Extracellular ATP-induced production of hydrogen peroxide in porcine thyroid cells

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ABSTRACT

Hydrogen peroxide (H₂O₂) is an essential substrate for the peroxidase reaction in thyroid hormone biosynthesis. We demonstrated the production of H₂O₂ from porcine thyroid cells stimulated with extracellular ATP, using a scopoletin–horseradish peroxidase (HRP) system. Incubation of isolated cells for 1 day in the presence of 10% (v/v) newborn calf serum was necessary for the detection of induction by ATP of H₂O₂ production. The rate of H₂O₂ production induced by the addition of ATP increased in a dose-dependent manner, and the concentration of ATP required for half-maximum stimulation was about 10 μmol/l. ADP and GTP were also effective, but only at higher concentrations than ATP. In the absence of extracellular Ca²⁺, the production rate was very low. Production of H₂O₂ from thyroid cells was also measured by a method which discriminated between H₂O₂ and superoxide anion (O₂⁻); in this, diacetyl-deuteroheme-substituted HRP was employed as the trapping agent for both O₂ metabolites. The thyroid cells produced H₂O₂, but not O₂⁻, when the cells were stimulated by extracellular ATP.

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INTRODUCTION

It has been established that thyroid peroxidase catalyses iodination of tyrosine residues in thyroglobulin and a subsequent coupling of the two iodotyrosine residues to form iodothyronine (Lamas, Dorris & Taurog, 1972; Nakamura, Yamazaki, Nakagawa et al. 1984). Hydrogen peroxide (H₂O₂) is essential as an oxidant in the peroxidase reactions. An excess amount of H₂O₂ is harmful to the cells; therefore, the production of H₂O₂ should be well regulated in vivo. Although various enzyme systems have been proposed for the production of H₂O₂, NADPH oxidase is now generally accepted as the H₂O₂ supplier (Deme, Virion, Hammou & Pommier, 1985; Nakamura, Ogihara & Ohtaki, 1987). The activity of the NADPH-oxidase is strictly dependent upon the Ca²⁺ concentration in a submicromolar range (Nakamura et al. 1987). Thyroid cells could generate the minimum amount of H₂O₂ needed for hormone synthesis.

Björkman & Ekholm (1984) found that H₂O₂ production in porcine thyroid open follicles is remarkably accelerated by the addition of a Ca ionophore. Takasu, Yamada & Shimizu (1987) established, using cultured thyroid cells, that an increase in the intracellular Ca²⁺ concentration stimulates H₂O₂ production. Okajima, Sho & Kondo (1988) found that intracellular Ca²⁺ was mobilized by extracellular ATP in a micromolar range in the rat thyroid cell line FRTL-5. In the present study we have demonstrated the production of H₂O₂ elicited by extracellular ATP and a Ca ionophore by primary cultured porcine thyroid cells.

MATERIALS AND METHODS

Thyroid cell preparation

Porcine thyroid cells were prepared as described by Sho & Kondo (1984) with slight modifications. Usually eight thyroid glands were minced and washed several times with Ca²⁺- and Mg²⁺-free Dulbecco's phosphate-buffered saline (DPBS). The minced tissue was treated with 150 ml 0·1% (w/v) collagenase (Wako Pure Chemicals, Osaka, Japan) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, U.S.A.) at 37°C for 1 h and filtered through a stainless-steel mesh. The follicles collected
were suspended in DBPS, and washed by repeated centrifugation with DPBS. The follicles were then dispersed by treatment with 20 ml 0·1% (w/v) trypsin (Difco Laboratories, Detroit, MI, U.S.A.) and 0·2 mg EDTA-4Na/ml in DPBS at 37 °C for 1 h. The treatment was stopped by adding 5 ml 10% (w/v) bovine serum albumin (Sigma, St Louis, MO, U.S.A.) and 80 ml ice-cold DPBS. The isolated cells were collected and repeatedly washed by centrifugation with DMEM containing 100 mg kanamycin/l. The viable cells were counted using the trypsin blue exclusion test. The cells (1·5 × 10^6/ml) were incubated as a suspension in DMEM supplemented with 10% (v/v) newborn calf serum (NbCS; Gibco) at 37 °C in a 5% CO_2:95% air, water-saturated atmosphere. After incubation for 20–26 h, the cells were collected and washed with DMEM and DPBS before experiments. A proportion of the cells aggregated during the 1-day incubation, and cell numbers in the suspension could not be counted with sufficient accuracy. Thus we prepared the cell suspension based on the number of cells counted before the incubation.

