Undetectable levels of tumor necrosis factor-α, nitric oxide and inadequate expression of inducible nitric oxide synthase in congenital hypothyroidism

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ABSTRACT. Objetive: To analyse the production of TNF-α and NO in euthyroid and hypothyroid newborns. Patients: A cross-sectional study was conducted involving 10 newborns diagnosed with primary congenital hypothyroidism (CH; group A) and 10 euthyroid children (group B).
Results: There were undetectable plasma levels of TNF-α and NO in the hypothyroid children, however plasma levels of TNF-α (5.5 ± 0.5 pg/ml) and NO (5.6 ± 1.7 μM) were detected at normal levels in all euthyroid children. Moreover, expression of iNOS mRNA in PBMC, determined by RT-PCR, was negative in both groups of infants. Conclusion: TNF-α and NO production are both impaired in hypothyroid newborns. We report for the first time evidence of undetectable levels of TNF-α and NO in infants with CH.

Keywords: TNF-α, NO, iNOS, congenital hypothyroidism

INTRODUCTION

Nitric oxide (NO) is a free-radical gas produced by many cell types, which has a broad repertoire of important pathophysiogical functions [1-3]. NO is synthesised from L-arginine by a family of three nitric oxide synthase (NOS) proteins: neuronal or NOS1, endothelial or NOS3, and inducible or NOS2 [4]. NO has a role as a neurotransmitter and regulator of blood pressure, it has vasodilator and antiplatelet, tumoricidal, and microbicidal activities. High NO production has been also associated with several important pathological situations [2, 3, 5]. Cytokines such as TNF-α induce NOS2 [6]. Moreover, it has been shown to modulate immune functions, although contrasting effects have been described [2, 7-9]. NO also has controversial effects on cytokine synthesis. It has been reported that the synthesis of TNF-α by human peripheral blood mononuclear cells (PBMC) [10] and lipopolysaccharide-stimulated neutrophil preparations [11] is increased by exogenous NO.

TNF-α and NO are involved in the normal development and pathology of the CNS. Neuronal differentiation is regulated by the nitregic plexus that produces NO, and NO induced by NOS2 is involved in the control of neuronal and glial activation, proliferation, differentiation and survival, thus influencing the development and regeneration of the CNS [12]. On the other hand, thyroid hormones are essential for both neuronal and glial differentiation and are required for the normal development of the CNS [13-15].

Thyroid hormones affect the NO synthase content in the developing cerebral cortex [16, 17]. Cerebral NO values are directly correlated with those of triiodothyronine (T3), active thyroid hormone produced at the target cell by deiodinative pathways from circulating plasma levels of free thyroxine (FT4) [18]. The expression of thyroid receptor isoforms [19] and the receptor of the thyroid-stimulating hormone [20], thyrotropin (TSH), have been demonstrated in human neurones and astrocytes.

Hypothyroidism impairs brain development principally at the level of myelination and oligodendroglial proliferation and differentiation [13-15]. Thus, early diagnosis and treatment of CH are crucial, and screening programs have proved to be very successful in preventing mental retardation [21, 22]. Our objective was to determine if the production of TNF-α and NO is the same in euthyroid and hypothyroid newborns.

PATIENTS AND METHODS

Patients

A cross-sectional study was conducted in twenty newborns, classified into two groups:

Group A: ten euthyroid infants, mean age 20.6 ± 2.4 days, monitored because their mothers had received L-thyroxine during pregnancy.

Group B: ten newborns diagnosed with primary congenital hypothyroidism (CH) by a screening program of the Community of Madrid, mean age 10.2 ± 1.1 days. Informed consent was obtained from the families and the study was approved by the Ethics Committe of the Hospital. Two ml of blood (EDTA tube) were obtained by venopuncture of a peripheral vein, and centrifuged at 0°C. Frozen serum and cells were conserved.

Thyroid function tests

Neonatal TSH (N < 5.0 uU/ml) and FT4 (N 1.0-2.0 ng/dl) levels were determined using by electrochemioluminiscence assays, with time resolved fluorescence (DELFIA).

NO and TNF-α production assays

NO levels were evaluated by the Greiss reaction (Bioxytech Nitric Oxide Assay; Portland, USA) [23]. Plasma TNF-α levels were determined by ELISA (INNOGENETICS N.V. Haven, Zwijnaarde, Belgium). TNF-α and NO concentrations were assayed in triplicate.

