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ORIGINAL PAPER

Hypothyroidism and hyperthyroidism modulates Ras-MAPK intracellular pathway in rat thyroids

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Abstract Thyrotrophin induces proliferation and function in thyroid cells acting through a seven transmembrane G protein-coupled receptor. The proliferative pathways induced by thyrotropin (TSH) in thyrocytes in vivo are not completely understood yet. The aim of this work is to evaluate if Ras can be induced by TSH in rat thyroids, and whether extracellular regulated kinase (ERK) may be involved in the subsequent intracellular signalling cascade. We induced hypothyroidism in Wistar rats by methimazole (MMI) treatment (0.03% in the drinking water for 21 days). A subset of the hypothyroid rats received T_4 (1 μg/100 g bw) during the last 10 days of MMI treatment. Hyperthyroidism was induced by subcutaneous injections of T_4 (10 μg/100 g bw) during 10 days in another group of rats. Our data show that in the hypothyroid rats there is a clear positive Ras modulation, but a decrease in pERK. In contrast, thyroidal pERK increases in T_4-induced hyperthyroidism, but without any change in RAS, although these changes did not reach statistical significance. Thus, while the rat thyroid proliferation induced by TSH may involve an increase in RAS signalling, the subsequent cascade does not involve ERK phosphorilation, which in fact, increases during T_4-induced hyperthyroidism.

Keywords ERK · Thyroid proliferation · TSH · Thyroid function · Hypothyroidism

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Introduction

Thyrotrophin (TSH), acting through a seven transmembrane G protein-coupled receptor, is the main activator of thyroid cell proliferation and function. The TSH receptor activation of the Gs protein increases intracellular cyclic AMP (cAMP), and activates protein kinase A (PKA). Others pathways may also be involved in TSH effects on thyrocytes. In fact, Van Sande et al. [1] recently showed that TSH can activate the phospholipase C cascade in human thyrocytes.

Thyroidal proliferation, but not function, can also be induced by growth factors [2]. Ras proteins are involved in the transduction of growth factor signals by surface receptors, and are key components of downstream signalling through several pathways [3]. Ras activation of the Raf serine/threonine kinases, and activation of the ERK mitogen-activated protein kinases (MAPKs) is an important signalling pathway for many Ras effects, the others being the activation of phosphatidylinositol-3 kinase (PI3K) or the Ral-small GTPases [4]. Ciullo et al [5] reported that Ras activity is required for the FRTL-5 cells cycle progression in the early-mid G1 phases, and that TSH, cAMP or PKA significantly stimulate the PI3K-Ras complex formation.

TSH, via cAMP, in cooperation with insulin, IGF-1 or other growth factors, stimulate cell cycle progression and proliferation in various thyrocyte culture systems, including rat thyroid cells lines (FRTL-5, WRT, PCCl3) and primary cultures of rat, dog, sheep and human thyroids. The often contradictory reports were critically reviewed by Kimura et al. [6], who concluded that some results are valid only for the specific experimental conditions used in a particular experiment. The proliferative pathways induced by TSH in thyrocytes in vivo are not completely under-

stood yet. The aim of this work is to evaluate a possible Ras induction by TSH, as well as to evaluate whether pERK is involved in the intracellular signal thus activated.

Results

As expected, hypothyroidism caused an expressive increase in thyroid size, 13.5 ± 0.7 mg in controls versus 29.0 ± 2.2 mg in the hypothyroid animals. After 10 days of replacement with a physiological amount of T_4 (1 μg T_4/100 g bw) the thyroid glands were still enlarged (34.6 ± 3.8 mg). The thyroid weight in the T4-induced hyperthyroidism group (10 μg T_4/100 g bw) did not differ from that of controls. The changes in thyroid gland weight reflect the changes in serum TSH that was significantly increased in the hypothyroid group, and decreased in the hyperthyroid rats. The MMI-induced TSH increase was only partially reverted by the physiological T_4 treatment (Table 1). Serum T_4 and T_3 were significantly decreased in the hypothyroid group, and serum T_3, but not T_4, was significantly increased in T_4-induced hyperthyroidism (Table 1).

As can be seen in Fig. 1, the average thyroid Ras expression was increased in the MMI-treated rats and in those treated with MMI and T_4, although the increases did not reach statistical significance ($p = 0.147$), but did not change in the hyperthyroid animals. The pERK expression almost doubled in the hyperthyroid animals, but tends to decrease in the hypothyroid group (Fig. 2), while the total ERK expression is greater in the hypothyroid group than in the hyperthyroid or control rats (Fig. 3). In all three cases the mean values of the hypothyroid T_4-treated rats were similar to the controls, halfway between the hypothyroid and the hyperthyroid groups. Thus, hypothyroidism decreases the amount of phosphorylated ERK protein while hyperthyroidism clearly increases ERK phosphorylation (Figs. 2 and 3).

