Inhibition of Thyrocyte Iodide Uptake by H^+K^+ ATPase Inhibitor, Timoprazole

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Abstract. The inhibitory effect of timoprazole on iodide uptake by cultured thyrocytes was observed using FRTL-5 cells. The inhibition was noticed to be dose dependent and was eliminated completely by rinsing. Timoprazole pretreated with acid had inhibitory effects, but [3H] leucine incorporation experiments indicated that acidified timoprazole inhibited protein synthesis to some degree. In addition, TSH-induced cAMP production was also inhibited by acidified timoprazole. Timoprazole pretreated in a neutral solution did not have any cytotoxic effects on TSH-induced cAMP production or protein synthesis at concentrations less than 1000 μM , suggesting that timoprazole by itself has specific effects on iodide uptake. The absence of any direct interaction of the drug with iodide was confirmed by separation of timoprazole and iodide on a C_{18} Sep-Pak column. It is concluded that timoprazole by itself could inhibit iodide transport through the thyroid cell membrane. This may provide a useful system in further investigating the mechanism of iodide transport.

Key words: H^+K^+ ATPase inhibitor, Iodide uptake, Thyroid

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TIMOPRAZOLE (TMP; an H^+K^+ ATPase inhibitor, Astra Pharmaceutical Co.) is a parent compound of the well-known group of pyridinylmethylsulfinyl benzimidazoles which block gastric acid secretion by inhibiting H^+K^+ ATPase. This inhibitory effect is associated with the generation of a highly reactive metabolite in acidic conditions, which binds covalently to the enzyme [1, 2]. In toxicological studies in animal experiment systems *in vivo*, it was found that in addition to the anti-secretory effect, TMP also inhibited the accumulation of iodide in the thyroid gland [3], but this work did not determine whether the inhibition results from TMP itself or a *de novo* derivative of TMP processed by gastric acid. In the present study, the effect of TMP on iodide uptake was stud-

ied *in vitro* by using FRTL-5 cells. FRTL-5 is a cloned cell line of rat thyroid origin which possesses several functional characteristics of normal thyroid cells including thyrotropin (TSH)-induced iodide uptake, but little iodide organification machinery.

Materials and Methods

Iodide uptake inhibition

Iodide uptake by FRTL-5 cells was measured by the modified method of Weiss [4]. In brief, cells were cultured in a 24 well-plate (1.5×10^5 cells/well) for 24 h in a 6 hormone medium with 5% calf serum (6H-5CS). The final concentrations of the hormones used were insulin (Sigma), 10 $\mu g/ml$; hydrocortisone (Calbiochem), 10 nM; transferrin (Sigma), 5 $\mu g/ml$; glycyl-L-histidyl-L-lysine acetate (Sigma), 10 ng/ml; somatostatin (Sigma), 10 ng/ml; and thyrotropin (Armour, Miles, Sigma), 1 mU/ml [5]. Then the cells were rinsed once with PBS

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and, in half of the wells, the medium was changed to 5H-5CS which lacks TSH found in 6H-5CS, while the others received fresh 6H-5CS. After 72 h, these media were replenished and the cells were incubated for another 48 h. Before application, TMP was dissolved in 99.5% ethanol to a concentration of 100 mM and adjusted to either pH 7.4 or pH 1.8 with 10 mM Tris or 0.1 N HCl, respectively. After 15 min, Hanks' buffer supplemented with 10 mM Hepes (pH 7.4) and 0.2% bovine serum albumin was added to neutralize and dilute the pretreated TMP solution. The pretreated TMP was added to FRTL-5 cells for 15 min and then incubated with Na[¹²⁵I] (Amersham, 134 mCi/mmol, 540 nCi/well), 10 μ M non-labeled NaI and bTSH (0 or 2.5×10^{-9} M) for another 40 min. After washing three times with ice-cold Hanks' buffer, the cells were solubilized by 0.1 N NaOH and the incorporated radioactivity was measured in a gamma counter (Aloka Co., Tokyo, Japan). When the solutions were treated with 5% trichloroacetic acid (TCA) to evaluate the iodine organification by FRTL-5 cells, less than 1% of radioactivity was collected in the precipitate. The time-variation in the inhibition of iodide uptake by FRTL-5 cells when pretreated by acidified and non-acidified TMP (1000 μ M) was studied after 5 and 40 min-incubation with Na[¹²⁵I].

