# Lack of prostaglandin involvement in the mitogenic effect of TSH on canine thyroid cells in primary culture

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The production of prostaglandin $E_2$ ($PGE_2$) by cultured dog thyroid cells was high in a serum-containing medium and low in a serum-free, completely defined medium. Thyrotropin (TSH) and epidermal growth factor (EGF), two mitogenic factors for these cells, did not stimulate $PGE_2$ release. Indomethacin, at a concentration which completely inhibited $PGE_2$ production, had no effect on thyroid cell multiplication and DNA synthesis stimulated by TSH and EGF. It is concluded that cyclooxygenase products are not involved in the proliferation of canine thyroid cells and its control by TSH.

*Thyroid* *Thyrotropin* *EGF* *Prostaglandin* *Indomethacin* *Proliferation* *Serum-free culture*

## 1. INTRODUCTION

Prostaglandin (PG)$F_{2\alpha}$ initiates DNA synthesis and cell proliferation in 3T3 fibroblast cultures [1]. This mitogenic action is synergistically enhanced by prostaglandin (PG) $E_1$ or $E_2$ [2]. Prostaglandin production has been suggested to have a mediator role in the stimulation of proliferation by various growth factors or tumor promoters [3–7]. In vivo and in vitro experiments have suggested that prostaglandins might play a role in the mitogenic action of thyrotropin (TSH) on the thyroid gland. Authors in [8] have observed that prostaglandin synthesis inhibitors abolished the stimulatory effect of TSH on rat thyroid cell multiplication in vivo. In [9] it was reported that indomethacin and aspirin inhibit the mitogenic effect of TSH on a cloned cell line derived from the rat thyroid. In addition, a chronic stimulatory effect of TSH on rat thyroid prostaglandin synthesis has been observed in [10]. In order to evaluate the general validity of these observations, we investigated the effects of TSH on prostaglandin synthesis and of cyclooxygenase inhibitors on the mitogenic action of TSH, in primary cultures of differentiated canine thyroid cells [11,12]. In this model system, TSH is sufficient to trigger the DNA synthesis and the proliferation of cells which are quiescent in serum-free, chemically defined culture conditions [12].

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Collagenase (130 units/mg) was purchased from Worthington Chemical Co. (Freehold, NJ). Basal medium of Eagle (BME), Dulbecco's modification of minimum essential medium (DMEM), Ham's F12 medium, MCDB 104 medium, glutamine, penicillin-streptomycin and amphotericin B (fungizone) were obtained from Flow Labs (Irvine). Foetal calf serum was from Eurobio (Paris). Bovine insulin, transferrin, murine epidermal growth factor (EGF) were purchased from Collab. Res. (Waltham, MA). Glycylhistidyllysyl acetate and somatostatin were Sigma products (St Louis, MO). Bovine TSH (1–3 IU/mg) was obtained from Armour Pharmaceutical Co. (Chicago, IL). [methyl-$^3$H]Thymidine (40 Ci/mmol) and tritium-labelled tracers were obtained from the Radiochemical Centre (Amersham). PG and $TxB_2$



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standards were purchased from Upjohn Diagnostics (Kalamazoo, MI). Anti-$PGE_2$ and anti-$PGF_{2\alpha}$ antisera were purchased, respectively, from Institut Pasteur de Paris and Clinical Assays. Anti-6-keto-$PGF_{1\alpha}$ and anti-$TxB_2$ antisera were gifts from Dr J. Beetens (Antwerp) and Dr J.M. Smith (Philadelphia), respectively. Optifluor scintillation fluid was obtained from Packard Instruments.

2.2. *Cell culture*

Dog thyroid cells were cultured as in [11,12]. Briefly, the thyroid tissue was digested by collagenase so that the resulting suspension consisted mainly of fragmented and intact follicles. These follicles were seeded in 35-mm tissue culture-treated plastic Petri dishes, and, in 1 day, adhered to the substratum while a monolayer developed. The seeding was realized so that $5 \times 10^4$ to $2 \times 10^5$ cells attached to the dish after 1 day and 1 medium change. Following indications, the cells were cultured in BME supplemented with 10% serum from dogs treated for 2 days with thyroid extracts in order to reduce the endogenous TSH level [11], 4% foetal calf serum, 2 mM glutamine, 100 units penicillin/ml, 100 $\mu$g streptomycin/ml, 2.5 $\mu$g amphotericin B/ml; or in a chemically defined serum-free medium: DMEM + $F_{12}$ + MCDB 104 (2:1:1) supplemented by 10 $\mu$g insulin/ml, 1.25 $\mu$g transferrin/ml, 10 ng glycylhistidyllysyl acetate/ml, 10 ng somatostatin/ml and 40 $\mu$g ascorbic acid/ml with the same glutamine and antibiotic supplement. The Petri dishes were maintained in a water-saturated incubator at 37°C in an atmosphere of 5% $CO_2$ in air. The medium was renewed every other day and TSH or other effectors were added as indicated.

