

Effect of iodide on Fas, Fas-ligand and Bcl-w mRNA expression in thyroid of NOD mice pretreated with methimazole

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Abstract

Nonobese diabetic (NOD) mice and a derived strain, NOD.H.2h4, have been used as a model for experimental spontaneous thyroiditis and thyroiditis induced by iodide excess after a goiter-inducing period. Some authors have proposed that iodide, given after methimazole or propylthiouracil, is capable of inducing apoptosis in thyroid cells and that anti-thyroid drugs can modulate the expression of apoptosis components such as Fas and its ligand (Fas-L). Here we evaluated the effect of potassium iodide (20 µg/animal for 4 days, *ip*) given to NOD mice at the 10th week of life after exposure to methimazole (1 mg/ml) in drinking water from the 4th to the 10th week of life. Fas, Fas-L and Bcl-w expression were analyzed semiquantitatively by RT-PCR immediately after potassium iodide administration (group MI44D) or at week 32 (MI32S). Control groups were added at 10 (C10) and 32 weeks (C32), as well as a group that received only methimazole (CM10). An increase in the expression of Fas-L and Bcl-w ($P < 0.01$, ANOVA) was observed in animals of group MI44D, while Fas was expressed at higher levels ($P = 0.02$) in group C32 (72.89 ± 47.09 arbitrary units) when compared to group C10 (10.8 ± 8.55 arbitrary units). Thus, the analysis of Fas-L and Bcl-w expression in the MI44D group and Fas in group C32 allowed us to detect two different patterns of expression of these apoptosis components in thyroid tissue of NOD mice.

Key words

- Thyroiditis
- Nonobese diabetic mice
- Apoptosis
- Methimazole
- Iodide
- Fas mRNA
- Fas-L mRNA
- Bcl-w mRNA

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Introduction

Over the last decade, apoptosis has become one of the main topics of interest in the biological sciences. This phenomenon is critical to life and disease processes, playing a key role in regulatory activities, including immunology and immunopathology (1). Fas (CD95) and its natural ligand (Fas-L) are

transmembrane proteins belonging to the family of tumor necrosis factor and ligands. The association of Fas-L with cells expressing Fas leads to apoptosis through the activation of a complex intracellular signaling pathway, resulting in cell death (2). Bcl-w is one of the members of the Bcl-2 family recently described as one of the anti-apoptotic proteins constitutively expressed in many

tissues (3-5).

Nonobese diabetic (NOD) mice have been developed as a model for type 1 diabetes, but also express lymphocytic infiltrates in other organs including the thyroid (6). Many et al. (7) have shown that NOD mice submitted to an overload of iodine after a goiter-inducing phase developed thyroid infiltrates similar to those observed in human Hashimoto's thyroiditis and also expressed autoantibodies directed at thyroid cell membrane antigens. NOD.H.2h4, a strain derived from NOD, is also prone to the development of thyroid infiltrates that are clearly related to the amount of iodine intake (8,9).

Iodine-containing compounds such as amiodarone (10) and radiographic contrast media (11) have also been implicated in the induction of apoptosis in thyroid and vascular epithelium, respectively. Recently, Vitale et al. (12) have shown that iodide induces apoptosis of thyroid cells in culture and Burikhanov and Matsuzaki (13) demonstrated that potassium iodide can do the same *in vivo* when given to rats with propylthiouracil- or methimazole-induced goiter.

In the present study we investigated the expression of messenger RNA specific for Fas, Fas-L and Bcl-w in goitrous NOD mice immediately after the administration of potassium iodide and at a later stage (32 weeks) by semiquantitative analysis.

Material and Methods

Animals and experimental groups

Male NOD mice used in this protocol were bred under germ-free conditions until the 4th week of life, when they were transferred to a specific pathogen-free animal facility in our laboratory. Control animals (5 per group) had free access to autoclaved water and food until 10 (group C10) or 32 weeks of life (group C32) when they were sacrificed. The animals in the experimental groups received methimazole (Tapazole[®],

Eli Lilly do Brasil Ltda., São Paulo, SP, Brazil) diluted in autoclaved drinking water (1 mg/ml) from the 4th to the 10th week of life when it was discontinued and then received potassium iodide by the intraperitoneal route (20 mg animal⁻¹ day⁻¹) for 4 days. Five animals were sacrificed immediately after the administration of potassium iodide (group MI44D) and five were kept alive until 32 weeks of age (group MI32S). Five animals were sacrificed at the end of the methimazole treatment without being injected with potassium iodide (group CM10).