Determination of iNOS mRNA

Determination of iNOS mRNA in PBMC from the infants was carried out by reverse transcription RT-PCR. PBMCs (10^6/ml) were washed in phosphate-buffered saline, and the pellet was frozen at – 70°C until further analysis. The mRNA from 10^5 cells was isolated using oligo(dT)-coated magnetic beads and by subsequent RT analysis (PolyAtract series 9600 mRNA isolation and cDNA synthesis system; Promega), according to the manufacturer's instruction. PCR analysis was carried out with an automatic thermal cycle (GeneAmp PCR system 9600; Perkin Elmer).

For amplification of the desired cDNA, the following gene-specific primers were used: iNOS sense (5'CGGTGCTGTATTTCCTTACGAGGCGCGAAGAAGG-3') and iNOS antisense (5'GGTGCTGCTTGTTAGGAGGTCAAGTAAAGGGC-3'). The reaction mixture contained 5 µl of cDNA (1/6 of the isolated cDNA), 1 µl sense and antisense primers, 200 µM deoxynucleotide triphosphates, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer) in a final volume of 50 µl. The cycle program was set to denature at 94°C for 45s, to anneal at 60°C for 45s, and to extend at 72°C for 2 min, for a total of 40 cycles. Electrophoresis of the PCR products was performed with 1.5 % agarose gels containing ethidium bromide 1 mg/ml. A 100-bp DNA ladder (GIBCO BRL) was used as a molecular weight marker. Glyceraldehyde-3-phosphate dehydrogenase mRNA was amplified as a control.

Statistical analysis

The non-parametric Mann-Whitney "U" test was performed to compare the data obtained in and between both groups of subjects.

RESULTS

The plasma levels of TSH, FT4, NO and TNF-α are summarised in Table 1. TSH levels (3.5 ± 0.5 µU/ml) (N < 5.0) and FT4 (1.5 ± 0.06 ng/dl) (N 1.0 – 2.0) were normal in Group A (euthyroid children), confirming normal thyroid function. High values of plasma TSH (371 ± 5 0.7 µU/ml) and low levels of FT4 (0.47 ± 0.1 ng/dl) confirmed the diagnosis of primary congenital hypothyroidism in patients of Group B.

Plasma levels of NO (5.6 ± 1.7 µM) and TNF-α (5.5 ± 0.5 pg/ml) in euthyroid children were also within the normal range [24, 25]; however, NO (< 0.2 µM) and TNF-α (< 4.0 pg/ml) were undetectable in the hypothyroid newborns. Interestingly, plasma levels of TNF-α and NO were detected at normal levels in all hypothyroid infants after treatment (data not shown). To test if iNOS expression in PBMC was responsible for the observed differences, we assayed expression of iNOS mRNA in PBMC from euthyroid and hypothyroid newborns, by RT-PCR. This was negative in both groups (Figure 1A and Figure 1B).

DISCUSSION

The transplacental transport of thyroid hormones from the mother to the fetus during pregnancy has been clearly demonstrated, both in experimental models [26, 27] and in humans [28, 29]. The fetal brain is specially rich in type-II iodothyronine 5'- deiodinase, and its activity increases in hypothyroid brains, enhancing local conversion of circulating plasma FT4 to T3 and thus partially compensating for the diminished levels of serum T4 [18, 30].

At birth the child has a relatively immature CNS. Thyroid hormones play an important role during late brain development. A lack of thyroid hormones during late brain development, in the postnatal period in patients with CH [26, 31], results in permanent deficits in brain functions, including intellectual development. Even a few days delay in high dose-replacement of thyroid hormone in congenitally hypothyroid newborns results in a measurable reduction in later intellectual development [21, 31]. The early detection by screening programs, allowing the diagnosis and initiation of appropiate replacement therapy (L-thyroxine) without delay, are mandatory to avoid mental retardation in children with primary CH.

The development of the human brain includes proliferation, migration and organization of neurons, and finally, ensheathment of this circuitry with the neural-specific membrane, myelin. These events span from the second month of gestation into adult life, with an important stage during the perinatal period [28]. Thyroid hormones are essential to normal development of the CNS, and appear to regulate those processes associated with terminal brain differentiation such as dendritic and axonal growth, synaptogenesis, neuronal migration and myelination [14, 15, 18], probably interacting with the synthesis of TNF-α and NO at local levels. Many actions of TNF-α on the CNS, such as controlling neuronal and glial activation, proliferation and survival, influencing neuronal plasticity and development of the CNS are probably mediated by locally produced, NOS2-derived NO [12].

