# Retinoic acid modulation of thyroid dual oxidase activity in rats and its impact on thyroid iodine organification

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## Abstract

The sodium–iodide symporter (NIS) mediates iodide uptake into the thyrocytes, which is important for the diagnosis and therapy of thyroid disorders. Decreased ability to uptake iodide in thyroid carcinomas reduces the efficacy of radioiodine therapy, and retinoic acid (RA) treatment reinduces iodide uptake. The effectiveness of treatment depends not only on iodide uptake but also on the ability of thyrocytes to organify iodine, which is catalyzed by thyroperoxidase (TPO) in the presence of $H_2O_2$. Our goal was to determine the influence of RA on thyroid iodide uptake, iodine organification, and TPO and dual oxidase (DuOx) activities. Normal rats were treated with all-*trans*-RA or 13-*cis*-RA (100 or 1500 μg/100 g body weight (b.w.), s.c.) for 14 and 28 days. The 2 h thyroid radioiodine content significantly decreased in rats treated with all-*trans*-RA (100 μg/100 g b.w.) for 14 days. In this group, NIS function and TPO activity were unchanged, whereas DuOx activity was significantly decreased, which might have contributed to the decrease in iodine organification. Both doses of 13-*cis*-RA for 28 days increased the 15 min thyroid radioiodine uptake, while the 2 h radioiodide uptake increased only in rats treated with the highest dose of 13-*cis*-RA. While TPO activity did not change, $H_2O_2$ generation was increased in this group, and serum thyroxine levels were normal. Since radioiodine half-life in the thyroid gland is important for treatment efficacy, our results highlight the importance of correctly choosing the RA isomer, the time and the dose of treatment, in order to improve the efficacy of radioiodine therapy.

*Journal of Endocrinology* (2010) **205,** 271–277

## Introduction

The content of iodine in the thyroid gland depends on the presence of proteins that are necessary for iodide uptake through the basolateral membrane of thyrocytes and its incorporation into the acceptor protein thyroglobulin (TG) at the apical surface of these cells (DeGroot & Niepomniszcze 1977, Kopp 2005). The sodium–iodide symporter (NIS) is responsible for iodide uptake through the thyroid cell basolateral membrane against its electrochemical gradient. At the apical pole of thyrocytes, intracellular iodide is transported through pendrin (PDS; Royaux *et al.* 2000) into the follicular lumen and then incorporated into Tg. Iodide oxidation and organification occur at the apical surface of the follicular cell, and these reactions are catalyzed by thyroperoxidase (TPO) in the presence of $H_2O_2$. Thus, thyroid iodide organification depends on TPO activity, which is modulated by the concentration of iodide and $H_2O_2$ (DeGroot & Niepomniszcze 1977, Kopp 2005). In the presence of sufficient amounts of iodine, the limiting step for thyroid hormone biosynthesis is the availability of $H_2O_2$, which is generated by thyroid dual oxidase (DuOx; Corvilain *et al.* 1991, Dupuy *et al.* 1999).

Treatment with retinoic acid (RA) was shown to increase *NIS*, *TPO*, and *TG* mRNA levels in cancer cell lines and to decrease *Nis* mRNA levels and iodide uptake in normal rat thyroid cell line (Schmutzler *et al.* 1997). In normal thyroid cell line, RA reduces *TSH* receptor mRNA levels and increases *TPO* and *TG* mRNA levels (Schmutzler *et al.* 1997, Kurebayashi *et al.* 2000). On the other hand, some authors demonstrated that RA suppresses the accumulation of *TPO* and *TG* mRNA stimulated by TSH in a time- and dose-dependent manner in cultures of human thyrocytes (Arai *et al.* 1991, Namba *et al.* 1993, Del Senno *et al.* 1993, 1994). However, the possible effect of RA on thyroid DuOx has not been assessed so far.

RA is widely used in dermatological treatment and thyroid cancer management (Verschoore *et al.* 1993, Coelho *et al.* 2004), although the effects of this drug on normal thyroid function are poorly defined. We have recently demonstrated that 13-*cis*-RA is able to increase thyroid radioiodide uptake in normal female rats (Silva *et al.* 2009); however, the effect on the thyroid of male rats was not evaluated.