To study the effect of extracellular Na⁺ or K⁺ ionic strength on TMP inhibitory function, the concentrations of Na⁺ or K⁺ in the Hanks' solution were varied from 20 to 140 mM and 1 to 10 mM, respectively, by equimolar substitutions of each ion with choline Cl according to the method of Weiss [4].

Washing-out experiments

FRTL-5 cells were incubated with acidified or non-acidified TMP (1000 μ M) for 15 min. Then half of the cells were washed three times with TMP free buffer. After incubation with ¹²⁵I solution for 40 min, the incorporated radioactivity was measured as described above.

Separation of iodide and TMP on a C₁₈ Sep-Pak column

Acidified or non-acidified TMP (100 μ M) was incubated with Na[¹²⁵I] (45.5 nCi) for 40 min then applied to a C₁₈ Sep-Pak column (Millipore Co., Milford) and eluted step-wise with 6 ml of 0–100%

methanol. The radioactivities in each eluted fraction were measured in a gamma counter. As a control, Na[¹²⁵I] and TMP were separately applied to the column. As for the measurement of TMP concentration, fractions of the solution were acidified by adding 1 ml 10 N HCl which turns TMP yellow. Dimerization or polymerization of TMP by acid treatment is not likely to occur [2, 6]. The colored TMP was quantified by absorbance at a wavelength of 410 nm with a spectrophotometer (Beckman Instrument, Inc., Fullerton).

Effect of TMP on protein synthesis

To determine the cytotoxic effect of TMP, FRTL-5 cells (1.5×10^5 cells/well) were cultured in a 24-well plate with 6H-5CS medium for 6 days. After that the medium was changed to MEM supplemented with 1/10 concentrated leucine and cultured overnight. Different concentrations of acidified or non-acidified TMP were added to the cells for 15 min and then incubated with 1 μ Ci/ml [³H] leucine (Amersham, 60Ci/mmol) for another 40 min. After rinsing with ice-cold PBS three times, the cells were solubilized with 0.1 N NaOH, and 5% TCA insoluble fractions were trapped on a Whatman glass filter. Their radioactivities were counted in a liquid scintillation counter (Beckman Instrument, Inc., Fullerton).

For the pulse-labeling study of [³H] leucine, FRTL-5 cells (8×10^5 cells/3.5 cm plate) were prepared as described above and treated with 1000 μ M TMP at 37 °C. After this, at 5, 10, 15, 20 and 30 min intervals, [³H] leucine was added and incubated for 5 min. The incorporated radioactivity of the [³H] leucine was assayed as above.

Effect of TMP on TSH-induced cAMP production

TSH-induced cAMP production was measured by the modified method of Vitti [7]. Briefly, FRTL-5 cells were seeded on a 96 well plate (4×10^4 cells/well) and grown to subconfluence in 6H-5CS medium. Then the cells were further cultured in the absence of TSH for 7 days. After rinsing with low salt Hanks' solution, the cells were incubated with both acidified and non-acidified TMP at 0 or 1000 μ M in low salt Hanks' with 0.5 mM 3-isobutyl-methyl-xanthine (IBMX) and TSH (0, 10 and 100 μ U/ml). The reaction was continued for 2 h at 37 °C. The concentration of cAMP was measured

with an RIA kit (EIKEN Chem., Japan).

Statistical analysis

For statistical analysis of the results, one way analysis of variance with pairwise comparison by the Bonferroni method was used.

