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Rapid communication

Thyroid hormone regulation of APP (β-amyloid precursor protein) gene expression in brain and brain cultured cells

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ABSTRACT

We have previously shown that the thyroid hormone triiodothyronine negatively regulates the transcriptional activity of the β-amyloid precursor protein gene (APP) in cultured murine neuroblastoma cells, by a mechanism that involves binding of the nuclear thyroid hormone receptor (TR) to DNA sequences located within the first exon of the gene. In this report we present results showing that the thyroid hormones also repress the expression of APP in human neuroblastoma cells and in primary cultures of rat neurons. In addition, and in agreement with the results obtained in cultured cells, APP messenger RNA and protein levels are significantly higher in the brain of hypothyroid rats and mice, and also in Alzheimer-related brain regions dissected from KO mice lacking TRs. These results show that binding of the thyroid hormones to their nuclear receptors mediate their repressive effect on APP gene expression in vivo.

1. Introduction

Alzheimer's disease is a degenerative disorder of the central nervous system which causes mental deterioration and progressive dementia, and is accompanied by neuropathologic lesions including the presence of senile plaques of which the β-amyloid protein, a hydrophobic 39–43 residue amino acid peptide, is the major component (Selkoe, 1994). The β-amyloid protein is proteolytically derived from a set of alternatively spliced β-amyloid precursor proteins (APP) that at physiological levels appear to be involved in neurotrophic events. In contrast, its overexpression might cause neuronal degeneration by a mechanism that likely involves an increased production of β-amyloid protein (Mattson et al., 1993). APP is proteolytically cleaved by the action of a set of enzymes referred to generically as secretases. The α-secretase cleaves the protein between residues of the Aβ domain and precludes the generation of amyloidogenic fragments. Alternatively, the successive action of the β-secretase, which cleaves the precursor at the amino terminus of the Aβ sequence, and the γ-secretase which in turn cuts at the positions 39–43 of this domain generates the Aβ peptides.

APP is ubiquitously expressed in mammalian tissues and its expression can be modulated by a variety of compounds, among others the thyroid hormones. The thyroid hormones, thyroxine (T4) and triiodothyronine (T3), play an important role in growth, differentiation and metabolism. The thyroid hormones are essential for the adequate development of the central nervous system and congenital hypothyroidism in humans is associated to mental deficiency and other neurological abnormalities (Morreale de Escobar et al., 2004). The thyroid hormones are also required for normal brain functioning in adult individuals, and hypothyroidism is accompanied by neurological symptoms that in a way might resemble those observed in Alzheimer patients. Although a definite link between thyroid hormones and this pathology has not been yet established, it has been suggested that a history of thyroid dysfunction might represent a risk factor for the development of this neurodegenerative disease (Tan and Vasan, 2009).

Data from our laboratory have demonstrated that T3 represses the expression of the APP gene in cultured murine neuroblastoma cells, by a mechanism that requires binding of the nuclear T3 receptor (TR) to specific sequences located in the first exon of the gene (Belandia et al., 1998; Villa et al., 2004). In addition, a reduction of TRα mRNA levels, which might contribute to an increase in the APP levels, had been found in the hippocampus of Alzheimer's patients, and increased expression of APP in the brain of hypothyroid mice has been also reported (O'Barr et al., 2006). These results strongly suggest that the thyroid hormones might play a role in the development of Alzheimer's disease by modulating the expression and content of APP in the brain cells. However, despite the potential relevance of these descriptions, up to now the repressive effect of thyroid hormones on APP expression in vivo has not been adequately analyzed.

In this work, we have determined the levels of APP, mRNA and/or protein, in human SH-SY5Y neuroblastoma cells and in primary cultures of rat cortical neurons treated with the thyroid hormone,

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and also in extracts obtained from total brain, or specific brain regions of euthyroid and hypothyroid rats, as well as from genetically modified mice lacking TRs. Our results confirm that the liganded thyroid hormone receptors not only repress APP gene expression in different in vitro models, but also in the brain of mice and rats.