DOI: 10.1677/JOE-09-0421
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Therefore, the aim of the present study was to analyze the influence of RA treatment on thyroid iodide uptake, serum thyroxine ($T_4$), and TPO and DuOx activities in male rats.

## Materials and Methods

### *Materials*

All-*trans*-RA and 13-*cis*-RA were purchased from Sigma Chemical Co. Tris(hydroxymethyl)aminomethane, glucose, and potassium iodide were purchased from Merck. Fetal bovine serum was purchased from Cultilab (Rio de Janeiro, RJ, Brazil). Glucose oxidase (grade I) was purchased from Boehringer, and Na-$^{125}$I was purchased from Amersham.

### *Animals*

Adult male Wistar rats weighing 250–300 g were housed under controlled conditions of temperature (24±1 °C) and light (12 h light starting at 0700 h) with water and food made available *ad libitum*. The study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Institutional Animal Welfare Committee (CAUAP/UFRJ).

Rats were randomly divided into the following groups: control, all-*trans*-RA and 13-*cis*-RA. RA was administered in the dose of 100 μg/kg body weight (b.w.) s.c. daily for 14 days according to Coya *et al*. (1997), and in the doses of 100 and 1500 μg/kg b.w. for 28 days. The dose of 1500 μg/kg b.w. is similar to that used in the treatment of thyroid cancer in humans (Coelho *et al*. 2004). Both all-*trans*-RA and 13-*cis*-RA were dissolved in DMSO and suspended in propylene glycol (1:1, v/v). Rats from all experimental groups were killed 24 h after the last injection.

### *TPO preparation*

TPO extraction from rat thyroids was performed as previously described (Moura *et al*. 1989, Ferreira *et al*. 2005). Pools of two rat thyroids were minced and homogenized in 0·5 ml of 50 mM Tris–HCl buffer, pH 7·2, containing 1 mM KI, using an Ultra-Turrax homogenizer (Staufen, Germany). The homogenate was centrifuged at 100 000 ***g*** at 4 °C for 1 h. The pellet was suspended in 0·5 ml digitonin (1%, w/v) and incubated at 4 °C for 24 h to solubilize the peroxidase. The digitonin-treated suspension was centrifuged at 100 000 ***g*** at 4 °C for 1 h, and the supernatant containing the solubilized TPO was used for the assays. Protein content was determined by the method of Bradford (1976).

### *Thyroid peroxidase activity*

The TPO iodide oxidation activity was measured as previously described (Nakashima & Taurog 1978, Pommier 1978, Moura *et al*. 1989, Carvalho *et al*. 1994, Ferreira *et al*. 2005). The assay mixture contained 1·0 ml of freshly prepared 50 mM sodium phosphate buffer, pH 7·4, containing 24 mM KI and 11 mM glucose, and increasing amounts (20–80 μl) of solubilized TPO. The final volume was adjusted to 2·0 ml with 50 mM sodium phosphate buffer, pH 7·4, and the reaction was started by the addition of 10 μl of 0·1% glucose oxidase (Boehringer Grade I). The increase in absorbance at 353 nm (tri-iodide production) was registered for 4 min on a Hitachi spectrophotometer (U-3300). The $\Delta A_{353\ nm}$/min was determined from the linear portion of the reaction curve, and one unit (U) of TPO corresponds to $\Delta A_{353\ nm}$/min = 1·0. The enzymatic activity is expressed as U/g protein.

### *DuOx preparation*

For DuOx preparation, the excised thyroid glands remained at 4 °C for 24 h in 50 mM sodium phosphate buffer, pH 7·2, containing 0·25 M sucrose, 0·5 mM dithiothreitol, 1 mM EGTA, 5 μg/ml aprotinin, and 34·8 μg/ml phenylmethylsulphonyl fluoride (PMSF) before homogenization. Then, the homogenate was centrifuged at 100 000 ***g*** for 35 min at 4 °C and resuspended in 0·5 ml of 50 mM sodium phosphate buffer, pH 7·2, containing 0·25 M sucrose, 2 mM $MgCl_2$, 5 μg/ml aprotinin, and 34·8 μg/ml PMSF, and stored at −20 °C until $H_2O_2$ generation measurements.