Results

Effect of TMP on iodide uptake

Iodide uptake by FRTL-5 cells at various concentrations of TMP is shown in Fig. 1. TMP, both acidified and non-acidified, inhibited TSH-stimulated iodide accumulation into FRTL-5 cells. The inhibition was observed to be dose dependent giving an IC_{50} of 289 μ M at acidic and 475 μ M at neutral conditions respectively. TMP pretreated with acid resulted in a slightly stronger inhibitory effect on iodide uptake. Iodide uptake was significantly inhibited by TMP at concentrations of 100 μ M ($P < 0.05$) and more than 200 μ M ($P < 0.01$)

Fig. 1. Dose dependent TMP inhibition of iodide uptake by FRTL-5 cells. TMP was pretreated in pH 7.4 (○) and pH 1.8 (●) solutions for 15 min, then neutralized and added to FRTL-5 cells. After incubation with [125 I] for 40 min, the incorporated radioactivity was measured. Bars indicate standard deviation (SD) of three different cell preparations. *, $P < 0.05$; **, $P < 0.01$ compared to basal value.

under both acidic and neutral conditions.

Figure 2 shows TMP induced iodide uptake inhibition after 5 and 40 minutes' incubation with Na[125 I]. Acidified and non-acidified TMP inhibited both the initial (5 min-) and steady state (40 min-) accumulation of iodide into FRTL-5 cells. This means that TMP inhibited the accumulation of iodide in the initial phase of iodide influx and persistently thereafter.

Wash-out experiment

As shown in Fig. 3, the inhibitory effect of 1000 μ M TMP on iodide uptake could be completely eliminated after removing TMP by rinsing with Hanks' solution. Therefore the inhibitory action was observed only in the simultaneous presence of both [125 I] and TMP. This evoked a possible

Fig. 2. Effect of TMP on the initial and steady state uptake of iodide. Acidified and non-acidified TMP was added to FRTL-5 cells. After periods of 5 and 40 min-incubation with [125 I], the cells were washed three times and the incorporated radioactivity was measured. To compare the inhibitory rates of TMP at 5 and 40 min, the absolute uptake of iodide by the neutral control was designated as 100%. Radioactivity actually incorporated by the neutral control was 14465.3 cpm at 5 min and 10870 cpm at 40 min. From the left, the columns show the treatment with acidified TMP (black), acidic control, neutral TMP, and neutral control. Bars indicate SD. **, $P < 0.01$.

Fig. 3. Reversibility of TMP inhibitory effect by washing. FRTL-5 cells [TMP (+)] were exposed to acidified or non-acidified TMP for 15 min, and then half were washed and half were not. A control group of cells [TMP (-)] unexposed to TMP were washed in the same manner as above. Subsequently, in each case [^{125}I] was added and incorporated iodide was measured. Black column, washing. Dotted column, without washing. Bars indicate SD. **, $P < 0.01$.

direct association of TMP with iodide. To evaluate this possibility, we applied mixture of TMP and iodide on a C_{18} Sep-Pak column.

Separation of iodide and TMP by means of a C_{18} Sep-Pak column

A C_{18} Sep-Pak column was used to define the elution patterns of TMP, iodide, and mixture of iodide and TMP. As shown in Fig. 4, TMP and inorganic iodide were eluted at separate positions. Non-acidified TMP was recovered mainly in the 30% methanol fraction and acidified TMP was found mainly in the 30% and 80–90% methanol fractions. The radioactivity of [^{125}I], when isolated from TMP, was collected in the 0% methanol fraction. For the mixture of TMP and [^{125}I], the radioactivity of the iodide appeared in the 0% methanol fraction only, while no radioactivity was found in the 30% or 90% methanol fractions. This indicated that neither acidified nor non-acidified TMP interacted directly with [^{125}I].

Fig. 4. Separation of radio-labeled iodide and TMP on a C_{18} Sep-Pak column. Acidified or non-acidified TMP, radio-iodide by itself, and mixtures of TMP with [^{125}I] were eluted by 0–100% methanol through the column. The radioactivity of [^{125}I] in each fraction was determined in a gamma counter. TMP concentrations were measured with a spectrophotometer after adding HCl to each fraction. □, Acidified TMP; ◆, non-acidified TMP; ●, radio-iodide only; ○, radio-iodide incubated with acidified TMP; ■, radio-iodide incubated with non-acidified TMP.