**Measurement of H_2O_2 using scopoletin**

Generation of H_2O_2 was measured using 7-hydroxy-6-methoxy-2H-1-benzopyran-2-one (scopoletin; Sigma) and horseradish peroxidase (HRP; Toyobo Co. Ltd, Osaka, Japan). The thyroid cells incubated for 1 day were suspended in Heps-buffered saline (HBS) containing 25 mmol Hepes/l, 125 mmol NaCl/l, 2·7 mmol KCl/l, 8·1 mmol Na_2HPO_4/l and 1·5 mmol KH_2PO_4/l at pH 7·4 adjusted by NaOH. The suspension (2·0–2·5 × 10^7 cells/ml) was kept on ice and used within 30 min. The cell suspension (0·2 ml) was added to 1·8 ml standard reaction mixture in a quartz cuvette containing 5 μmol scopoletin/l, 80 μg HRP/ml, 1 mmol CaCl_2/l, 1 mmol MgCl_2/l and 5·5 mmol glucose/l in HBS at 37 °C, then the reaction was started by adding ATP (Sigma) and/or ionomycin (Calbiochem, La Jolla, CA, U.S.A.). Fluorescence was recorded with excitation and emission wavelengths of 370 and 465 nm respectively, in a spectrofluorophotometer (Shimadzu RF-500, Kyoto, Japan) with constant stirring. The effects of ATP analogues, adenosine (Wako Pure Chemical), AMP, cyclic AMP, ADP, adenylylimidodiphosphate and GTP (Sigma) were examined on the H_2O_2 production system. The effects of superoxide dismutase from bovine erythrocyte, bovine thyrotrophin (TSH), acetylcholine and noradrenaline (Sigma) were also examined.

**Measurement of H_2O_2 using diacetyl-HRP**

Generation of H_2O_2 was also measured by a method which discriminates between H_2O_2 and the superoxide anion, O_2^−, and in which diacetylenitroheme-

substituted HRP (diacetyl-HRP) is employed as the trapping agent for both O_2 metabolites (Makino, Tanaka, Iizuka et al. 1986; Nakamura, Ohtaki, Makino et al. 1989). Diacetyl-HRP was prepared as described by Makino & Yamazaki (1972). The standard reaction medium contained 3 × 10^6 cells/ml, 93 μmol diacetyl-HRP/l, 1 mmol CaCl_2/l, 1 mmol MgCl_2/l and 5·5 mmol glucose/l in DPBS at 28 °C. The reaction was started by adding ionomycin or ATP, and spectral changes of the reactions were monitored by a double-beam spectrophotometer (Shimadzu MPS-2000) with constant stirring.

**RESULTS**

**Detection of H_2O_2 by scopoletin in thyroid cells treated with ATP and ionomycin**

Isolated thyroid cells without incubation in the culture medium were damaged, and the spontaneous production of H_2O_2 was very high under the standard conditions described in Materials and Methods. Incubation for 1 day in medium supplemented with 10% NbCS prevented this damage, and the cells produced H_2O_2 after addition of ATP as shown in Fig. 1. The production was further enhanced by adding ionomycin, a Ca ionophore. The maximum production rate occurred with a concentration of ionomycin of 0·2 μmol/l or more. The production rate varied between cell preparations, being 0·24–1·3 μmol/l per min under the standard conditions. We therefore added 0·4 μmol ionomycin/l after the test of ATP induction, and the ratio of the rate in the presence of ATP to the maximum induced by ionomycin was also evaluated.

**Effects of ATP and ATP analogues on H_2O_2 production**

Figure 2 shows the dependence of H_2O_2 production rate on the concentration of ATP. As the concentration of ATP was raised, the production rate increased in a dose-dependent manner and reached a plateau at about half the production rate induced by 0·4 μmol ionomycin/l. The ATP concentration giving half-maximal stimulation was about 10 μmol/l.

The effects of ATP analogues on H_2O_2 production were examined. High concentrations of ADP (0·1 mmol/l), adenylylimidodiphosphate (0·3 mmol/l) and GTP (0·1 mmol/l) were able to induce H_2O_2 production, however, the capability of these analogues was less than half that of ATP. Little production was observed by the additions of AMP, cyclic AMP and adenosine.

Addition of TSH (up to 10 mU/ml), acetylcholine (0·1 mmol/l) and noradrenaline (0·1 mmol/l) into the
cell mixture induced no detectable production of H₂O₂ within the observation time of about 15 min in our cell preparation and detection system (data not shown). Addition of 0-1 mg superoxide dismutase/ml in the reaction medium also had no effect on the production of H₂O₂ which had been induced by ATP and ionomycin (data not shown).

**Effect of extracellular Ca²⁺ on H₂O₂ production**

In the presence of 1 mmol EGTA/l instead of 1 mmol CaCl₂/l addition of ATP induced almost no H₂O₂ production, and subsequent addition of 2 mmol CaCl₂/l induced production at a very low rate compared with the control experiment (Fig. 3). When ionomycin was finally added, however, the production rate was almost the same as that in the control experiment.