2.3. *Proliferation assays*

Cell multiplication curves were obtained from cell DNA measurements. Our cell DNA assay described in [11] uses the increase of fluorescence of ethidium bromide when complexed with nucleic acids. It has been verified that an increase in cell DNA amount per dish reflects an increase in cell number.

DNA synthesis was evaluated by the frequency of the [$^3$H]thymidine-labelled nuclei as estimated by autoradiography [12]. The quiescent cells, after 4-day culture in the chemically defined medium, were incubated with TSH in the presence of [$^3$H]thymidine (10 $\mu$Ci/ml, $3 \times 10^{-5}$ M) and deoxycytidine ($10^{-4}$ M) for the indicated times. The cells in the Petri dishes were fixed by methanol, extensively washed and autoradiographies were performed as in [13]. The cells were stained with toluidine blue and the proportion of labelled nuclei was evaluated by counting at least 500 nuclei from different microscopical fields.

2.4. *Active transport of iodide [14]*

The trapping of iodide was evaluated by the uptake of radioiodide at equilibrium. The cells in the Petri dishes were incubated for 2 h with $Na^{131}I$ (2 $\mu$Ci/ml) in $10^{-6}$ M KI in BME at 37°C. Mercaptomethylimidazole (1 mM) was added to block iodide organification. The cells were then rapidly rinsed with BME 3 times, scraped and counted in a $\gamma$-counter. The radioactivity was normalized to the cellular DNA measured from the same dishes.

2.5. *Prostaglandin assays*

The radioimmunoassay of $PGE_2$, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and thromboxane $B_2$ released by the cells was performed as in [15]. The specificity of antisera used was reported in [15]. The following criteria of validity for these assays were fulfilled: good interassay reproducibility (c.v. = 5% for 10 serial measurements over one month), good correlation between values obtained at two different dilutions of the samples ($r = 0.92$) and complete inhibition by indomethacin of the release of immunoreactive material. The concentration of $PGE_2$ in dog serum was 6.5 ± 2.0 ng/ml (mean ± SD; $n = 6$). All the results were corrected by subtracting the amount of $PGE_2$ present in the culture medium.

## 3. RESULTS

Dog thyroid cells, cultured in the presence of serum, released large amounts of $PGE_2$ in the culture medium (fig.1). During the first 24-h period, starting one day after seeding, the output of $PGE_2$ was 19 ± 3 ng/$\mu$g DNA (mean ± SE, 9 experiments): this represents about 96 ± 17 ng/$10^6$ cells, on the basis of a nuclear content of DNA equal to 5 pg. $PGE_2$ production gradually declined during the following days (fig.1). TSH (10 munits/ml), which stimulated the proliferation of

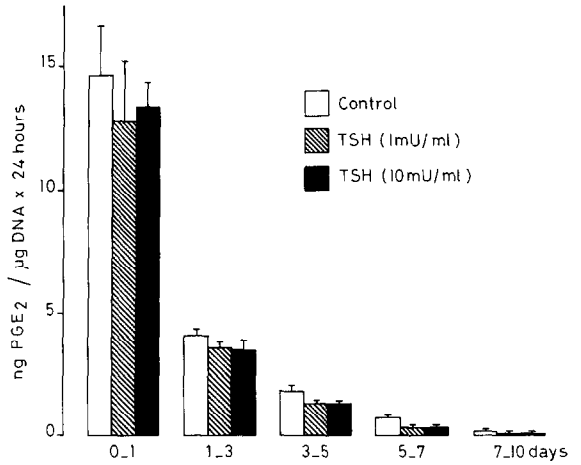


Fig.1. Kinetics of $PGE_2$ release by dog thyroid cells cultured in a serum-containing medium (BME + 10% dog serum and 4% foetal calf serum). $PGE_2$ was measured in the medium in which thyroid cells grew for the indicated periods. Results are expressed as the mean ± range of measurements on duplicate dishes in 1 representative experiment out of 9.