Total RNA extraction

Mice were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (20 mg/kg) and then sacrificed by careful cervical dislocation. Thyroids were dissected and the tissue immediately placed into RNase-free plastic tubes containing 4 M guanidine thiocyanate, 0.1 M 2-mercaptoethanol, 25 mM sodium citrate, pH 7.0, and 0.5% sarcosyl, frozen in liquid nitrogen and maintained at -80°C until use. Total RNA extraction was performed according to the method of Chomczynski and Sacchi (14) and all aliquots were analyzed by spectrophotometry (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) before use. The ratio of readings at 260 nm/280 nm was determined and only samples presenting a ratio between 1.6 and 1.8 were used.

Reverse transcriptase-polymerase chain reaction

cDNA was synthesized from 5 mg of total RNA using 0.5 ml of Oligo d(pt) and ultrapure water to complete 31 ml. Tubes were heated to 65°C for 10 min and then refrigerated at 4°C for 5 min when 18 ml of reaction solution (10 µl Superscript[™] II buffer, 2 µl 0.5 mM dNTP mix, 5 µl 0.1 M DTT and 1 µl RNAsin; GibcoBRL, Life Technologies, Gaithersburg, MD, USA) was

added. The temperature was then set at 42°C for 2 min and 1 ml Superscript™ II (GibcoBRL) was added to each tube. The reaction was developed at 42°C for 50 min followed by 15 min at 70°C and then 4°C at the end. cDNA samples were stored at -20°C until use.

The polymerase chain reaction (PCR) was performed in 200-ml tubes containing 2 ml cDNA, 100 ng sense primer, 100 ng anti-sense primer, and 43 ml reaction solution (5 ml PCR buffer, 5 ml 0.5 mM dNTP mix, 1.5 ml 50 mM MgCl₂, and 31.5 ml ultrapure water). Each sample was overlaid with mineral oil (Sigma, St. Louis, MO, USA) and incubated in a thermocycler (GeneAmp 9700, Perkin-Elmer, Foster City, CA, USA) using one cycle at 94°C for 3 min followed by 40 cycles of 94°C for 60 s, 58°C for 45 s and 72°C for 90 s. PCR fragments were visualized by agarose gel electrophoresis and ethidium bromide staining. Sample contamination by genomic DNA was verified submitting the RNA sample to PCR amplification omitting the reverse transcriptase step. Cyclophilin (housekeeping gene) was co-amplified as an internal control. The primers used are listed in Table 1.

Semiquantitative measurement of reverse transcriptase-PCR products

PCR products were submitted to electrophoretic analysis in 1.5% agarose gel with ethidium bromide (2 ml/50 ml). Samples contained 8 ml cDNA, 1.5 ml PCR loading buffer and 5.5 ml ultrapure water and migrated in the presence of TBE buffer containing 2 ml/100 ml ethidium bromide for 45 min (70 V, 150 mA). PCR products were visualized by excitation of ethidium bromide under ultraviolet light, digitally recorded with a Nucleovision® system (NucleoTech, San Mateo, CA, USA) and their molecular weight and band pixel area were calculated using the Gel Expert® Software (Nucleo Tech).

Semiquantitative measurements of expression (SE) of apoptosis components were calculated for each sample by the following formula and expressed as arbitrary absorbance units (AU): SE (AU) = pixel area of the product to be analyzed/pixel area of cyclophilin x 100.

Statistical analysis

All results are reported as means ± SD. Comparisons of semiquantitative measurements of expression were made with ANOVA or the Student *t*-test when appropriate.

Results

Frequency of expression of PCR products

All cDNA samples used for the study expressed the product of the housekeeping gene cyclophilin (276 bp), confirming their quality. The expected products for the Fas-L (206 bp) and Bcl-w (230 bp) genes were expressed in all animals studied. Expression of the Fas product (267 bp) was observed in all animals from groups C10, MI44D and C32, but only in four among five animals from group CM10 and three among five animals from group MI32S.