### *$Ca^{2+}$- and NADPH-dependent $H_2O_2$-generating activity: DuOx activity*

$H_2O_2$ production was measured as previously described (Leseney *et al*. 1999, Cardoso *et al*. 2001). We incubated samples of thyroid particulate fractions at 30 °C in 1 ml of 170 mM sodium phosphate buffer, pH 7·4, containing 1 mM sodium azide, 1 mM EGTA, 1 μM FAD, and 1·5 mM $CaCl_2$. The addition of 0·2 mM NADPH started the reaction; aliquots of 100 μl were collected at intervals up to 20 min, and mixed with 10 μl of 3 M HCl to stop the reaction and destroy the remaining NADPH. Initial rates of $H_2O_2$ formation were determined from eight aliquots of each assay by following the decrease in 0·4 μM scopoletin fluorescence in the presence of HRP (0·5 μg/ml) in 200 mM phosphate buffer, pH 7·8, in a Hitachi spectrofluorimeter (F 4000). The excitation and emission wavelengths were 360 and 460 nm respectively. Specific activities were expressed per milligram protein (nmoles $H_2O_2$/h per mg protein).

### *Thyroid radioiodide content*

We have previously shown that the measurement of radioiodide uptake 15 min after $^{125}$I-NaI administration (short-term iodide uptake) reflects iodide transport through the NIS, without influence of *in vivo* thyroid iodine organification activity (Ferreira *et al*. 2005, Lima *et al*. 2006). Thus, in order to evaluate the *in vivo* NIS function, the animals received Na-$^{125}$I (3700 Bq, i.p., Amersham) 15 min before decapitation. We also administered Na-$^{125}$I (3700 Bq, i.p., Amersham)



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2 h before decapitation, in order to evaluate both iodide transport and *in vivo* organification activities (thyroid iodine accumulation). In the thyroid, iodine organification is catalyzed by TPO and consists of binding of iodine to tyrosyl residues of TG, an essential step of thyroid hormone biosynthesis. The radioactivity of the thyroid glands was measured using a gamma counter (LKB) and expressed as percentage of total $^{125}I$ injected per mg of thyroid.

### *$T_4$ measurement*

Serum total $T_4$ concentrations were measured using commercial kits (RIA, Diagnostic Systems Laboratories Inc., TX, USA, or eletrochemiluminescence, Roche Diagnostics). $T_4$ RIA sensitivity was of 0·4 μg/dl, and inter- and intra-assay coefficients of variation varied from 5·6 to 8·6% and 4 to 5·1% respectively. $T_4$ eletrochemiluminescence assay sensitivity was of 0·42 μg/dl, and intra-assay coefficients of variation were of 4·2%.

### *Statistical analysis*

The experiments were repeated at least two times using at least three animals per group in each experiment, so a total number of at least six animals per group were achieved. Results are expressed as mean ± S.E.M. and were analyzed by unpaired *t*-test. Differences were considered significant when $P<0·05$.

## Results

### *Fourteen days of RA treatment*

Short-term (15 min) thyroid radioiodide uptake was unchanged by the treatment with both isomers of RA in the low dose administered (100 μg/100 g b.w.) for 2 weeks (Fig. 1A), suggesting that RA did not affect NIS function in the period of time and dose used. On the other hand, treatment with all-*trans*-RA for 2 weeks produced a significant decrease in thyroid iodine accumulation (2 h; Fig. 1B). TPO activity was not affected by the treatment with either isomers of RA for 2 weeks when compared to the control group, as shown in Fig. 1C. However, administration of the low dose of all-*trans*-RA (100 μg/100 g b.w.) for 2 weeks led to a significant decrease in $H_2O_2$ formation, while treatment with 13-*cis*-RA had no effect (Fig. 1D), suggesting an inhibitory effect of all-*trans*-RA on DuOx activity. Despite decreased DuOx activity after 14 days of RA treatment, total serum $T_4$ concentration remained unchanged (Table 1).