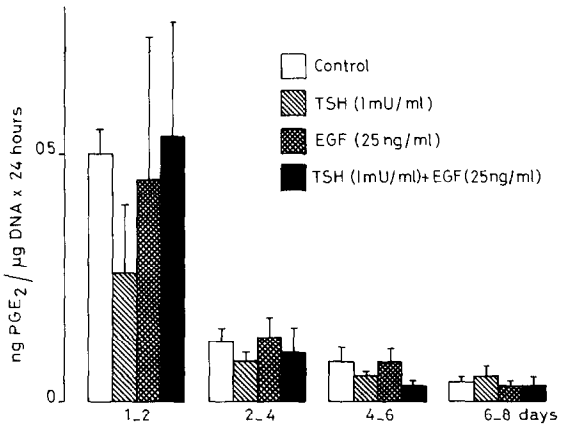


Fig.2. Kinetics of $PGE_2$ release by dog thyroid cells cultured in a serum-free, completely defined medium. TSH or EGF were added at day 1 and their presence maintained in the culture medium which was renewed every other day. Results are expressed as the mean ± SD of 4 determinations (duplicate dishes in 2 separate experiments).

these cells, did not increase their release of $PGE_2$ (fig.1). In most experiments, TSH produced a significant inhibition of this release: during the first 24-h period, TSH-treated cells released 30 ± 4% less $PGE_2$ than control cells (mean ± SE, 9 experiments; range: 4–39%). When canine thyroid cells were cultured in a serum-free completely defined medium, they released much lower amounts of $PGE_2$ (fig.2). This release declined with time and was stimulated neither by TSH, nor by Epidermal Growth Factor (EGF), which were both mitogenic for these cells ([16], submitted) (fig.2). The thyroid cells produced amounts of 6-keto-$PGF_{1\alpha}$ similar to those of $PGE_2$ and lower quantities of $PGF_{2\alpha}$, whereas $TxB_2$ was below the detection limit. The release of 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ was characterized by the same temporal pattern and lack of stimulation by TSH, as $PGE_2$ release (not shown).

Indomethacin, at a concentration of 0.56 $\mu$M or 1.4 $\mu$M which completely inhibited the production of $PGE_2$ (table 1), had no effect on the multiplication of canine thyroid cells measured either in a serum-containing medium or in a serum-free completely defined medium. The stimulatory effect of TSH or of the TSH-EGF combination on thyroid cell multiplication (fig.3) and DNA synthesis (table 1) was not altered by indomethacin. The enhancement of iodide uptake by TSH was also insensitive to indomethacin (table 1).

## 4. DISCUSSION

The amount of $PGE_2$ produced by the dog

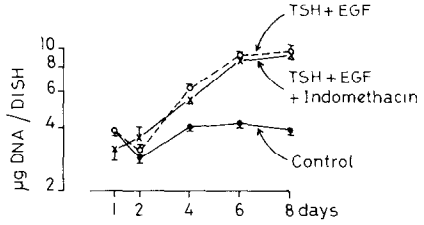


Fig.3. Lack of effect of indomethacin on the multiplication of dog thyroid cells stimulated by a combination of TSH and EGF. TSH (1 munit/ml) and EGF (25 ng/ml) were added to control medium at day 1 and their presence maintained throughout the culture period, while medium was renewed every other day. Indomethacin (1.4 $\mu$M) was present from the seeding time (day 0). Results are expressed as the mean ± range of measurements on duplicate dishes.