Discussion

The mechanisms by which proto-oncogenes interfere with thyroid differentiation are still unknown. Rearrangements of the proto-oncogene RET resulting in its constitutive activation are found in a significant proportion of papillary carcinomas of the thyroid. Nevertheless, the conditional expression of variants of RET rearrangements in PCCL3 cells, a well-differentiated rat thyroid cell line, showed it to be a weak tumour-initiating event [7]. In human tumourigenesis, Kimura et al. [8] showed that the BRAF protein V600E (formerly designed V599E) mutation is the most common genetic change in thyroid papillary cancer. Xing [9], in a metanalysis of 29 studies, found an overall prevalence of the related T1799A mRNA BRAF mutation in 44% of sporadic adult thyroid tumours. Recently, De Vita et al. [10] reported that a high RAS oncogene expression in FRTL-5 cells inhibits thyroid differentiation, by inhibiting Tiff1 and Pax8 expression, and induces TSH-independent growth. A low expression of the same RAS oncogene induces TSH-independent growth, but does not affect Tiff1 and Pax8 expression.

The role of the ERK mitogen-activated protein kinases (MAPK) in TSH-induced thyrocyte proliferation is still not completely understood. Pomerance et al. [11] demonstrated that TSH activates the p38 MAPK in Chinese hamster ovary cells stably transfected with human TSH receptor (hTSHR-CHO), but their data was not confirmed by Vandeput [12] who found no TSH-induced activation of c-Jun-N terminal kinases (JNK1 and JNK2) or p38 MAPK in proliferating primary culture of human and dog thyrocytes. Iacovelli et al. [13] showed that TSH increases ERK phosphorylation in FRTL-5 cells by a mechanism mediated by cAMP, but independent of PKA. Differences in the thyrocyte culture systems may be responsible for many of the conflicting data in the literature [6, 14].

Our data suggest that TSH may stimulate Ras in rat thyroids, but the signalling thus induced does not involve pERK. On the other hand, hyperthyroidism increases pERK expression, be it through a decreased TSH stimulus or by a direct effect of thyroid hormones, without any increase in Ras expression. An increase in total ERK was induced by hypothyroidism and partially reverted by a physiological dose of T_4 (Fig. 3), although these changes did not reach statistical significance.

Table 1 TSH, T_4 and T_3 serum levels [Mean ± SEM]

Experimental groups	Serum TSH[a] (ng/ml)	Serum T4 (μg/dl)	Serum T3 (ng/dl)
Control	1.42 (1.25–1.62) ($n = 8$)	3.20 ± 0.25 ($n = 12$)	25.45 ± 2.66 ($n = 6$)
Hypothyroid (MMI)	28.4* (24.0–32.3) ($n = 8$)	0.77 ± 0.15* ($n = 10$)	12.78 ± 0.50* ($n = 6$)
MMI + T_4 (1 μg T_4/100 g BW)	10.4* (8.97–12.1) ($n = 7$)	2.84 ± 0.45 ($n = 10$)	25.98 ± 5.34 ($n = 6$)
Hyperthyroid (10 μg T_4/100 g BW)	0.60* (0.55–0.67) ($n = 8$)	2.71 ± 0.39 ($n = 10$)	39.02 ± 4.62* ($n = 5$)

[a] limits determined from log-transformed values

*$p < 0.05$ or less from control

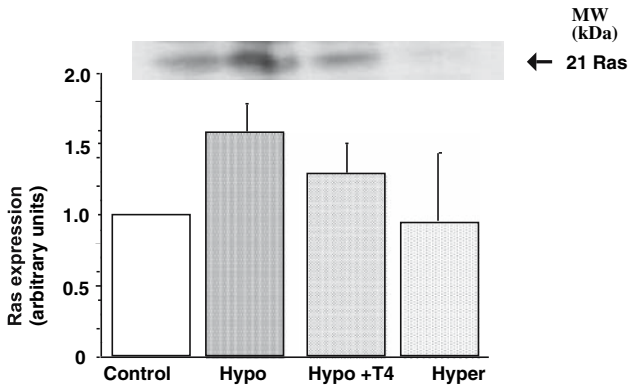

Fig. 1 Thyroidal Ras modulation by thyroid hormones. To induce hypothyroidism animals received MMI, 0.03%, in drinking water for 21 days. A subset of them was treated with a physiological replacement T4 dose (1 mg/100 g body weight, sc; hipo + T4) during the last 10 days of protocol. A subset of non MMI treated animals were given supraphysiological T4 dose (10 mg/100 g, bw, sc; hyper) for 10 days; whereas their controls (control), as well the T4 not treated hypothyroidism (hypo) were given daily NaCl 0.9%. Data are expressed as arbitrary units, related to the control animals. Each bar represents mean ± SEM of three separate experiments, including 4 animals per pool. $p = 0.147$, Friedman's test