**Detection of H₂O₂ by the diacetyl-HPD method in thyroid cells treated with ATP and ionomycin**

The spectral changes of diacetyl-HPD from its ferric form to compound II, an H₂O₂ adduct of the peroxidase, and to compound III, an O₂⁻ adduct, were easily distinguishable. Using this method we found that the plasma membrane fraction prepared from porcine thyroid homogenate generated O₂⁻, but not H₂O₂, in the presence of Ca²⁺ and NADPH.
were 30 treatment stimulated 1 HRP (Nakamura et al. 1989). However, isolated thyroid cells produced $H_2O_2$, but not $O_2^-$, when the cells were stimulated by a Ca ionophore (Y. Nakamura, R. Makino, T. Tanaka, Y. Ishimura & S. Ohtaki, unpublished results). In the present study, we examined which $O_2$ metabolite is liberated from cells stimulated by extracellular ATP.

Figure 4 shows the spectral changes of diacetyl-HRP induced by the addition of 30 μmol ATP/l to incubated cells. The resultant new species exhibited absorption peaks at 530 and 565 nm and troughs at 495 and 645 nm. These characteristics were the same as those observed when the cells were incubated with 0.5 μmol ionomycin/l, and agreed with those of compound II of diacetyl-HRP, an $H_2O_2$ adduct of the enzyme (Makino & Yamazaki, 1972; Makino et al. 1986).

DISCUSSION

The production of $H_2O_2$ is enhanced by a Ca ionophore, A-23187, in open thyroid follicles (Björkman & Ekholm, 1984) and in isolated cells (Takasu et al. 1987). Addition of a Ca ionophore induced an influx of a large amount of $Ca^{2+}$ from the medium into the cells and elicited irreversible changes in numerous, normally well-regulated, physiological activities, leading to cell death. Although a Ca ionophore is a useful drug for the elucidation of regulatory mechanisms involving intracellular $Ca^{2+}$, the changes induced by the Ca ionophore are not the same as those under physiological conditions. In particular, $H_2O_2$ production is essential for hormone synthesis, being a substrate for thyroid peroxidase. Excess $H_2O_2$, however, is harmful to biological tissues. To understand the mechanism of hormone synthesis, therefore, it is important to elucidate the physiological agonist for intracellular $Ca^{2+}$ mobilization and also how $Ca^{2+}$ mobilization and $H_2O_2$ production are connected.

Acetylcholine, noradrenaline and a high concentration of TSH have been proposed as agonists for $Ca^{2+}$ mobilization (Corda, Marcocci, Kohn et al. 1985; Rani, Boyd & Field, 1985; Bone, Alling & Grollman, 1986). However, we could not detect induction of $H_2O_2$ production by these agonists.

In the present study, $H_2O_2$ production from thyroid cells was induced by micromolar concentrations of extracellular ATP, a possible physiological agonist of $Ca^{2+}$ mobilization. Extracellular ATP has been found to enhance the inositol phosphate turnover and iodide efflux in FRTL-5 cells (Okajima et al. 1988), results compatible with those of the present study. Selectivity of ATP analogues in our study was also similar to theirs. However, ATP was able to induce $Ca^{2+}$ mobilization irrespective of the presence of extracellular $Ca^{2+}$ (Sho, Okajima, Akiyama et al. 1989). In this respect, our observation is inconsistent with the above. In the absence of extracellular $Ca^{2+}$, addition of ATP induced almost no production of $H_2O_2$, and the rate of production was still low after subsequent addition of $Ca^{2+}$. When the Ca ionophore was added, however, the production rate was as high as that in the control experiment (Fig. 3). The following possibilities are suggested. (1) The differences in the cells used in this and previous studies (primary cultured cells vs cell line, and porcine vs rat) were responsible for the different results. (2) In porcine cells, an irreversible change occurred during the incubation with EGTA. (3) The porcine thyroid cells we used were still damaged after the 1-day incubation and the storage capacity of intracellular $Ca^{2+}$ was much smaller than that of FRTL-5 cells.

Interestingly, when we added 0.3 mU/ml TSH in the NbCS medium for the 1-day incubation, the
production rate of \( H_2O_2 \) upon the addition of ATP and ionomycin was only 5–20% of that in the cells incubated without TSH. The viability of the cells was almost the same as that in cells incubated without TSH (90%), but the proportion of the cells in cluster formation was higher than preparations incubated without TSH. The reason for the low production of \( H_2O_2 \) in the TSH-preincubated cells is not clear at present; however, the following possibility is suggested. Mauchamp, Margotat, Chambard et al. (1979) reported that the isolated hog thyroid cells form a follicle-like structure during cultivation, and the right-side-out and the inside-out follicles are formed with and without TSH in the medium respectively. If this is the case, the cells incubated with TSH for 1 day produced \( H_2O_2 \) and liberated it into the lumen of the follicle-like structure, and hence \( H_2O_2 \) was not detectable.

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