2. Materials and methods

2.1. Chemicals and antibodies

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were purchased from Biowhittaker. Resin AG1X8 came from BioRad. Polyclonal antibody against the cytoplasmic domain of APP was from Sigma–Aldrich quimica. The second biotinylated anti-rabbit antibody and the peroxidase-conjugated streptavidine used in Western blot analysis were from Amersham International plc. Reverse transcriptase and other reagents used for the PCR analysis of DNA were from Promega.

2.2. Cell culture

SH-SY5Y cells, a neuroblastoma cell line of human origin, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and maintained at 37 °C in a 5% CO_2 atmosphere. Previous to the experiments, the culture medium was replaced with a similar medium containing serum depleted of thyroid hormones by treatment with resin AG1X8, and the cells were then incubated in this medium for an additional 24 h period before the beginning of the experiments. At the times indicated, cells were collected and processed, or stored frozen for posterior analysis.

2.3. Primary culture

Primary cultures of rat cortical neurons were prepared as previously described (Cuesta et al., 2009b). Briefly, the plates were treated with poly-L-lysine (100 μg/ml) and laminin (4 μg/ml) overnight at 37 °C before seeding. Cerebral cortices from 18-day-old rat embryos were dissected and mechanically dissociated in Eagle's minimum medium supplemented with 28.5 mM $NaHCO_3$, 22.2 mM glucose, 0.1 mM glutamine, 5% fetal bovine serum, and 5% donor horse serum. The cells were seeded at a density of 0.3×10^5 cells/cm^2 in the same medium.

2.4. Animals: Ethics statement and preparation of animals

Wistar rats and mice, were treated in accordance with the protocols issued by the 'Ethics Committee for Animal Experimentation' of the Instituto de Investigaciones Biomédicas (CSIC-UAM), which followed National (normative 1201/2005) and International recommendations (normative 609/86 from EU).

Hypothyroid rats: To induce neonatal hypothyroidism, drinking water containing 0.02% methyl-mercaptoimidazol (MMI) and 1% $KClO_4$ was administered to dams starting from the 9th day after conception and was continued until the end of the experiments. Control euthyroid and hypothyroid animals were killed 8–12 h (P0), 5, 10 or 15 days after birth. For in vivo hormonal treatments thyroxine, which crosses the blood–brain barrier more efficiently than triiodothyronine, was administered as a single daily intraperitoneal injection (1.8 μg/100 g body weight) starting at P1.

Hypothyroid mice: Hypothyroidism in mice was induced by administration during 16 weeks of 0.05% MMI and 1% $KClO_4$ in the drinking water, starting in 1-month-old animals. Body weight was significantly reduced and circulating T3 and T4 levels were reduced by more than 80%, confirming the efficacy of the treatment (Contreras-Jurado et al., 2011).

TRα1/TRβ double knockout mice (TRα1$^{-/-}$/TRβ$^{-/-}$) – referred to as KO group – were generated as previously described (Gothe et al., 1999), and supplied with food and water *ad libitum* in our animal facility for 8 months, together with the corresponding wild type control animals.

2.5. Quantitative real-time PCR assays

RNA was extracted using TRI Reagent (Sigma–Aldrich). RNA from 5 to 7 animals was pooled and the APP mRNA levels were analyzed by quantitative RT-PCR. Reverse transcription was performed using 2 μg of RNA and the SuperScript™ First-Strand Synthesis System (Invitrogen Life Technologies). PCRs were performed using a MX3005P instrument (Stratagene) and detected with Sybr Green. Data analysis was done using the comparative CT method and data were corrected with the control GAPDH mRNA levels, which were not affected by any of the treatments used. The following PCR primers were used,

APP: Forward: 5′-GGCCCTCGAGAATTACATCA-3′, and reverse: 5′-GTTCATGCGCTCGTAGATCA-3′

GAPDH: Forward: 5′-ACAGTCCATGCCATCACTGCC-3′, and reverse: 5′-GCCTGCTTCACCACCTTCTTG-3′.