Table 1. Primers for mouse cyclophilin, Fas, Fas-ligand (Fas-L) and Bcl-w reverse transcriptase-PCR.

Gene	Primer sequences
Cyclophilin	
Sense	5' GAC AGC AGA AAA CTT TCG TGC 3'
Antisense	5' GGT TCT GAC TCA CCG ACC T 3'
Fas	
Sense	5' GGC GAT TTC TGG GAC TTT GTT TCC T 3'
Antisense	5' GCA CAG AAG GGA AGG AGT A 3'
Fas-L	
Sense	5' CTA GAG GGC CGG ACC AAA GGA GAC C 3'
Antisense	5' AGG TGG AAG AGC TGA TAC ATT CCT AAT CCC 3'
Bcl-w	
Sense	5' GAG TTT GAG ACC CGT TTC CGC CGC ACC TTC 3'
Antisense	5' CCA CCA TCC AAT CCT GCA CTT GTC CCA CCA 3'

Semiquantitative measurement of reverse transcriptase-PCR products

Mean Fas expression was higher ($P = 0.01$) for group C32 (72.89 ± 47.09 AU) than for group C10 (10.8 ± 8.55 AU). No difference was detected when group C10 was compared to group CM10. The expression of Fas-L and Bcl-w gene products was more

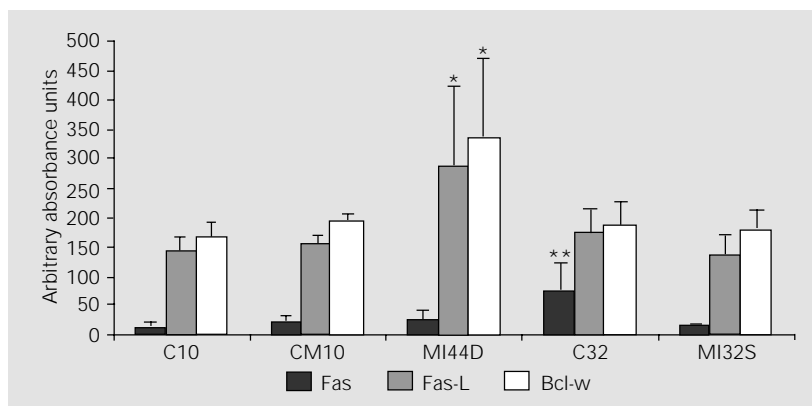


Figure 1. Semiquantitative measurement of the expression of Fas, Fas-ligand (Fas-L) and Bcl-w in control NOD mice (C10 or C32), treated with methimazole (CM10) or potassium iodide after methimazole (groups MI44D and MI32S), from the 4th to the 10th week of life. * $P < 0.01$ compared to the other groups (ANOVA); ** $P = 0.02$ compared to C10 (Student t-test).

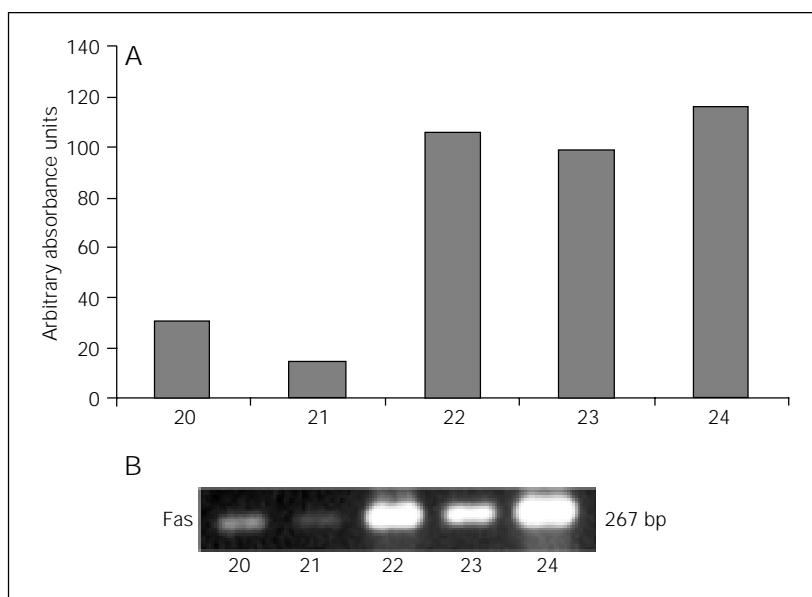


Figure 2. Individual levels of Fas expression in control NOD mice at the 32nd week of life. A, Results of semiquantitative measurements expressed as arbitrary absorbance units. B, Representative results of PCR analyzed in 1.5% agarose gel.

