

## RESEARCH

# Bisphenol A increases hydrogen peroxide generation by thyrocytes both *in vivo* and *in vitro*

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## Abstract

Bisphenol A (BPA) is the most common monomer in polycarbonate plastics and an endocrine disruptor. Though some effects of BPA on thyroid hormone (TH) synthesis and action have been described, the impact of this compound on thyroid H<sub>2</sub>O<sub>2</sub> generation remains elusive. H<sub>2</sub>O<sub>2</sub> is a reactive oxygen species (ROS), which could have deleterious effect on thyrocytes if in excess. Therefore, herein we aimed at evaluating the effect of BPA exposition both *in vivo* and *in vitro* on H<sub>2</sub>O<sub>2</sub> generation in thyrocytes, besides other essential steps for TH synthesis. Female Wistar rats were treated with vehicle (control) or BPA 40 mg/kg BW for 15 days, by gavage. We then evaluated thyroid iodide uptake, mediated by sodium-iodide symporter (NIS), thyroperoxidase (TPO) and dual oxidase (DOUX) activities (H<sub>2</sub>O<sub>2</sub> generation). Hydrogen peroxide generation was increased, while iodide uptake and TPO activity were reduced by BPA exposition. We have also incubated the rat thyroid cell line PCCL3 with 10<sup>-9</sup> M BPA and evaluated *Nis* and *Duox* mRNA levels, besides H<sub>2</sub>O<sub>2</sub> generation. Similar to that found *in vivo*, BPA treatment also led to increased H<sub>2</sub>O<sub>2</sub> generation in PCCL3. *Nis* mRNA levels were reduced and *Duox2* mRNA levels were increased in BPA-exposed cells. To evaluate the importance of oxidative stress on BPA-induced *Nis* reduction, PCCL3 was treated with BPA in association to N-acetylcysteine, an antioxidant, which reversed the effect of BPA on *Nis*. Our data suggest that BPA increases ROS production in thyrocytes, what could lead to oxidative damage thus possibly predisposing to thyroid disease.

## Key Words

- ▶ bisphenol A
- ▶ endocrine disruptor
- ▶ reactive oxygen species
- ▶ hydrogen peroxide
- ▶ dual oxidase
- ▶ thyroperoxidase
- ▶ sodium-iodide symporter
- ▶ iodide
- ▶ thyroid

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## Introduction

Bisphenol A (BPA) is the most common monomer in polycarbonate plastic composition. It is used not only in the manufacture of polycarbonate-made products, such as beverage and food containers, but also in the synthesis of epoxy resins, found in the covering of canned food (1). When submitted to acidic pH or high temperature, the polymer undergoes breakdown of the ester bonds and

BPA can be released from the matrix, contaminating food, beverages and environment (2, 3).

BPA is an endocrine disruptor, being considered a selective estrogen receptor modulator (SERM) (4, 5), with important effects on reproductive function (4, 6). Besides that, BPA was shown to have positive association with serum TSH (7) and negative association with free T4

levels (8), thus suggesting an impact also in thyroid axis. Furthermore, BPA has been shown to increase oxidative stress, leading to oxidative damage of proteins, lipids and nucleic acids in liver, central nervous system, reproductive system and kidney (9, 10).

For thyroid hormone (TH) synthesis, thyrocytes require iodide, which is transported through the basolateral plasma membrane by  $\text{Na}^+/\text{I}^-$  symporter (NIS) (11, 12, 13). Thyroperoxidase (TPO), the key enzyme in TH biosynthesis, oxidizes iodide in the presence of  $\text{H}_2\text{O}_2$ , produced by dual oxidases (DUOX1 and DUOX2) (14, 15, 16, 17). However, an excessive production of hydrogen peroxide could lead to oxidative stress, since  $\text{H}_2\text{O}_2$  is a reactive oxygen species (ROS) and thyroid gland damage (18, 19, 20).

Since BPA has been shown to increase oxidative damage in several tissues and since thyrocytes are exposed to high levels of ROS due to TH biosynthesis, herein we hypothesized that BPA exposure could lead to an increased ROS production in thyrocytes thus affecting thyroid function. Therefore, our aim was to investigate the effect of BPA exposure on thyroid  $\text{H}_2\text{O}_2$  production both *in vivo* and *in vitro*, besides the effect of BPA treatment on two proteins essential for TH synthesis: NIS and TPO.