2.6. Western blot analysis

Cellular proteins were extracted from cultured cells, brain, or tissues (hippocampus and cortex) dissected from animal brains by lysis with a buffer (150 mM NaCl, 50 mM Tris pH 8, 2 mM EDTA, 1% Triton, 0.1% SDS) containing the protease inhibitors PMSF (1 mM) and leupeptin (10 μg/ml). The protein content of extracts was determined by using the BCA assay. Equal amounts, 40 μg, of cell extracts were electrophoresed in an 8% SDS–polyacrylamide gel and transferred to an immobilon polyvinylidine difluoride membrane. Non specific binding was blocked with 5% nonfat dried milk in TBS-T (Tris buffered saline, 0.1% Tween 20) for 2–3 h at room temperature and the cellular APP detected with a 1/4000 dilution of the rabbit polyclonal antibody, raised against the carboxy-terminal domain of human APP. After 1 h incubation at room temperature the membrane was washed and incubated with a second biotinylated anti-rabbit antibody (1/2000) for an additional hour, washed again and finally incubated for 1 h with 1/2000 peroxidase-conjugated streptavidine. All incubations took place at room temperature, and detection by enhanced chemiluminescence (ECL, Amersham International plc.) was carried out according to manufacturer's indications.

2.7. Statistical analysis

Results are expressed as mean ± SD. The differences between means were evaluated by a Student's *t*-test and considered statistically significant at $p < 0.05$.

3. Results

3.1. Triiodothyronine decreases intracellular APP levels in SH-SY5Y cells and in primary cultures of rat cortical neurons

We have previously reported that T3 represses the APP gene transcriptional activity (Villa et al., 2004), decreasing APP levels in N2aβ cells, a murine neuroblastoma cell line (Cuesta et al., 2009a). Now, we have determined intracellular APP protein and APP mRNA levels in SH-SY5Y cells, a human neuroblastoma cell line, and in primary cultures of rat cortical neurons, incubated for 48 h in the presence or in the absence of 200 nM T3. The results obtained are illustrated in Fig. 1. As previously observed in N2aβ

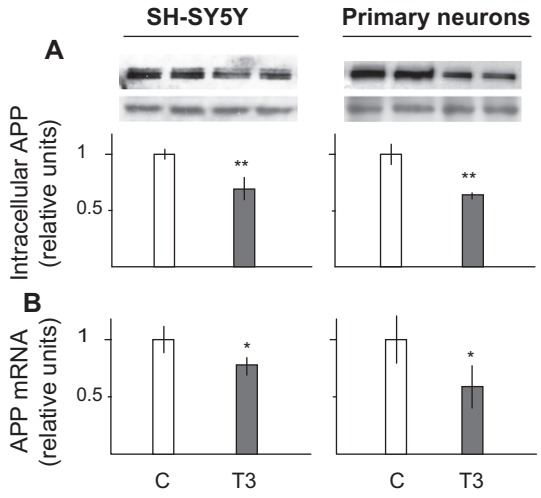

Fig. 1. T3 decreases APP levels in human neuroblastoma SH-SY5Y cells and in primary cultures of rat cortical neurons. APP, intracellular protein (panel A) and mRNA (panel B), levels were determined in extracts obtained from cells incubated for 48 h in the presence, or in the absence (control), of 200 nM T3. Data (mean ± SD) were obtained from four independent samples (two separate experiments performed in duplicate). Significance, $p < 0.05$(*) or $p < 0.01$(**), is indicated.

cells, T3 caused a reduction of the intracellular APP protein content (panel A) and APP mRNA levels (panel B) in both the cells of human origin and in the rat primary cultures, thus discarding a N2aβ cell-specific hormonal effect.

3.2. Increased levels of APP mRNA and protein in the brain of hypothyroid rats

To further confirm the inhibitory effect of thyroid hormones on APP gene expression we next examined the APP mRNA levels in the brain of euthyroid and hypothyroid newborn rats. Brains were disrupted from control and hypothyroid rats sacrificed at different postnatal times (0, 5, 10 and 15 days after birth), the total RNA was isolated and the APP mRNA was analyzed by using quantitative RT-PCR. As illustrated in Fig. 2A, mRNA levels were higher in all groups of hypothyroid animals. Moreover, this increase was clearly reversed in a group of 5 days old hypothyroid rats that were injected for 4 days with a replacement dose of T4.

