicles after exposure to DMNQ or menadione. Follicular cells were exposed to 0–20 μM DMNQ or menadione for 2 hr in the presence of 0.1 μM KI. Cellular TPO activity was measured in the sonicate of 100,000*g* pellet by guaiacol assay. The results are the means ± SD of triplicate samples of one of the representative experiments.

When DMNQ or menadione was used, O_2^- formation was demonstrated in the medium (Table I); this was confirmed by the EPR experiment (Fig. 1C). The mechanism of quinone-mediated O_2^- generation is different from the classic NADPH oxidase system. Quinones are reduced by intracellular redox enzymes and transfer one electron to oxygen for formation of O_2^- (16). In addition, DMNQ and menadione were the potent agents to augment H_2O_2 production in the thyroid cell (Table I). The most important finding in this study was that the two quinones displayed antithyroid activity by inhibiting iodine organification, a step of thyroid hormone formation, and its catalytic enzyme, TPO. How did the two quinones inactivate cellular TPO? The action of menadione can be derived from the generation of reactive oxygen species and/or arylation of critical nucleophil, whereas the action of DMNQ is specifically mediated solely through the generation of reactive oxygen species (15, 16). Thus, inactivation of TPO by DMNQ indicates that reactive oxygen species are responsible for TPO inactivation. How do reactive oxygen species inhibit TPO? Two mechanisms are possible: formation of compound III due to excessive H_2O_2 or O_2^-, and free radical-mediated TPO inactivation (28). The former causes reversible TPO inactivation and the latter causes irreversible TPO inactivation (28). Compound III formation is impossible to demonstrate in the cellular system. Furthermore, removal of quinone by washing follicles did not restore TPO activity, suggesting that this is irreversible TPO inhibition. We speculate that oxygen free radicals attacked the active site of TPO, causing inactivation of the catalytic site of the enzyme. Theoretically, this is quite possible. In the TPO molecule, proximal and distal histidine residues play a critical role for catalytic activity of TPO because the former is linked to the iron center of the heme and the latter is close to the peroxide-binding pocket (29). Vicious free radical (hydroxyl radical) formation is facilitated by the presence of iron known as Fenton reaction; thus, histidine residues linked to the heme can be the specific target by oxygen free radicals. Jenzer *et al.* (10, 28) also proposed the same mechanism for inactivation of lactoperoxidase by free radicals in their cell-free experiments. Nevertheless, the generation of excessive amounts of reactive oxygen species can have a negative effect on thyroid hormone formation. Thus, the presence of SOD and other antioxidants in the thyroid cell becomes important for maintenance of thyroid hormone formation if oxygen free radials are produced accidentally. We have not examined the effects of SOD and catalase on TPO activity in our culture system because the two enzymes are not permeable to the membrane and quinones produce oxygen free radicals primarily intracellularly. Reactive oxygen species did not have inhibitory effect on TPO mRNA levels or electrophoretic mobility of TPO molecule as long as the exposure time was short. This suggests that transcription process and antibody binding sites of TPO molecules are not inhibited by reactive oxygen species. Although the two quinones are not the products of human body, they are useful to examine the effect of oxygen free radicals *in vitro*.

Are there any clinical conditions involving O_2^- and its related radicals in the thyroid gland? Ionized radiation releases reactive oxygen species from the water molecule (11). Thus, neck cancer patients who undergo radiation therapy may be exposed to significant amounts of reactive oxygen species in the thyroid gland. Administration of ^{131}I-iodine to rats showed an increase in lipid peroxidation in the thyroid gland, suggesting the generation of oxygen free radicals by β radiation (12). Activated leukocytes due to infection generate reactive oxygen species from the leukocyte NADPH oxidase system, and this phenomenon is known as respiratory burst (13). Thus, the thyroid gland of patients with acute bacterial thyroiditis is exposed to reactive oxygen species. Mutation of the *ras* oncogen is one of the most common events in initiating tumors, including thyroid tumors (30). It has been shown that activated *ras* oncogen forms a large amount of O_2^- as its message signal (14). Thus, it is conceivable that thyroid tumors linked to the mutated *ras* oncogen produce oxygen free radicals. There are many naturally occurring quinones isolated from biological tissues (16). Also, chemotherapeutic drugs (adriamycine, daunorubicin, and mitomycine), acetaminophen (Tylenol), and air pollutants (cigarette smoke and automobile exhaust) are common source of quinones. Some of the quinones have a great potential to induce the generation of oxygen free radicals (16). Whether these quinones or quinone products exhibit organ-specific toxicity to the thyroid gland is unknown.

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