TNF-α and NO production are both impaired in all the hypothyroid newborns in our study. These findings corroborate experimental *in vitro* data that demonstrate that thyroid hormones affect the NOS content in the develop-

Table 1
Plasma levels of TSH, FT4, NO and TNF-α in euthyroid (Group A) and hypothyroid (Group B) newborns.

Group A					
Euthyroid newborns	Age (days)	TSH μU/ml	FT_4 ng/dl	NO μM	TNF-α pg/ml
1	17	4.0	1.6	7	5.3
2	13	2.1	1.7	5	4.9
3	21	5.0	1.5	3	4.8
4	26	2.2	1.4	5	6.4
5	21	3.6	1.6	1	4
6	27	4.9	1.7	19	8.3
7	30	4.8	1.5	9	7.2
8	11	3.0	1.8	4	6.1
9	30	3.2	1.1	2	4.4
10	11	2.2	1.6	1	4.0
Mean ± s.e.m.	20.6 ± 2.4	3.5 ± 0.4	1.5 ± 0.06	5.6 ± 1.7	5.5 ± 0.5
Group B					
Hypothyroid newborns	Age (days)	TSH μU/ml	FT_4 ng/dl	NO μM	TNF-α pg/ml
1	5	330	0.8	< 0.2	< 4
2	12	240	1.0	< 0.2	< 4
3	7	340	0.5	< 0.2	5.1
4	7	690	0.5	< 0.2	< 4
5	11	500	0.2	< 0.2	< 4
6	16	110	0.5	< 0.2	< 4
7	8	280	0.1	< 0.2	< 4
8	11	400	0.2	< 0.2	< 4
9	10	330	0.8	< 0.2	< 4
10	15	490	0.1	< 0.2	< 4
Mean ± s.e.m.	10.2 ± 1.1	371 ± 50.7	0.47 ± 0.10	< 0.2	< 4

ing cerebral cortex and hippocampus of postnatal rats. Our study supports the notion that NO is an essential negative regulator of neuronal precursor proliferation during vertebrate brain development [16], and strengthens the role of thyroid hormones and their interaction with the nitrergic plexus in regulating brain development. As NO plays an important role in the development of the CNS [12], this may help to explain some of the mechanisms implicated in the neuronal disturbances associated with thyroid hypofunction [13-15, 32]. On the other hand, the undetectable TNF-α levels in infants with CH could be related to the role of transforming growth factors, important regulators of neural cell growth and differentiation of brain development [33]. TNF-α is an excellent inducer of iNOS, which is also inhibited by growth hormone [34].

In summary, our study reports, for the first time, evidence of non-production of TNF-α and NO in infants with CH, and suggests a novel mechanism that could explain, in part, the effect on brain development of congenital hypothyroidism.

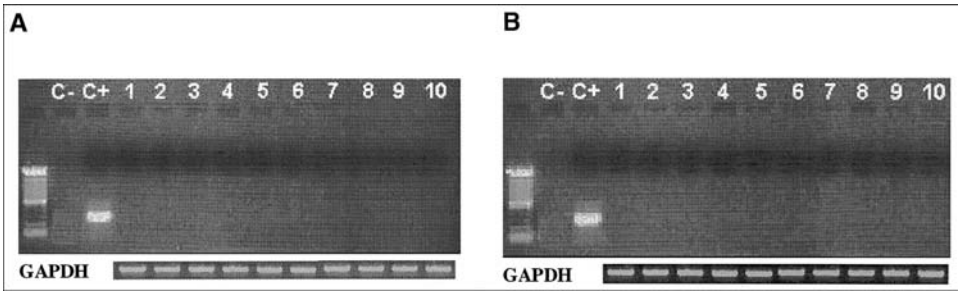

Figure 1
Expression of iNOS mRNA in PBMC from euthyroid (A) and hypothyroid (B) newborns.
Lane C: negative control. Lane C +: positive control. A) Lanes 1-10. PBMC from each euthyroid infant. B) Lanes 1-10. PBMC from each hypothyroid infant. iNOS mRNA was detected by RT-PCR with specific primers.

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