ACTIVATION OF THYROID ORNITHINE DECARBOXYLASE (ODC) *IN VITRO* BY HYPOTONICITY; A POSSIBLE MECHANISM FOR ODC INDUCTION

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SUMMARY: Thyrotropin, dibutyryl cyclic AMP, and Graves' immunoglobulin G activate mouse thyroid ornithine decarboxylase *in-vitro*. We now report that hypotonicity of the incubation medium, *per se*, also stimulates the enzyme. The hypotonic-induced increase was due to osmotic, not ionic changes and was linearly related to medium dilution. When near maximal hypotonicity was combined with various thyroid stimulators, a synergistic effect on ornithine decarboxylase was seen. Thus, changes in membrane configuration (as induced experimentally by hypotonicity) may be an important primary event in thyroid activation by thyrotropin or other stimulators.

INTRODUCTION: Ornithine decarboxylase (ODC) (EC 4.1.1.17), the rate-limiting enzyme in polyamine synthesis (1), is regulated in thyroid by thyrotropin (TSH) and may serve as a marker for the growth-promoting effects of TSH and/or other thyroid stimulators (2). During studies in which mouse thyroid ODC activity was induced *in-vitro* by TSH, dibutyryl cyclic AMP (DBC), and certain thyroid-stimulating immunoglobulins (ODC-SA) (3), it was observed that hypotonicity of the incubation medium, *per se*, also stimulates thyroid ODC. It has previously been shown that putrescine content of Hela cells in culture is inversely related to osmolality of the growth medium, and that changes in putrescine content could be correlated with changes in ODC activity (4).

We now report that thyroid ODC *in-vitro* is activated by medium hypotonicity and that the stimulation is due to osmotic rather than ionic changes.

MATERIALS: DL-[1-^{14}C] ornithine monohydrochloride (60 mCi/mmole) was purchased from Amersham-Searle (Arlington Heights, IL). DL-[2-^{14}C] ornithine hydrochloride (3.5 mCi/mmole) was purchased from New England Nuclear (Boston, MA). Basal Medium (Eagle) with Earle's Salts (BME) (osmolality approximately 285 mOsm/Kg (5)) was purchased from Grand Island Biochemicals (Grand Island, NY). TSH (in the form of Thytropar$^{(R)}$) was purchased from Armour Pharmaceutical (Kankakee, IL). 1,3-diaminopropane was purchased from Aldrich Chemical Co. and was neutralized with HCl before use. Dibutyryl cyclic AMP, putrescine dihydrochloride, spermidine trihydrochloride, and cycloheximide, were purchased from Sigma Chemical Co. (St. Louis, MO).

Abbreviations used in text: TSH = thyrotropin; DBC = Dibutyryl cyclic AMP; ODC-SA = Graves' immunoglobulin G with ODC-stimulating activity; ODC = ornithine decarboxylase; BME = Basal Medium (Eagle).

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METHODS

Tissue Preparation: Five Sprague-Dawley mice were used for each determination. The animals were killed by cervical dislocation and the thyroids removed *en bloc* while still attached to the trachea. After rinsing in BME and removal of any excess connective tissue, the intact thyroid/tracheas were incubated in 3.5 ml of BME-Earle's Salts (control) or with the various stimulators under an atmosphere of 95% O_2 - 5% CO_2 for 4 h at 37 C. The thyroids were then carefully trimmed off the tracheas, and homogenized with a Polytron (Brinkman Inst.) homogenizer in 1.1 ml of 0.05 M Na-K-phosphate buffer, pH 7.2 containing 10 mM tetrasodium EDTA and 5 mM dithiothreitol at 4 C. The homogenate was centrifuged at 20,000 x g for 20 min at 4 C, and the supernatant fraction immediately assayed for ODC.

Hypotonic Induction of ODC: ODC activity was induced *in-vitro* by incubating intact mouse thyroid/tracheas for 4 h at 37 C in media of varying degrees of hypotonicity. This was effected by varying the ratio of BME and water in the incubation medium (*e.g.* 1.75 ml H_2O + 1.75 ml BME = 0.5 isotonic). In other experiments, the amount of BME was kept constant throughout and changes in osmolality were achieved by different ratios of 0.25 M sucrose and water. After incubation, the glands were homogenized and treated as above.