In addition, the APP protein content was determined in hippocampus and cerebellum of 1-month-old rats. Of interest, the results obtained show that hypothyroidism increased the APP protein content in hippocampus, a brain region severely affected in Alzheimer's disease, but not in cerebellum, a tissue minimally involved in this pathology (Fig. 2B).

3.3. Intracellular APP protein and APP mRNA levels are increased in hippocampus and cortex of hypothyroid and TR-KO mice

Hippocampus and cortex were obtained from the brain of euthyroid control mice and aged paired animals rendered hypothyroid by treatment with MMI/$KClO_4$, and also from TRα1/TRβ-knockout (KO) mice devoid of the α1 and β isoforms of the thyroid hormone nuclear receptor, and the corresponding wild type age-paired control animals. APP mRNA and protein levels were analyzed by quantitative RT-PCR and Western blot, respectively. As expected, the results illustrated in Fig. 2 confirm that in both brain regions, hippocampus (panel C) and cortex (panel D), the levels of APP mRNA and APP protein were clearly increased not only in hypothyroid animals, with reduced circulating hormone levels, but also in the TR-KO animals devoid of the corresponding nuclear receptors.

4. Discussion

Dementia and thyroid dysfunction are both prevalent conditions in the elderly population, and therefore widely extended in developed countries where the life expectancy has increased significantly. Moreover, an association between thyroid function and cognitive performance during aging has been suggested in a

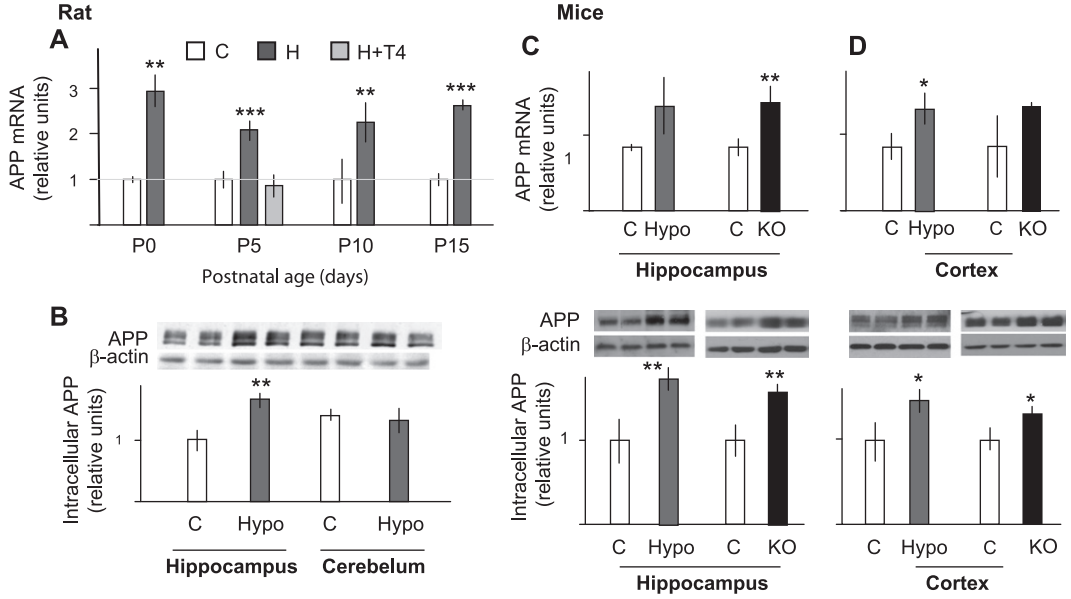

Fig. 2. Increased expression of APP in the brain of hypothyroid and TR-KO animals. (A) APP mRNA levels in the brains of control and hypothyroid (Hypo) rats sacrificed at 0, 5, 10 and 15 days after birth, and in the brain of 5 days old hypothyroid rats treated with T4 for the previous 4 days (H + T4). Data are expressed relative to those obtained in the corresponding euthyroid animals. (B) APP intracellular protein levels in hippocampus and cerebellum of 1-month-old control and hypothyroid animals. (C and D) APP mRNA and cellular protein determined in hippocampus (C) and cortex (D) of hypothyroid and TR-KO mice. The results represent the mean ± SD of four determinations by group and are expressed relative to the values of corresponding control mice. Significance, $p < 0.05$(*), $p < 0.01$(**) or $p < 0.001$(***), is indicated.