intense in group MI44D ($P < 0.01$, ANOVA) (Figure 1).

Analysis of the individual responses showed that in all groups except group MI32S some animals overexpressed Fas, suggesting a different behavior of the signaling for the synthesis of this apoptosis component among the NOD mice studied. Specifically, two animals in group C10 and one in groups CM10 and MI44D expressed Fas at clearly higher levels than the other components of the group. This fact was more prominent in group C32, where three animals had semiquantitative levels of expression of about 100 AU, while the other two were below 30 AU (Figure 2).

The expression of Fas-L and Bcl-w was uniform, except for group MI44D, where high standard deviations (40.69 and 40.68% of the mean, respectively) reflected the existence of scattered values. Mean levels of Fas-L and Bcl-w expression were higher ($P < 0.01$, ANOVA) in group MI44D than in the others.

When animals from group MI44D were analyzed individually, it was possible to observe two patterns of expression of Fas-L and Bcl-w (Figure 3A). Animals number 6, 8 and 9 showed higher amplification of the PCR product for both Fas-L and Bcl-w than animals number 7 and 10. Animal number 6 overexpressed Fas, as detected visually in 1.5% agarose gel (Figure 3B).

Discussion

Iodine has been linked to thyroid physiology and pathology since the beginning of endocrinology. Some investigators have related the increase in the prevalence of autoimmune thyroid diseases (Hashimoto's thyroiditis and Graves' disease) to iodine supplementation in deficient areas (15), although these findings have recently been questioned (16). Experimental models of thyroiditis have been classically based on administration of thyroglobulin whole molecule

(17) or its related peptides (18) associated or not with adjuvants. In both cases, iodination of tyrosines is considered fundamental for the development of experimental autoimmune thyroiditis.

Experimental thyroiditis induced by administration of supraphysiologic doses of iodine has been described in NOD mice or their derived strains such as NOD.H.2h4 (6), and for other models of thyroid autoimmunity such as BB/W rats (19) and obese strain chickens (20). The mechanisms involved have been related to direct toxicity as a consequence of high peroxide levels due to the strong expression of thyroid peroxidase in goiter-inducing conditions (7,21). Recently, many authors have related iodine to the development of apoptosis in humans (10,11) or in experimental models (22).

We submitted NOD mice to methimazole in drinking water for 6 weeks in order to promote goiter-inducing conditions and then administered high doses of potassium iodide to create the conditions usually observed in experimental models of thyroiditis in order to evaluate the changes in expression of Fas,

Fas-L and Bcl-w.

We did not detect differences in the mean levels of Fas-L expression when control 10-week-old animals were compared to those receiving methimazole without an iodine overload. This result is in contrast with those observed by Mitsiades et al. (23) who reported an increase in Fas-L expression in patients treated with methimazole for Graves' disease.

Ours is the first study to evaluate the expression of Bcl-w in an experimental model of thyroiditis. In accordance with what is described in the literature for other organs (24), Bcl-w was expressed in all animals studied, although we observed higher levels of its messenger RNA in animals sacrificed 4 days after iodine administration.