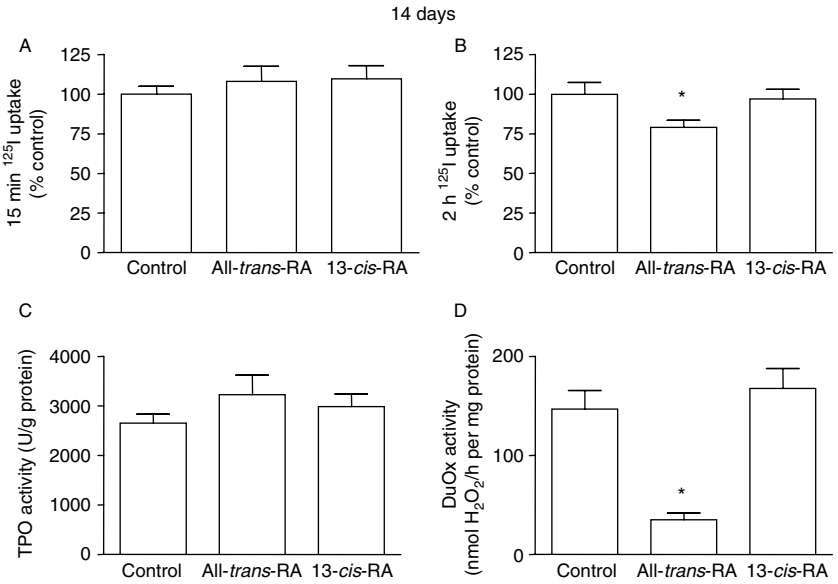


**Figure 1** Effect of all-*trans*-retinoic acid and 13-*cis*-retinoic acid administered s.c. for 14 days in the dose of 100 μg/kg b.w., daily injections, on some parameters of thyroid function. (A) Short-term iodide uptake 15 min after radioiodide administration (control ($n=16$), all-*trans*-RA ($n=19$), and 13-*cis*-RA ($n=19$)). (B) Thyroid iodine accumulation 2 h after radioiodide administration (control ($n=11$), all-*trans*-RA ($n=13$), and 13-*cis*-RA ($n=13$)). *$P<0·05$ versus control and versus 13-*cis*-RA of 100 μg/kg b.w. (C) Thyroperoxidase iodide oxidation activity (control ($n=27$), all-*trans*-RA ($n=32$), and 13-*cis*-RA ($n=31$)). (D) DuOx $Ca^{++}$-dependent $H_2O_2$-generating activity (control ($n=12$), all-*trans*-RA ($n=10$), and 13-*cis*-RA ($n=12$)). *$P<0·0001$ versus control and 13-*cis*-RA. Measurements were performed as described in Materials and Methods. Data are expressed as mean ± S.E.M.



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**Table 1** Effect of all-*trans*-retinoic acid (RA) and 13-*cis*-RA for 14 days in the dose of 100 μg/kg body weight (b.w.) on serum total thyroxine ($T_4$) levels. Results are expressed as mean±S.E.M.

| | $T_4$ (μg/dl) |
|---|---|
| **Treatment** | |
| Control | 6·0±0·4 (*n*=16) |
| 100 μg/kg b.w. | |
| All-*trans*-retinoic acid | 6·2±0·2 (*n*=19) |
| 13-*cis*-retinoic acid | 6·0±0·3 (*n*=18) |

*Twenty-eight days of RA treatment*

All-*trans*-RA treatment did not affect 15-min thyroid radioiodide uptake (Fig. 2A). However, treatment with 13-*cis*-RA for 28 days significantly increased NIS function in both doses (Fig. 2B), as previously described for thyroid cancers (Coelho *et al*. 2004) and female rats (Silva *et al*. 2009).

Treatment with 13-*cis*-RA at the higher dose (1500 μg/100 g b.w.) for 4 weeks produced a significant increase in the 2 h thyroid radioiodine content (Fig. 2D), which was unchanged by treatment with all-*trans*-RA (Fig. 2C).

While TPO activity was not affected by RA treatment in both doses (Fig. 3A and B), DuOx activity was significantly increased in the thyroid of rats treated with 13-*cis*-RA in the dose of 1500 μg/100 g b.w. for 4 weeks (Fig. 3D), but not with all-*trans*-RA (Fig. 3C). Total serum $T_4$ did not differ among groups (Table 2).