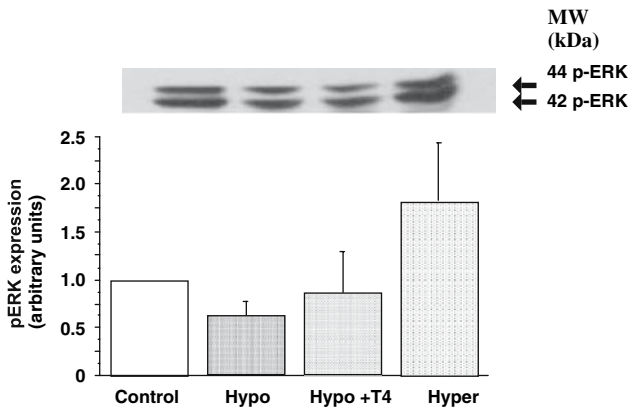

Fig. 2 pERK modulation by thyroid hormones. To induce hypothyroidism animals received MMI, 0.03%, in drinking water for 21 days. A subset of them was treated with a physiological replacement T4 dose (1 mg/100 g body weight, sc; hipo + T4) during the last 10 days of protocol. A subset of the non MMI treated animals were given supraphysiological T4 dose (10 mg/100 g, bw, sc; hyper) for 10 days; whereas their controls (control), as well the T4 not treated hypothyroidism (hypo) were given daily NaCl 0.9%. Data are expressed as arbitrary units, related to the control animals. Each bar represents mean ± SEM of three separate experiments, including 4 animals per pool. $p = 0.074$, Friedman's test

In agreement with Vandeput et al. [12], ERK phosphorylation was not increased in the hypothyroid rat thyroid, in fact it decreased by 30%. Thus even very high levels of TSH are unable to increase thyroid ERK phosphorylation in vivo.

Regulation of ERK phosphorylation by thyroid hormones has been reported in HeLa and CV-1 cells. T_4, T_3

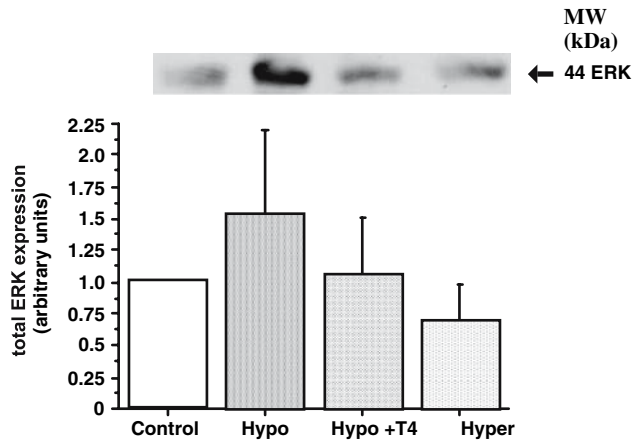

Fig. 3 Total ERK expression in murine thyroid tissue after T4 treatment. To induce hypothyroidism animals received MMI, 0.03%, in drinking water for 21 days. A subset of them was treated with a physiological replacement T4 dose (1 mg/100 g body weight, sc; hipo + T4) during the last 10 days of protocol. A subset of the non MMI treated animals were given supraphysiological T4 dose (10 mg/100 g, bw, sc; hyper) for 10 days; whereas their controls (control), as well the T4 not treated hypothyroidism (hypo) were given daily NaCl 0.9%. Data are expressed as arbitrary units, related to the control animals. Each bar represents mean ± SEM of three separate experiments, including 4 animals per pool. P = 0.3, Friedman's test

and rT_3 can activate the mitogen-activated protein kinase (ERK1/2) signal transduction cascade in 15–30 min, and thus enhance the activity of several nuclear transactivator proteins through serine phosphorylation [15]. The time span involved discards a genomic effect of the thyroid hormones, but in the present study we cannot discard them since the animals received T_4 for a prolonged period (10 days). Treatment with a high dose of T_4 produced a clear increase in thyroid ERK phosphorylation and this seems to be a direct thyroid hormone effect, since these animals had low TSH serum levels. Woodmansee et al. [16] also detected an ERK phosphorylation increase in a thyrotrophic tumour cell line (TtT-97) after 1–2 days of thyroid hormone treatment. This increase in ERK phosphorylation was reverted, and the fraction of cells in S-phase increased, when the thyroid hormone was withdrawn, clearly showing the impact of ERK phosphorylation on the proliferation of thyrotrophs.