Effect of TMP on protein synthesis

To rule out the non-specific cytotoxic action of the drug, the effect of different concentrations of TMP on protein synthesis by FRTL-5 cells was studied. As shown in Fig. 5, protein synthesis by FRTL-5 cells was reduced at concentrations of more than 200 μM of acidified TMP but there was no significant cytotoxicity at concentrations of less than 1000 μM of non-acidified TMP. According to the time-course of the [^3H] leucine incorporation test, high doses (1000 μM) of acidified TMP produced the maximal inhibition of protein synthesis after 15 min, but the maximum inhibition of iodide uptake occurred within 5 min, indicating that there is a time lag between the inhibition of iodide uptake and the inhibition of protein synthesis. Non-acidified TMP did not affect the protein synthesis of the cells even after a 30 min-exposure. Iodide uptake inhibition by non-acidified TMP is therefore thought to result from TMP-specific effects at concentrations of less than 1000 μM .

Fig. 5. Effect of TMP on protein synthesis by FRTL-5 cells. FRTL-5 cells were exposed to acidified (●) and non-acidified (○) TMP for 15 min. Then [^3H] leucine was added and incubated with the cells for 40 min, after which the incorporated radioactivity of [^3H] leucine was measured. Bars indicate SD. **, $P < 0.01$ compared to basal value.

Effect of TMP on TSH-induced cAMP production

As shown in Fig. 6, TSH-induced cAMP production by FRTL-5 cells was markedly inhibited by acidified TMP at 1000 μM . Non-acidified TMP at the same concentration did not have any significant inhibitory effect on cAMP production.

Effect of extracellular Na^+ or K^+ on inhibition of iodide uptake by TMP

When the Na^+ or K^+ concentration was decreased in the extracellular fluid, the iodide uptake was reduced even in the absence of TMP. The ratio of iodide uptake with non-acidified TMP to that without it was observed to be similar for Na^+ concentrations from 20 to 140 mM (Fig. 7a) and K^+ concentrations from 1 to 10 mM (Fig. 7b), respectively, in the extra cellular medium.

Fig. 6. Effect of TMP on TSH-induced cAMP production. FRTL-5 cells were cultured in the absence of TSH for 7 days before the assay. Acidified (■) and non-acidified TMP (▲) (1000 μM) in low salt Hanks' containing 0, 10 and 100 $\mu\text{U}/\text{ml}$ of TSH were added to the cells and incubated for 2 h. Acidic (●) and neutral (○) control solutions without TMP were applied in the same way. The cAMP content in the medium was determined with an RIA kit. The vertical bars represent the SD of three determinations.

Fig. 7. Effect of extracellular Na⁺ and K⁺ on the inhibition of iodide uptake by TMP. FRTL-5 cells were incubated at the indicated final concentrations of Na⁺ [shown in (a)] or K⁺ [shown in (b)] Hanks' buffer (○), in which NaCl and KCl were replaced by equimolar concentrations of choline Cl. Non-acidified TMP (●) (1000 μM) dissolved in the same buffer was added to the cells. Steady state (40 min) iodide uptake was determined as described in the Materials and Methods. The results shown are the mean ± SD of three determinations.

Discussion

The effect of TMP (an H⁺K⁺ATPase inhibitor) on iodide uptake was studied *in vitro* systems by using FRTL-5 cells. Since the action of TMP on acid secretion in gastric parietal cells is associated with the generation of a highly reactive metabolite in conditions of low pH, the present study concerned on the effect of TMP pretreated under neutral and acidic conditions.