## Discussion

We have shown herein that RA may affect thyroid function not only by interfering with NIS function, but also by modulating DuOx activity. It is well documented that RA stimulates thyroid iodide uptake in human thyroid follicular carcinoma cells (Schmutzler 2001), thyroid cancer (Coelho *et al*. 2004), and thyroid gland of female rats (Silva *et al*. 2009). We now demonstrate that although 28 days of treatment with 13-*cis*-RA stimulates radioiodide uptake, in fact, 14 days

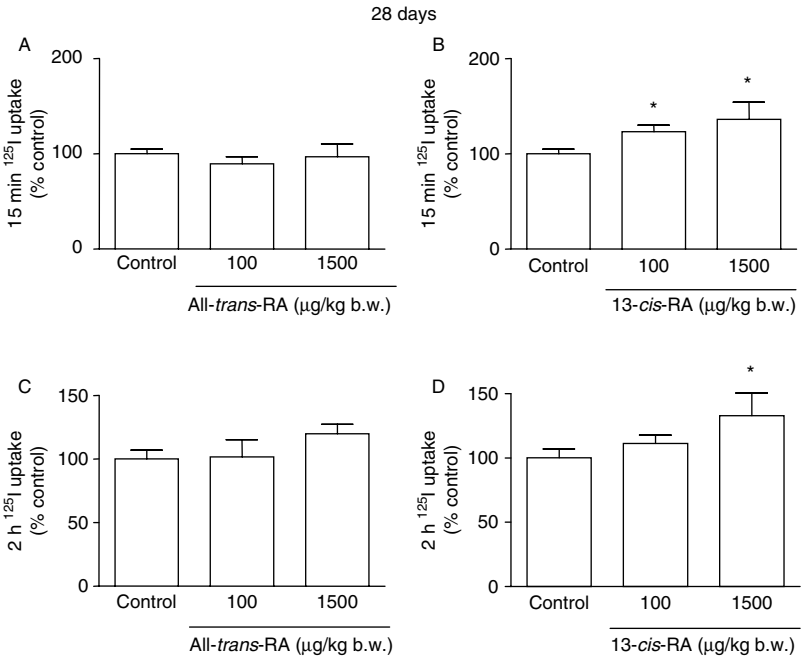


**Figure 2** Effect of all-*trans*-retinoic acid and 13-*cis*-retinoic acid administered s.c. for 28 days in the doses of 100 and 1500 μg/kg b.w., daily injections, on thyroid iodide uptake (15 min) and accumulation (2 h). (A) Short-term iodide uptake 15 min after radioiodide administration in rats treated with all-*trans*-retinoic acid (control (*n*=17), all-*trans*-RA of 100 μg/kg b.w. (*n*=11), and all-*trans*-RA of 1500 μg/kg b.w. (*n*=7)). (B) Short-term iodide uptake 15 min after radioiodide administration in rats treated with 13-*cis*-retinoic acid (control (*n*=17), 13-*cis*-RA of 100 μg/kg b.w. (*n*=11), and 13-*cis*-RA of 1500 μg/kg b.w. (*n*=7)). *$P$<0·05 versus control. (C) Thyroid iodine accumulation 2 h after radioiodide administration in rats treated with all-*trans*-retinoic acid (control (*n*=24), all-*trans*-RA of 100 μg/kg b.w. (*n*=12), and all-*trans*-RA of 1500 μg/kg b.w. (*n*=14)). (D) Thyroid iodine accumulation 2 h after radioiodide administration in rats treated with 13-*cis*-retinoic acid (control (*n*=24), 13-*cis*-RA of 100 μg/kg b.w. (*n*=12), and 13-*cis*-RA of 1500 μg/kg b.w. (*n*=14)). *$P$<0·05 versus control. Measurements were performed as described in Materials and Methods. Data are expressed as mean±S.E.M.