## Materials and methods

### Animals

In all experiments, adult (4–5 months old) female Wistar rats were kept in controlled temperature (22–25°C) animal room, with a 12-h light:12-h darkness cycle. We have chosen to study female rats since the prevalence of thyroid diseases is higher in women than in men (20, 21). Pelleted commercial chow (Paulinea, São Paulo, Brazil) and water were available *ad libitum*. The Institutional Committee for Use of Animals in Research approved the study (number: IBCCF167), and the procedures were in compliance with the International Guiding Principles for Biomedical Research Involving Animals of the Council for International Organizations of Medical Sciences (Geneva, Switzerland), and the guiding principles for care and use of animals from the American Physiological Society.

### Experimental design

Animals were divided into two groups: Control (C) and bisphenol A (BPA). Control rats received vehicle (corn oil:isopropanol 20% v/v) and BPA group received

bisphenol A in the dose of 40mg/kg body weight (BW) (22, 23, 24), dissolved in vehicle. Both were administered daily by gavage. After 15 days of treatment, animals were weighed and killed. The dose of BPA was chosen based on the non-observable adverse effect level (NOAEL) for BPA, established in 50mg/kg BW (25, 26). Thus, we decided to use a dose lower than NOAEL.

### Histological analysis

The thyroids were collected and fixed in 10% formaldehyde (pH 7.4) for 48h, followed by dehydration in ethanol (Vetec, Rio de Janeiro, Brazil) and clarification in xylene (Vetec, Rio de Janeiro, Brazil). Tissues were embedded in paraffin and cut at the thickness of 5  $\mu\text{m}$ . Sections were stained with hematoxylin and eosin for topographic analysis according to Andrade *et al.* (27). High-resolution images (1600  $\times$  1200 pixels) were obtained using digital camera (QImaging Retigar-2000R Fast 1394 Mono, Surrey BC, Canada) coupled to light microscope (Olympus BX50). High-quality images were captured with the QCapture Pro7 (QImaging).

### Radioiodide uptake

We have previously shown that the measurement of radioiodide uptake 15 min after  $^{125}\text{I}$ -NaI administration (short term iodide uptake) reflects iodide transport through the sodium-iodide symporter without the influence of *in vivo* thyroid iodine organification activity (28). Thus, in order to evaluate the *in vivo* NIS function using thyroid radioiodine uptake measurements, the animals received  $\text{Na-}^{125}\text{I}$  (3700Bq i.p., Amersham, Buckinghamshire, England) 15 min before decapitation. Thyroids were removed and weighed. The radioactivity of the thyroid glands was measured using a gamma counter (LKB), and the percentage of the  $^{125}\text{I}$  in the gland relative to the total  $^{125}\text{I}$  injected was calculated. Results were expressed as relative to control.

### Thyroid peroxidase activity

TPO extraction and activity measurement were performed as previously described (28, 29). Rat thyroids were minced and homogenized in 0.5 mL of 50mM Tris-HCl buffer, pH 7.2, containing 1mM KI, using an Ultra-Turrax homogenizer (Staufen, Germany). The homogenate was centrifuged at 100,000g, 4°C for 1h. The pellet was suspended in 0.5 mL triton (0.1% v/v) and incubated at 4°C for 24h to solubilize TPO. The suspension was

centrifuged at 100,000g, 4°C for 1 h, and the supernatant containing solubilized TPO was used for the assays.

The assay mixture contained: 1.0 mL of freshly prepared 50 mmol/L sodium phosphate buffer, pH 7.4, containing 24 mmol/L KI and 11 mmol/L glucose, and increasing amounts of solubilized TPO. The final volume was adjusted to 2.0 mL with 50 mmol/L sodium phosphate buffer, pH 7.4, and the reaction was started by the addition of 10  $\mu$ L of 0.1% w/v glucose oxidase (Boehringer Grade I). The increase in absorbance at 353 nm (tri-iodide production) was registered for 3 min on a Hitachi spectrophotometer (U-3300). The  $\Delta A_{353\text{nm}/\text{min}}$  was determined from the linear portion of the reaction curve and related to protein concentration. Protein concentration was determined by the Bradford assay (30). Results were expressed as relative to control.