The levels of Fas gene expression measured semiquantitatively were more prominent in the control group at 32 weeks of life, although this was not a homogeneous observation. In fact, two distinct levels of expression were demonstrable, with three NOD mice presenting more Fas messenger RNA than the other two, a fact that could also be

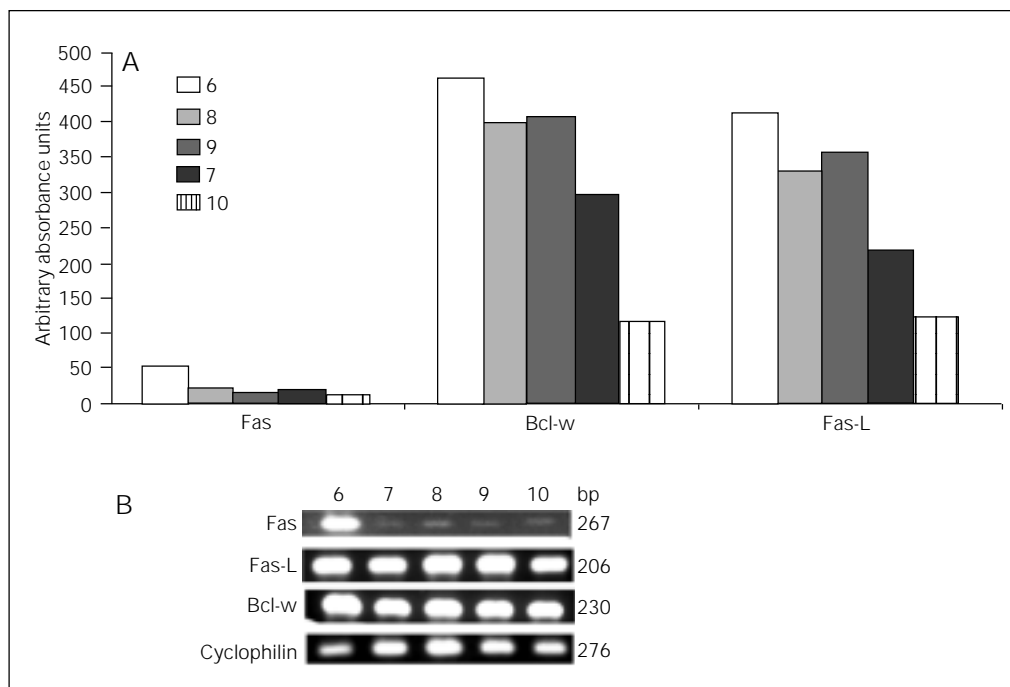


Figure 3. Individual levels of expression of Fas, Fas-ligand (Fas-L) and Bcl-w in NOD mice treated with potassium iodide (20 mg/animal) after receiving methimazole in drinking water from the 4th to the 10th week of life. A, Semiquantitative measurement for each animal (numbers 6-10), showing different patterns of response for Fas-L and Bcl-w. B, Representative 1.5% agarose gel electrophoresis.

observed in one animal of group MI44D and in two control animals at 10 weeks.

Higher levels of Fas-L and Bcl-w expression have been observed in animals sacrificed 4 days after iodine administration (25). Fas-L seems to be expressed constitutively in human and murine thyroid tissue and its association with Fas in neighboring cells seems to lead to apoptosis in a fratricidal way (26). Accordingly, the iodine-induced elevated expression of this apoptosis-related gene could play an important role also in the model of experimental thyroiditis in NOD mice. Our results agree with those of Blucher et al. (22), who described high expression of Fas-L in BB/W rats after iodide administration. However, these authors have also detected an increase in Fas expression in comparison with control animals. This difference may be related to the design of the study, since these investigators administered iodide for longer periods of time. On the other hand, Burikhanov and Matsuzaki (13) identified maximum DNA fragmentation after 3 h of potassium iodide doses, with a

decrease in apoptosis after 12 h. This indicates a very fast process, perhaps not observed in our study design.

The NOD mice used in the present study presented different levels of response to Fas-L and Bcl-w signaling after iodide administration. This was evident in the animals observed immediately after the injection of iodide. Fas is considered to be constitutively expressed in thyroid tissue as described by Giordano et al. (26). However, some animals overexpress the signal for the Fas gene, as clearly observed in group C32. Concerning the present results, we have observed that, at least with respect to thyroid tissue, the NOD mice in our colony present two different patterns of Fas, Fas-L and Bcl-w expression. Extension of this analysis in future research must include other organs, mainly pancreas, in order to evaluate thyroid specificity. Also, future confirmation of these biological responses to iodide excess could allow us to characterize a subgroup of NOD mice susceptible to iodide-induced experimental thyroiditis.

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