Davis et al. [17] showed that 10–20 min after 293T cells were exposed to T_4, the mitogen-activated protein kinase was complexed with the nuclear thyroid hormone receptor (TR), resulting in its serine phosphorilation. Furthermore, Chen et al. [18] reported that the nuclear TR phosphorylation is critical for its stability and transcriptional activity. Similar effects were observed by Tang et al. [19] who showed that the T_4-induced ERK phosphorylation increases oestrogen receptor phosphorylation in human breast cancer (MCF-7) cells, suggesting that T_4 may mimic

E_2 action on its nuclear receptor. Our data show that hypothyroidism can increase RAS expression in the thyroid gland, and that this increase is partially reversed by T_4. Thus Ras expression in the thyroid is stimulated by TSH, as are other thyroid marker proteins. Thyroid ERK phosphorylation seems not to be stimulated by TSH or Ras but rather to increase in response to T_4. It remains to be seen how this T_4- induced MAPK phosphorylation increase relates to the decrease in proliferation generally expected in a thyroid not stimulated by TSH. Activation of the ERK pathways is generally related to growth-promoting actions, however, growth inhibition may also be a consequence of ERK activation in the thyroid, as well as in the thyrotroph [16], and the hepatocyte [20].

Materials and methods

Animals

The study was approved by the CAUAP/IBCCF^{o} (Institutional Committee for Use of Animals in Research), and the procedures used were in compliance with the International Guiding Principles for Biomedical Research Involving Animals, Council for International Organizations of Medical Sciences (Geneva, Switzerland), and the guiding principles for care and use of animals from American Physiological Society.

Male Wistar rats were kept from birth in a temperature-controlled (22–25°C) animal room, with a 12 h light-12 h darkness cycle, and received pelleted commercial chow (Paulínea, São Paulo, Brazil; iodine content 2 mg/kg) and water ad libitum.

Induction of hypothyroidism and hormone measurements

To induce hypothyroidism 24 adult Wistar male rats (200–250 g) were treated with 2-mercapto-1-methylimidazole (MMI, Sigma USA), 0.03% in the drinking water, for 21 days. Half of the MMI-treated animals received a physiological replacement dose of T_4 in saline (1 μg/100 g body weight, sc; hypo+T_4), while the other 12 received NaCl (0.9% solution, sc; hypo), during the same period Another group (12 animals) was given a supraphysiological amount of T_4 in saline (10 μg/100 g bw, sc; hyper), during the last 10 days of the protocol. The 12 controls (control) received only NaCl 0.9% solution, sc, during the same period. The animals were killed, the thyroids were excised, weighed and quick-frozen in liquid N_2, blood samples were collected and the sera were stored at –20°C. Serum T_3 and T_4 were determined by specific Coated-Tube Radioimmunoassay (RIA) kits (Diagnostic Systems Laboratories. Inc, DSL), T_3 and T_4 sensitivity were, respectively 4.3 ng/dl and 0.4 μg/dl. Serum T4 inter- and intraassay coefficients of variation were 7.4% and 5.0%, respectively, and serum T_3 inter- and intraassay coefficients of variation were 4.2% and 6.5%, respectively. Serum TSH RIA measurement were done, as previously described [21] using a kit supplied by the National Hormone and Peptide Program, NIDDK (Bethesda, MD, USA) and expressed in terms of the preparation (RP-3) provided. TSH, sensitivity was 0.50 ng/ml.

Western blotting analysis

Pools of 4 thyroid glands were homogenized in Tris-HCl buffer 0.0625 M, pH 6.8, containing 10% glycerol, 5% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 3% sodium dodecyl sulphate (SDS) and 0.01% bromophenol blue. Protein concentration was determined by the method of Bradford [22] before the addition of β-mercaptoethanol and bromophenol blue. 50 μg of the homogenate were separated according to molecular mass by polyacrilamide gel electrophoresis (SDS-PAGE): 7.5% gel for pERK, and 12% gel for Ras. After transfer to a PVDF membrane (Millipore), and hybridization with specific anti-H-Ras, anti-pERK (p42 and p44) or anti-ERK 1 (44, and with less affinity, 42) antibodies (Santa Cruz Biothechnogy, Inc), the immunoblots were detected by chemiluminescence using a ECL kit (Amersham International).

Statistical analysis

Hormone measurements are presented as mean ± SEM. Parametric analysis of variance (ANOVA) and post-hoc Newman–Keuls test were used for statistical evaluation of serum hormonal data; serum TSH analysis was performed on log-transformed values. Densitometric data are expressed as arbitrary units, related to the control animals. Non-parametric ANOVA (Friedman test) was used to evaluate densitometric data [23].

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