### Thyroid H<sub>2</sub>O<sub>2</sub> production

H<sub>2</sub>O<sub>2</sub> generation was quantified in thyroid particulate fractions by the Amplex red/horseradish peroxidase assay (Molecular Probes, Invitrogen), which detects the accumulation of a fluorescent oxidized product, as previously described (18, 31). To measure H<sub>2</sub>O<sub>2</sub> production from particulate fraction, the excised thyroid glands remained at 4°C for 24 h in 50 mmol/L sodium phosphate buffer (pH 7.2) containing 0.25 mol/L sucrose, 0.5 mmol/L dithiothreitol (DTT), 1 mmol/L EGTA, 5 mg/mL aprotinin and 34.8 mg/mL phenylmethylsulphonyl fluoride (PMSF) before homogenization. Then, the homogenate was centrifuged at 100,000g for 35 min at 4°C and suspended in 0.25 mL of 50 mmol/L sodium phosphate buffer (pH 7.2) containing 0.25 mol/L sucrose, 2 mmol/L MgCl<sub>2</sub>, 5 mg/mL aprotinin and 34.8 mg/mL PMSF (32). This particulate fraction was incubated in 150 mmol/L sodium phosphate buffer (pH 7.4) containing 100 U/mL superoxide dismutase (SOD) (Sigma), 0.5 U/mL horseradish peroxidase (HRP) (Roche), 50  $\mu$ mol/L Amplex red (Molecular Probes), 1 mmol/L EGTA, with or without 1.5 mmol/L CaCl<sub>2</sub>. Then, 0.1 mg/mL NADPH was added to start the reaction and the fluorescence was immediately measured in a microplate reader (Victor X4; PerkinElmer, Norwalk, CT) at 30°C, using excitation wavelength at 530 nm and emission wavelength at 595 nm. H<sub>2</sub>O<sub>2</sub> production was quantified using standard calibration curves (31).

The specific enzymatic activity was obtained as nmol of H<sub>2</sub>O<sub>2</sub> per hour per milligram of protein (nmol/h/mg protein) and calcium-dependent H<sub>2</sub>O<sub>2</sub> generation was obtained by subtracting H<sub>2</sub>O<sub>2</sub> generation in the absence

of calcium from that obtained in the presence of calcium. Then, the results were expressed as relative to control. Protein concentration was determined by the Bradford assay (30).

### Radioimmunoassays

Serum T3 and T4 were determined by specific coated tube RIA kits (MP Biomedicals, Orangeburg, NY, USA). Intra- and interassay coefficients of variation for T3 were 4.4–5.6% and 5.3–7.5%, respectively, and sensitivity was 6.7 ng/dL. For T4 intra- and interassay coefficients of variation were 3.3–8.1% and 5.3–11.4%, respectively, and sensitivity was 0.76  $\mu$ g/dL. All procedures were performed following the fabricant recommendations.

### Cell culture

Rat thyroid cell line PCCL3 (donated by Prof. Roberto Di Lauro, Stazione Zoologica Anton Dohrn) was maintained in Coon's modified Ham's F-12 medium (HiMedia Laboratories, Mumbai, India), which contains 10 mM glucose, supplemented with 5% v/v fetal bovine serum and a six-hormone mixture (1 mIU/mL TSH, 10 mg/mL insulin, 5 mg/mL transferrin, 10 nM hydrocortisone, 10 ng/mL somatostatin, and 10 ng/mL glycyl-L-histidyl-L-lysine acetate) and maintained in a humidified 5% v/v CO<sub>2</sub> incubator at 37°C.

### Cell treatment and cell viability assay

In order to evaluate the possible toxic effect of bisphenol A on PCCL3 cells, we treated the cells with different concentrations of BPA (10<sup>-9</sup>, 10<sup>-7</sup>, 10<sup>-5</sup> and 10<sup>-3</sup> mol/L BPA) or vehicle (ethanol 0.1% v/v) for 24 h. As an index of cell viability, we used the commercially available MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich), according to the manufacturer's recommendations. This is a colorimetric assay to determine the number of viable cells. The assay is based on the cellular conversion of the tetrazolium salt into formazan that is soluble in tissue culture medium, and it is measured directly at 490 nm in 96-well assay plates. Absorbance is directly proportional to the number of living cells in culture. After treatment, cells were stained with MTT (0.5 mg/mL) for 3 h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Then, cells were lysed with DMSO (P A). All determinations were done in triplicates. Since we did not find reduction of cell viability with 10<sup>-9</sup> mol/L BPA, suggesting that BPA is not toxic for

PCCL3 cells at this concentration, we decided to perform the next experiments using this concentration. Moreover, literature data suggest that the concentration of BPA in the serum is in nmol/L range in humans (33, 34). Results were expressed as relative to control.

In order to evaluate the involvement of the prooxidant effect of BPA on *Nis* and *Tpo* mRNA regulation, PCCL3 cells were incubated for 24 h with or  $10^{-9}$  mol/L BPA in association or not with 1 mmol/L N-acetylcysteine (NAC), which was added 30 min before BPA addition (35).

### Real-time PCR

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen), following the manufacturer's instructions. After DNase treatment, reverse transcription was followed by real-time PCR, as previously described (36). Specific oligonucleotides, as described in Table 1, were purchased from Applied Biosystems (Foster City, California, USA). GAPDH was used as internal control. Results were expressed as relative to control.