Assay of ODC Activity: ODC activity was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C]-ornithine as previously described (2), except that incubation temperature was 44 C. Enzyme activity is expressed as pmoles $^{14}CO_2$ formed/g tissue/30 min incubation.

^{14}C-putrescine formation from DL-[2-^{14}C]-ornithine was assayed by the method of Raina (6). For comparison of $^{14}CO_2$ formation and ^{14}C-putrescine formation, "cold" ornithine was added to the DL-[1-^{14}C]-ornithine to give a final specific activity of 3.5 mCi/mmole, *i.e.* equal to that of DL-[2-^{14}C]-ornithine.

Protein concentration was measured by the method of Lowry, *et al* (7).

All data were analyzed for statistical significance by Student's *t* test.

RESULTS: Figure 1 shows the effect of increasing dilution of the incubation medium with water on mouse thyroid ODC activity. ODC activity was linearly related to medium dilution and was still rising at the highest dilution tested (0.43 isotonic). Significant activation was already evident at 0.85 isotonic. Under these conditions there should be no cell damage, for Roti (8) has shown that mouse leukemic cells are viable up to 0.44 isotonic. When the incubation medium was made hypertonic by the addition of sucrose, no activation of ODC was seen (data not shown).

Since hypotonic-induced increase in ODC could be due either to a reduction in osmolality or a reduction of ions in BME, in a second set of experiments the amount of BME was kept constant throughout and changes in osmolality were achieved by varying the relative amounts of 0.25 M sucrose and water, as described in "Methods". Under these conditions, the increase in ODC activity with decreasing osmolality was identical to the results shown in Figure 1, even though ionic concentrations were the same throughout (data not shown). In additional ex-

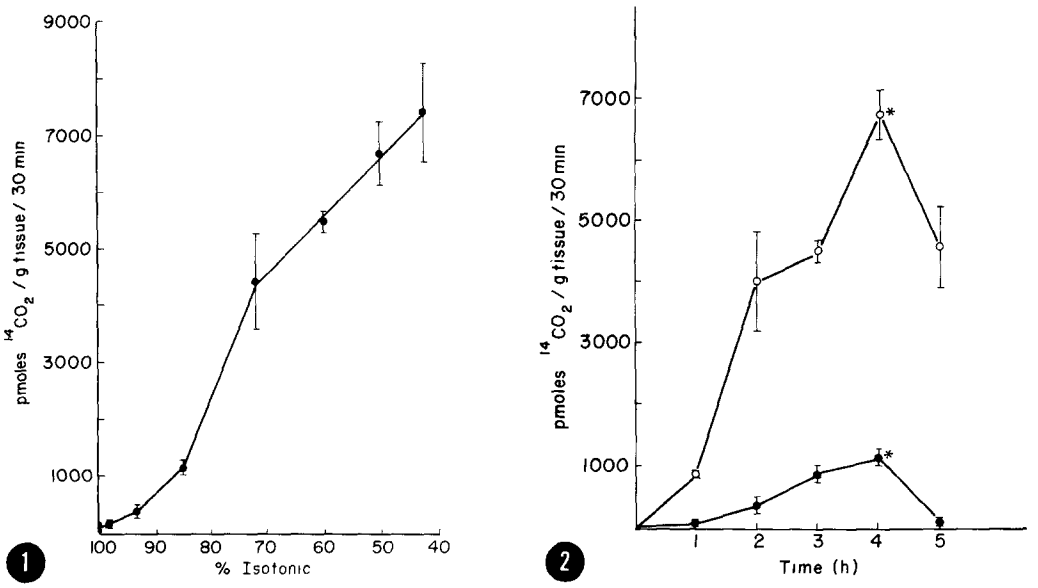

FIGURE 1. Hypotonic induction of ODC activity in mouse thyroid. Intact thyroid/tracheas were incubated for 4 h at 37 C in BME-Earle's Salts of varying degrees of hypotonicity, as described in "Methods" and then assayed for ODC activity. Each point represents the mean ± SD of three determinations.

FIGURE 2. Time-course of TSH-induced and hypotonic-induced ODC activity in mouse thyroid. Intact thyroid/tracheas were incubated with 2 mU/ml TSH (●—●) or in 0.60 isotonic BME (o—o) for various times and then assayed for ODC activity. Each point represents the mean ± SD of three determinations. *Significantly ($p < .005$) greater than 0 h.

periments where 0.25 M sucrose and BME were varied up to 44% BME and 56% 0.25 M sucrose, no increase in ODC activity was seen (data not shown).