Table 1

Effect of indomethacin on labelling of nuclei by [$^3$H]thymidine, iodide uptake and $PGE_2$ release in dog thyroid cells cultured in a defined serum-free medium

| | Labelled nuclei (%) | Iodide uptake (cpm $^{131}$I/ng DNA) | $PGE_2$ release (ng/$\mu$g DNA per 24 h) |
|---|---|---|---|
| Control | 2.2 ± 0.5 | 5.4 ± 0.4 | 0.23 ± 0.03 |
| TSH (1 munits/ml) | 54.1 ± 0.8 | 43.4 ± 1.0 | 0.17 ± 0.05 |
| Indomethacin (1.4 $\mu$M) | 1.6 ± 0.2 | 5.6 ± 0.6 | <0.02 |
| TSH (1 munit/ml) + indomethacin (1.4 $\mu$M) | 52.6 ± 0.3 | 38.7 ± 1.3 | <0.02 |

The cells were seeded and cultured for 4 days in the complete control serum-free medium, with or without indomethacin (1.4 $\mu$M). At day 4, TSH (1 munit/ml) was added to cells cultured in the presence or in the absence of indomethacin and the presence of the effectors was maintained for the rest of the culture. DNA synthesis was evaluated by the accumulation of [$^3$H]thymidine-labelled nuclei between day 5 and day 6, revealed by autoradiography. Iodide uptake was estimated 48 h after TSH addition. $PGE_2$ accumulation in the medium was estimated for a 56-h period starting at day 4. Results are expressed as the mean ± range of measurements on duplicate dishes

thyroid cells was critically dependent on the culture conditions: cells cultured in a defined medium released 10–30 times less $PGE_2$ than cells cultured in a serum-containing medium. Serum is a well known stimulus of prostaglandin synthesis in cell culture [17,18].

TSH did not stimulate the release of $PGE_2$, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$ or thromboxane $B_2$ by cultured dog thyroid cells. In most experiments performed in serum-containing medium, $PGE_2$ output was significantly decreased by TSH. TSH has also been shown to inhibit $PGE_2$ production by non-growing cultures of dog thyroid cells [19,20]. The chronic stimulatory effect of TSH on rat thyroid prostaglandin synthesis observed in vivo [10] has thus not been reproduced in this in vitro model of chronic stimulation by TSH. The lack of stimulatory effect of TSH on $PGE_2$ production by thyroid cells is in contrast with the delayed stimulation of prostaglandin synthesis in graafian follicles by LH and HCG, which share with TSH a similar glycoprotein structure and a common mediator, cyclic AMP [21,22]. EGF increases prostaglandin synthesis in some cell cultures [3]; on the contrary, in dog thyroid cells, it produced a mitogenic response but no stimulation of $PGE_2$ release.

Indomethacin, at a concentration which completely inhibited $PGE_2$ production by the thyroid cells, did not depress the basal proliferation stimulated by serum and the mitogenic effects of TSH and EGF, isolated or in combination, evaluated as cell multiplication (measurement of DNA content). The triggering by TSH of DNA synthesis by quiescent cells in serum-free conditions was not affected by indomethacin.

On the other hand, we have failed to detect any mitogenic effect of exogenously added prostaglandins E (1–3 $\mu$g/ml) or $F_{2\alpha}$ (1 $\mu$g/ml) both in high serum or serum-free conditions (unpublished). The discrepancy between our results and those in [9] on a rat thyroid cell line, might be due to a species difference, but might be also inherent to the experimental system used: in our hands, primary culture of cells, which have little proliferated in vitro in defined conditions before TSH addition, may not be suspected to be genetically transformed or selected by their culture conditions. In contrast, the rat thyroid cell line FRTL 5 has been selected by cloning from the FRTL cells in order to be quiescent in the presence of 5% serum and mitogenically stimulated by TSH [23]. The parental FRTL line itself appears actually immortal and is cloned and propagated in the continuous presence of TSH. TSH is not only required for the proliferation, but also for the survival of these cells [24]. Such lines may have developed new mechanisms of growth control or be submitted to new rate controlling effectors, the general relevance of which is questionable.

In conclusion, our study shows that in primary cultures of dog thyroid cells, TSH and EGF do not stimulate prostaglandin production and in-

domethacin does not inhibit their mitogenic action. This is true whatever the culture conditions used: either in a serum-containing medium or in a serum-free, completely defined medium. The interpretation of the results obtained in a completely defined medium is straightforward, since it is not complicated by the presence of prostaglandins contained in serum. It can thus be stated that cyclooxygenase products are not necessary for the expression of the mitogenic response to TSH (or to EGF) in dog thyroid cells in primary culture. The trapping of iodide and its stimulation by TSH, a marker of the differentiated state of these cells, is also independent of cyclooxygenase products. These findings are consistent with the view that cyclic AMP is a sufficient signal to mediate the stimulatory effect of TSH on both the differentiated functions and the proliferation of thyroid cells [12].

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