number of laboratory and epidemiological studies, and determination of the thyroid stimulating hormone (TSH) levels has been routinely used as a marker for cognitive impairments (Tan and Vasan, 2009). In particular, an apparent association between hypothyroidism, as well as hyperthyroidism, and Alzheimer's disease has been reported (Tan and Vasan, 2009), localized hypothyroidism of the central nervous system has been described in some patients with AD (Sampaolo et al., 2005), a reduced conversion of T4 to T3 has been detected in post mortem samples of patients with AD and a reduction of TR levels has been observed in Alzheimer hippocampus. Moreover, thyroid hormone has been reported to prevent the cognitive deficit in a mouse model of AD. However, the precise mechanisms by which thyroid hormones may affect and interfere with the development of neurodegenerative processes in Alzheimer's disease have been not yet clarified.

According to former data obtained in our laboratory, and latter confirmed by other laboratories (O'Barr et al., 2006; Belakavadi et al., 2011), one of these mechanisms could be related to the inhibitory effect of the thyroid hormones on APP gene expression. In fact, expression of APP plays a central role in Alzheimer's disease, and it has been suggested that an increase in the production of this protein might actively contribute to the development of this pathology. Overexpression of APP has been shown to specifically induce the activity of the alternative processing that leads to the generation of β-amyloid peptides (Mattson et al., 1993), and therefore it could be considered as a risk factor for AD. Accordingly, thyroid hormones, which decrease the transcriptional activity of the APP gene promoter and reduce the intracellular levels of this precursor (Belandia et al., 1998), might play a preventing role in the development of Alzheimer, by reducing the intracellular levels of APP, the generation and release of β-amyloid isoforms, and the subsequent formation of amyloid deposits and induction of neurodegenerative signals.

To further confirm that the repressive effect of the thyroid hormones on APP expression originally observed in murine N2aβ neuroblastoma cells is not restricted to this cell line, we have now analyzed the hormonal regulation in cultured human neuroblastoma cells, in primary cultures of rat cortical neurons, and also in cerebral tissues obtained from rats and mice depleted of thyroid hormones (hypothyroidism) or thyroid hormone receptors (KO). Validating our previous results and confirming more recent studies showing that thyroidal status can regulate APP gene expression (O'Barr et al., 2006; Belakavadi et al., 2011) and processing (O'Barr et al., 2006), the negative effect of the thyroid hormones on APP was reproduced in each one of these models. Thus, APP protein and mRNA levels were reduced in cultured cells exposed to T3, and were increased in the brain of hypothyroid mice and rats. Interestingly, this increase was observed even during the neonatal period and could be reversed by administration of a physiological dose of thyroxine to hypothyroid animals. Furthermore, elevated APP levels were found in brain regions such as hippocampus or cortex with functional relevance in AD, but not in cerebellum, not affected by the disease. The key role of TRs in mediating repression of the APP gene by the thyroid hormones was demonstrated by the finding that APP protein and mRNA levels were also increased in hippocampus and cortex of double KO mice lacking both TRα1 and TRβ, the main receptor isoforms that bind thyroid hormones. The phenotype of hypothyroid and TR KO mice is not always the same, and some alterations are even more intense in the hypothyroid animals (Forrest and Vennstrom, 2000). This has been ascribed to the actions of the unoccupied receptors that in the absence of ligand can bind corepressors and act as constitutive repressors (Aranda and Pascual, 2001). However, this does not appear to be the case with regulation of APP, since APP mRNA and protein levels was similarly increased in the brains of hypothyroid and TR KO animals. Therefore, binding of thyroid hormone to the nuclear receptor appears to be required for the maintenance of adequate levels of APP gene transcription in key brain areas. All together, these results reinforce the role of the thyroid hormones as a neuroprotective factor, and open the possibility that these hormones might be applied in the design of future therapeutic strategies.

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