Figure 2 shows the time-course of hypotonic (0.60 isotonic)-induced as well as TSH-induced (2 mU/ml) ODC activity. Both activities peaked at 4 h after stimulation, and it is evident that the hypotonic response is much greater than that induced by TSH. Additionally, the TSH response returns to basal values at 5 h, whereas the hypotonic response is still very much evident.

To insure that "authentic" ODC was being induced by hypotonicity, *i.e.* a reaction which resulted in putrescine formation, and not some non-specific decarboxylase reaction, concomitant ^{14}C-putrescine and $^{14}CO_2$ formation from DL-^{14}C-ornithine was measured for both TSH-stimulated and hypotonic-induced ODC. As can be seen from Table I, in both instances, increased $^{14}CO_2$ formation is also re-

TABLE I

COMPARATIVE AMOUNTS OF $^{14}CO_2$ FORMATION
AND [^{14}C]-PUTRESCINE FORMATION FROM DL-[^{14}C]-ORNITHINE

Treatment	pmoles $^{14}CO_2$ formed/g tissue/30 min‡	pmoles [^{14}C]-putrescine formed/g tissue/30 min‡
1. Control	0	0
2. TSH (2 mU/ml)	1786 ± 83	2438 ± 680
3. 0.8 isotonic	1630 ± 235	3414 ± 680
4. 0.6 isotonic	17508 ± 567	23908 ± 2960

Intact mouse thyroid/tracheas were incubated for 4 h in BME. ODC was then assayed by measuring ^{14}C-putrescine formation from DL-[2-^{14}C]-ornithine and $^{14}CO_2$ formation from DL-[1-^{14}C]-ornithine as described in "Methods".

‡ Each point represents the mean ± SD of triplicate ($^{14}CO_2$) or duplicate ([^{14}C]-putrescine) determinations.

flected in a stoichiometric increase in ^{14}C-putrescine formation. Additionally, non-specific mitochondrial ODC activity, seen in the 20,000 x g pellet (< 600 pmoles $^{14}CO_2$/g tissue/30 min) was unaffected by hypotonicity (data not shown). Thus, "authentic" ODC resulting in increased putrescine formation is induced by hypotonicity.

When cycloheximide in concentrations as low as 0.5 µg/ml was included in the hypotonic (0.44 isotonic) incubation medium, no induction in ODC activity was seen (data not shown). Inhibition by cycloheximide was total; thus, continued protein synthesis is necessary for hypotonic induction of ODC.

Addition of exogenous polyamines to the incubation medium inhibit *in-vitro* ODC activity (9,10). As shown in Table II, when 2 mM putrescine, spermidine, or 1,3-diaminopropane [a close structural analogue of putrescine (11)] were included in the incubation medium, the hypotonic-induced ODC response was inhibited by 80-96%. TSH-induced ODC activation was similarly inhibited (data not shown). Thus, in this respect, also, hypotonic induction of thyroid ODC is similar to TSH-induced enzyme activation.

Figure 3 details a representative experiment where minimal or intermediate medium hypotonicity was combined with ODC-SA (3a), TSH (3b), and DBC (3c), and

TABLE II

INHIBITION OF HYPOTONIC-INDUCED ODC BY POLYAMINES

Treatment	pmoles $^{14}CO_2$ formed/g tissue/30 min‡	% Inhibition
1. Control	72 ± 45	
2. 0.7 isotonic	8376 ± 816	
3. 0.7 isotonic + 2 mM putrescine	1653 ± 147*	80
4. 0.7 isotonic + 2 mM spermidine	336 ± 42*	96
5. 0.7 isotonic + 2 mM 1,3-diaminopropane	1116 ± 75*	86

Intact mouse thyroid/tracheas were incubated for 4 h in 0.7 isotonic medium, with and without added polyamines (2 mM). ODC activity was then assayed as described in "Methods".

‡ Each point represents the mean ± SD of triplicate determinations.

* Significantly ($p < .005$) less than 0.7 isotonic.

the resultant effect on ODC activation. The same pattern was observed for all three stimulators, *i.e.* in the presence of minimal medium hypotonicity, the effects were additive, and with intermediate (or near-maximal) hypotonicity, the effects were synergistic.