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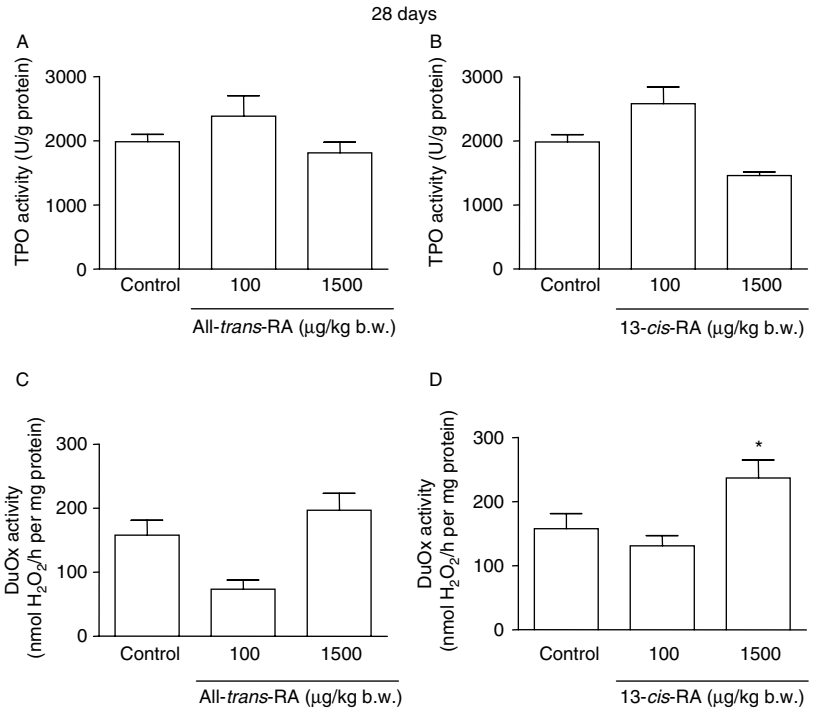


**Figure 3** Effect of all-*trans*-retinoic acid and 13-*cis*-retinoic acid administered s.c. for 28 days in the doses of 100 and 1500 μg/kg b.w., daily injections, on thyroperoxidase and DuOx activities. (A) Thyroperoxidase iodide oxidation activity in the thyroid of rats treated with all-*trans*-retinoic acid (control ($n$=26), all-*trans*-RA of 100 μg/kg b.w. ($n$=23), and all-*trans*-RA of 1500 μg/kg b.w. ($n$=7)). (B) Thyroperoxidase iodide oxidation activity in the thyroid of rats treated with 13-*cis*-retinoic acid (control ($n$=26), 13-*cis*-RA of 100 μg/kg b.w. ($n$=22), and 13-*cis*-RA of 1500 μg/kg b.w. ($n$=7)). (C) DuOx $Ca^{++}$-dependent $H_2O_2$-generating activity in the thyroid of rats treated with all-*trans*-retinoic acid (control ($n$=17), all-*trans*-RA of 100 μg/kg b.w. ($n$=9), and all-*trans*-RA of 1500 μg/kg b.w. ($n$=10)). (D) DuOx $Ca^{++}$-dependent $H_2O_2$-generating activity in the thyroid of rats treated with 13-*cis*-retinoic acid (control ($n$=17), 13-*cis*-RA of 100 μg/kg b.w. ($n$=7), and 13-*cis*-RA of 1500 μg/kg b.w. ($n$=10)). *$P$<0·05 versus control and versus 13-*cis*-RA of 100 μg/kg b.w. Measurements were performed as described in Materials and Methods. Data are expressed as mean±S.E.M.

of treatment with all-*trans*-RA decreased thyroid iodide content, which has been shown to be related to decreased thyroid iodide organification. DuOx activity was reduced in this group, while NIS function and TPO activity were not affected. It is known that the availability of $H_2O_2$ is a limiting step for iodine organification (Corvilain *et al.* 1991), so the decreased radioiodine content 2 h after its administration might be related to the decreased DuOx activity. This result highlights the importance of the duration of treatment with RA and the isomer used in order to achieve the desirable effect. It is known that radioiodide uptake *per se* is important although not sufficient for its therapeutic effectiveness.