TSH-induced iodide uptake by FRTL-5 cells was inhibited by both acidified and non-acidified TMP. The uptake of iodide determined at 5 and 40 min, respectively, is used as an indicator of the initial iodide influx and the steady state of the transport process [4]. It was noted that TMP inhibited both the initial and steady state accumulation of iodide. Since protein synthesis is an important intermediate process in TSH-stimulated iodide uptake [8], the decrease in protein synthesis due to acidified TMP may cause the inhibition of iodide uptake. However, the time-course inhibition of iodide uptake resulted in maximal inhibition within 5 min, while [³H] leucine incorporation tests indicated that maximal inhibition of protein synthesis by acidified TMP occurred after 15 min. This implies that protein synthesis inhibition may not be the main cause of iodide uptake inhibition and therefore that acidified TMP may have a direct inhibitory effect on iodide uptake. Acidified TMP also inhibited TSH-induced cAMP production. This inhibition may produce another mechanism of inhibition of iodide uptake by acidified TMP. In contrast, non-acidified TMP did not appear to have any effect on protein synthesis or TSH-induced cAMP production at concentrations less than 1000 μM. Column chromatography revealed that acidified TMP has a different chemical structure from non-acidified TMP. Their respective inhibitory mechanisms of iodide uptake may therefore be different from each other, but this remains to be elucidated.

Iodide transport is an independent step in the biosynthesis of thyroid hormones and involves a complex series of processes. One established mechanism for iodide transport is Na-dependency and requires the functional integrity of the Na⁺K⁺ATPase which exchanges the intracellular Na⁺ cotransported with iodide for extracellular K⁺. Previous studies have shown that iodide uptake

depends linearly on the external sodium concentration and requires the presence of external K^+ [4, 9]. This implies that some ions are important in iodide transport processes. Our experiments were planned to use differently pretreated TMP, with other conditions being the same, so that the systems with acidified and non-acidified TMP had identical Na^+ and K^+ ionic strength. The ratio of iodide uptake with and without non-acidified TMP was also similar at 20–140 mM Na^+ and 0–10 mM K^+ concentrations. This suggests that TMP does not inhibit iodide uptake via local ionic depletion on the cell surface, but the data can not completely rule out the pharmacological effect of TMP on Na^+K^+ ATPase. Another associated mechanism of iodide transport is intracellular acidification [10]. Recently the presence of a specific iodide channel in bovine thyroid [11] and H^+ -dependent ATPase activity in the FRTL-5 cell line has been reported [12]. With porcine thyroid, the immunoreactivity of H^+K^+ ATPase [13] and a weak inhibition of TSH-stimulated [^{125}I] organification by another H^+K^+ ATPase inhibitor, omeprazole, were observed [14]. In this study, we measured the H^+K^+ ATPase activity according to the method of Beil and Kornberg [15, 16] and failed to detect any K^+ -dependent ATPase activity on FRTL-5 cells (data not shown). It has been reported that K^+ -dependent ATPase in gastric parietal cells constitutes 4.2% of the total ATPase [15]. The distribution of H^+K^+ ATPase is predominately limited to gastric parietal cells with a small amount of activity in the colon. If H^+K^+ ATPase exists in thyrocytes, the activity is supposed to be a quite small proportion of total ATPase activities. Proton pump inhibitors do not appear to have any significant inhibitory effect on

Mg^{2+} -dependent ATPase or Na^+K^+ ATPase activity [17]. Further, our experiments indicated that the final production of cAMP by FRTL-5 cells was not significantly altered by neutral TMP. This suggested that the change in the total amount of intracellular ATP following the addition of TMP to FRTL-5 cells was quite small, even if any change existed. In gastric parietal cells, the H^+ pump inhibitor works through covalent binding to H^+K^+ ATPase by acidified TMP, but the inhibitory effect of TMP on iodine uptake was observed in its neutral form. The washing-out experiment in this report showed that the inhibitory effect depended on the simultaneous incubation of TMP with iodide and was reversible, suggesting that the inhibition in our system was not due to covalent binding of TMP with any cell components. The mechanism by which TMP inhibits iodide uptake is therefore unlikely to operate via H^+K^+ ATPase inhibition.

In summary, TMP could inhibit TSH-stimulated iodide uptake by FRTL-5 cells. Acidified and non-acidified TMP might generate this inhibitory effect in different ways. The inhibitory mechanisms of this drug in iodide transport may provide a new evaluation system to use in the study of thyroid iodide uptake.