DISCUSSION: This study shows that mouse thyroid ODC activity is induced *in-vitro* by reduction of the osmolality of the incubation medium. Munro, *et al* (12) previously reported that putrescine content of E.coli is inversely related to osmolality of the growth medium, and subsequently showed that a similar phenomenon occurs in mammalian cells (4). These changes in putrescine concentration were correlated with ODC activity, in that sudden increases in osmolality produced rapid decreases in ODC activity, and sudden decreases in osmolality produced substantial increases in ODC activity (4). Our findings are thus an extension of Munro's in that this phenomenon is also demonstrable in intact thyroid lobes.

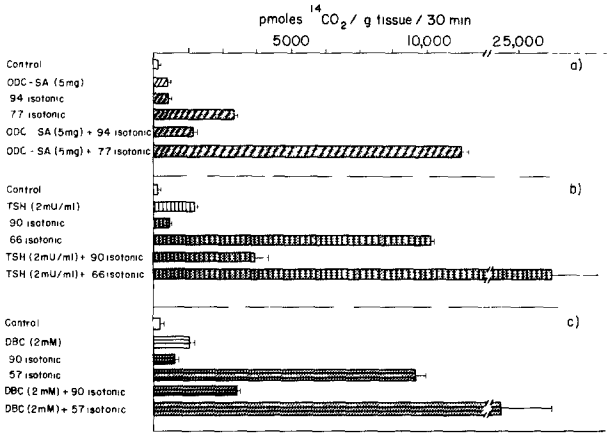

FIGURE 3. Effect of combining a minimal and intermediate dose of medium hypotonicity with a) ODC-SA, b) TSH, and c) DBC, and resultant effect on ODC activation. Intact thyroid/tracheas were incubated in BME of varying hypotonicity with and without ODC-SA (5 mg), TSH (2 mU/ml), or DBC (2 mM) for 4 h, after which ODC activity was assayed. Each bar represents the mean ± SD of three determinations.

Hypotonic-induced ODC activity in mouse thyroid (based on $^{14}CO_2$ formation) is truly representative of "authentic" ODC in that: i) stoichiometric amounts of ^{14}C-putrescine are also formed, ii) exogenous polyamines inhibit the ODC activity (9), and, iii) continued *de novo* protein synthesis is a requirement for enzyme induction. In all these respects, hypotonic-induced ODC is similar to TSH-activated thyroid ODC. The observation that the polyamines inhibit hypotonic induced ODC activity would argue against the possibility of lack of product inhibition in these stimulated cells as a cause for the greater magnitude of hypotonic-induced ODC activity as compared to ODC activation by authentic thyroid stimulators.

Chen, *et al* (13) have shown that addition of Na^+, K^+, or Mg^{++} to the medium completely inhibits ODC activity in different cell lines and that the effect is due to ionic rather than osmotic changes. While it is possible that changes in induced ODC and in basal ODC activity are separate phenomena, our data show that osmotic rather than ionic changes in the incubation medium are responsible for ODC activation. However, the data do not rule out changes in <u>intracellular</u> concentrations of cations (specifically, K^+) as the triggering mechanism of hy-

potonic activation of ODC. In this regard, Roti (8) has shown that dilution of isotonic medium to 0.5 isotonic results in a specific loss of K^+ from the cell (up to 50%).

Phagocytosis of latex beads by isolated thyroid cells results in a variety of stimulatory effects on thyroid cell function (14). In these studies, it was postulated that perturbation of the thyroid cell membrane, as induced experimentally by phagocytosis, could trigger the increased thyroid cell activity. Since ODC activity of L 1210 cells (15) and rat thyroid (2) is inhibited by agents which disrupt the microtubule-microfilament cytoskeleton of the cell, it would appear that intracellular ODC activity may be sensitive to perturbation of the cell membrane. It is therefore proposed that *in-vitro* activation of thyroid ODC by hypotonicity is, in fact, a related phenomenon, for Roti (16) has shown that mouse leukemic cell membranes stretch significantly after initial exposure to hypoosmotic conditions. Since hypotonicity potentiates, in a synergistic manner, stimulated thyroid ODC, the possibility that changes in cell membrane configuration (as induced experimentally by hypotonicity) may be an important primary event in ODC activation by TSH or other thyroid stimulators warrants further consideration and study.

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