On the other hand, 13-*cis*-RA significantly increased radioiodide uptake in both doses used when the rats were treated for 28 days. Increased NIS function after RA treatment is in agreement with data shown by Schmutzler *et al.* (2002). However, the increased radioiodine content was only detected in rats treated with the highest dose of 13-*cis*-RA. This result reinforces the concept that an increase in NIS function does not necessarily lead to increased thyroid radioiodine content. In this sense, $H_2O_2$ generation was increased in rats treated with the highest dose of 13-*cis*-RA for

**Table 2** Effect of all-*trans*-retinoic acid (RA) and 13-*cis*-RA for 28 days in the doses of 100 and 1500 μg/kg body weight (b.w.) on serum total thyroxine ($T_4$) levels. Results are expressed as mean±S.E.M.

| | **$T_4$** (μg/dl) |
|---|---|
| **Treatment** | |
| Control | 5·5±0·3 ($n$=19) |
| 100 μg/kg b.w. | |
| All-*trans*-retinoic acid | 5·9±0·4 ($n$=11) |
| 13-*cis*-retinoic acid | 6·1±0·7 ($n$=11) |
| 1500 μg/kg b.w. | |
| All-*trans*-retinoic acid | 5·0±0·3 ($n$=11) |
| 13-*cis*-retinoic acid | 5·4±0·3 ($n$=10) |



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28 days, showing again the importance of DuOx activity as a limiting step for iodine organification.

The effects of RA treatment on the thyroid of male rats were similar to those found in female rats, as previously described by our group (Silva *et al*. 2009), suggesting no gender-specific difference regarding thyroid response to RA treatment.

To our knowledge, we have reported herein for the first time the regulatory effect of RA on DuOx activity. As reviewed by Lambeth *et al*. (2007), DuOx1 and DuOx2 expression are regulated in both thyroid and non-thyroid cells by a promoter region within −150 bp of the first exon of DuOx1 and within −250 bp of DuOx2. In dog and pig thyrocytes, cAMP, which is a pathway downstream of the TSH receptor, increases DuOx protein and mRNA (Dupuy *et al*. 1999, De Deken *et al*. 2000, Morand *et al*. 2003). However, no data about a RA-responsive element in DuOx promoter are available. Pailler-Rodde *et al*. (1991) have shown that RA induces an increase in PKC activity. Since DuOx is a calcium-dependent NADPH oxidase (Carvalho *et al*. 1996, Dupuy *et al*. 1999, Ferreira *et al*. 2003, Ginabreda *et al*. 2008), 13-*cis*-RA could also increase DuOx activity by a stimulatory effect on the calcium pathway. Nevertheless, a direct effect of RA on DuOx promoter cannot be excluded. Further experiments are needed to elucidate the mechanism underlying the effect of RA on DuOx activity.

Even though thyroid iodine organification was significantly decreased in rats treated with all-*trans*-RA for 14 days and increased in rats treated with 13-*cis*-RA for 28 days, serum $T_4$ levels remained unchanged. Our data suggest that compensatory mechanisms might take place in order to avoid significant changes in serum $T_4$ levels.

In conclusion, our data suggest that treatment with RA produces direct effects on thyroid function that are dose, isomer and time dependent, suggesting that the efficacy of RA to improve radioiodine therapy might be influenced by these factors. Treatment with 100 μg/100 g b.w. of all-*trans*-RA for 2 weeks reduced thyroid iodine content, while treatment with 1500 μg/100 g b.w. of 13-*cis*-RA increased thyroid iodine content. So it might be important to evaluate serum thyroid hormones and TSH levels in patients under treatment with RA for dermatological and oncological purposes, especially in areas with a low iodine intake and in patients with possible subclinical thyroid dysfunction.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## Funding

This work was supported by grants from CNPq, Departamento de Tireóide/SBEM, FAPERJ/SUS, and FAPERJ/Pensa Rio.

## Acknowledgements

We are grateful for the technical assistance of Norma Lima de Araújo Faria, Advaldo Nunes Bezerra, and Wagner Nunes Bezerra. MM, ACMS, and MPM were recipients of fellowships from CAPES and CNPq.

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Received in final form 26 February 2010
Accepted 8 March 2010
Made available online as an Accepted Preprint
8 March 2010



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