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References

1. Beil W, Hannemann H, Mädge S, Sewing K-Fr (1987) Inhibition of gastric K^+/H^+ -ATPase by acid-activated 2-((2-pyridylmethyl)sulphonyl)benzimidazole products. *European J Pharmacol* 133: 37–45.
2. Ruwart MJ, Nezamis JE, Rush BD, Lancaster C, Davis JP, Nichols NM, Ochoa R (1984) Timoprazole is a unique cytoprotective agent in the rat. *Digestion* 30: 33–40.
3. Lindberg P, Brändström A, Wallmark B, Mattsson H, Rikner L, Hoffman KJ (1990) Omeprazole: the first proton pump inhibitor. *Medicinal Research Reviews* 10: 1–54.
4. Weiss SJ, Philp NJ, Grollman EF (1984) Iodide transport in a continuous line of cultured cells from rat thyroid. *Endocrinology* 114 1090–1098.
5. Ambesi-Impiombato FS, Parks LAM, Coon HG (1980) Culture of hormone-dependent functional epithelial cells from rat thyroids. *Proc Natl Acad Sci USA* 77: 3455–3459.
6. Rackur G, Bickel M, Fehlhaber HW, Herling A, Hitzel V, Lang HJ, Rösner M, Weyer R (1985) 2-((2-pyridylmethyl)sulfinyl)benzimidazoles: acid

- sensitive suicide inhibitors of the proton transport system in the parietal cell. *Biochem Biophys Res Commun* 128: 477–484.
7. Vitti P, Rotella CM, Valente WA, Cohen J, Aloj SM, Laccetti P, Ambesi-Impimbato FS, Grollman EF, Pinchera A, Toccafondi R, Kohn LD (1983) Characterization of the optimal stimulatory effects of Graves' monoclonal and serum immunoglobulin G on adenosine 3', 5'-monophosphate production in FRTL-5 thyroid cells: a potential clinical assay. *J Clin Endocrinol Metab* 57: 782–791.
 8. Weiss SJ, Philp NJ, Ambesi-Impimbato FS, Grollman EF (1984) Thyrotropin-stimulated iodide transport mediated by adenosine 3', 5'-monophosphate and dependent on protein synthesis. *Endocrinology* 114: 1099–1107.
 9. Bagchi N, Fawcett DM (1973) Role of sodium ion in active transport of iodide by cultured thyroid cells. *Biochim Biophys Acta* 318: 235–251.
 10. Marcocci C, Grollman EF (1988) Sodium/proton exchange in the maintenance of intracellular pH in FRTL-5 thyroid cells. *Endocrinology* 123: 1705–1711.
 11. Golstein P, Abramow M, Dumont JE, Beauwens R (1992) The iodide channel of the thyroid: a plasma membrane vesicle study. *Am J Physiol* 263: C590–C597.
 12. Woods DJ, Soden J, Bidey SP (1992) H⁺-dependent ATPase and K⁺ channel activities in the rat thyroid cell strain FRTL-5. *J Mol Endocrinol* 8: 79–86.
 13. Saccomani G, Helander HF, Crago S, Chang HH, Dailey DW, Sachs G (1979) Characterization of gastric mucosal membranes X. Immunological studies of gastric (H⁺ + K⁺)-ATPase. *J Cell Biology* 83: 271–283.
 14. Atterwill CK, Brown CG, Fowler KL, Jones CA (1989) Studies on the effects of omeprazole on thyroid function in the rat. *J Pharm Pharmacol* 41: 733–735.
 15. Beil W, Sewing K-Fr (1984) Inhibition of partially purified K⁺/H⁺-ATPase from guinea-pig isolated and enriched parietal cells by substituted benzimidazoles. *Br J Pharmacol* 82: 651–657.
 16. Kornberg A, Scott JF, Bertsch LL (1978) ATP utilization by rep protein in the catalytic separation of DNA strands at a replicating fork. *J Biol Chem* 253: 3298–3304.
 17. Lorentzon P, Jackson R, Wallmark B, Sachs G (1987) Inhibition of (H⁺ + K⁺)-ATPase by omeprazole in isolated gastric vesicles requires proton transport. *Biochim Biophys Acta* 897: 41–45.