### H<sub>2</sub>O<sub>2</sub> production in intact cells

Extracellular H<sub>2</sub>O<sub>2</sub> generation was quantified by the Amplex red/horseradish peroxidase assay, which detects the accumulation of a fluorescent oxidized product, as previously described (18, 31). Cells ( $1 \times 10^5$ ) in Dulbecco's PBS (D-PBS) containing CaCl<sub>2</sub> and MgCl<sub>2</sub> were incubated with D-glucose (1 mg/mL), superoxide dismutase (100 U/mL),

horseradish peroxidase (0.5 U/mL), and Amplex red (50 μmol/L), in the presence or absence of 1 μmol/L ionomycin, a calcium ionophore, since DUOX, the enzyme responsible for H<sub>2</sub>O<sub>2</sub> generation associated to TH synthesis, is a calcium-dependent NADPH oxidase (19). The fluorescence was immediately measured in a microplate reader (Victor3, Perkin Elmer) for 30 min (excitation wavelength=530 nm and emission wavelength=595 nm). H<sub>2</sub>O<sub>2</sub> generation was determined using standard calibration curves. Results were obtained as nmols of H<sub>2</sub>O<sub>2</sub> per hour per 10<sup>5</sup> cells. In order to obtain calcium-dependent H<sub>2</sub>O<sub>2</sub> generation, which is associated to DUOX activity, H<sub>2</sub>O<sub>2</sub> generation obtained in the presence of ionomycin was subtracted by that found in the absence of ionomycin. Then, results were expressed as relative to control.

### Statistical analyses

All the results are expressed as mean ± S.E.M. and were analyzed by Unpaired *t*-test. Experiments were performed at least twice and at least two animals/cell replicates per group per experiment were used. Results of cell viability assay were analyzed by One-way ANOVA followed by the Newman-Keuls multiple comparison test, since there were more than two groups. Statistical analyses were conducted using the software GraphPad Prism (version 5, GraphPad Software Inc, San Diego, California), and the level of significance was established at *P* < 0.05.

## Results

### Body and thyroid weight

BPA treatment led to a slight though significant increase in BW, as shown in Table 2. This result suggest that the energetic homeostasis was affected by BPA what is in accordance with the well-known obesogenic effect of BPA (37). On the other hand, absolute and relative thyroid weight remained unchanged (Table 2).

### Histological analysis

The thyroid glands from control rats showed a normal tissue organization: follicles with simple cuboidal epithelial cells surrounding a cavity rich in eosinophilic material or colloid, besides a rich vascular stroma (Fig. 1). In BPA-treated group, we observed many hypoactive follicles, with squamous epithelium surrounded by interfollicular connective tissue.

**Table 1** Primers used for real-time PCR assay.

<i>Nis</i>	Forward	5' GCT CAT CCT GAA CCA AGT GA 3'
	Reverse	5' ACG AGC ATT ACC ACA ACC TG 3'
<i>Duox1</i>	Forward	5' ATT TCT TGG GAG GTA CAG CG 3'
	Reverse	5' GTT AGG CAG GTA GGG TTC TTT C 3'
<i>Duoxa1</i>	Forward	5' TGA CCA GCT TAT TCA TCG GG 3'
	Reverse	5' CTG TGA GGG TGA TGT TGA GTC 3'
<i>Duox2</i>	Forward	5' AGG AGT GGC ATA AGT TTG AGG 3'
	Reverse	5' CCT TGT CAC CCA GAT GAA GTA G 3'
<i>Duoxa2</i>	Forward	5' TGG TAT TCT TGT CCT TGG CTG 3'
	Reverse	5' GGA GGT ACT GAA GGC TTT GTA G 3'
<i>Tpo</i>	Forward	5' GAA TGA GGA ACT GAC CGA GAG 3'
	Reverse	5' TGA CAA GCC ACA GAA CTC TC 3'
<i>β-Tsh</i>	Forward	5' TCT GCG CTG GGT ATT GTA TG 3'
	Reverse	5' CGG TAT TTC CAC CGT TCT GT 3'
<i>Gapdh</i>	Forward	5' TGA TTC TAC CCA CGG GAA GT 3'
	Reverse	5' AGC ATC ACC CCA TTT GAT GT 3'

*β-Tsh*, beta subunit of thyroid stimulating hormone (TSH); *Duox1*, dual oxidase 1; *Duox2*, dual oxidase 2; *Duoxa1*, dual oxidase maturation factor 1; *Duoxa2*, dual oxidase maturation factor 2; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Nis*, sodium iodide symporter; *Tpo*, thyroperoxidase.

**Table 2** Effect of BPA treatment on body weight, absolute and relative thyroid weight of female rats.

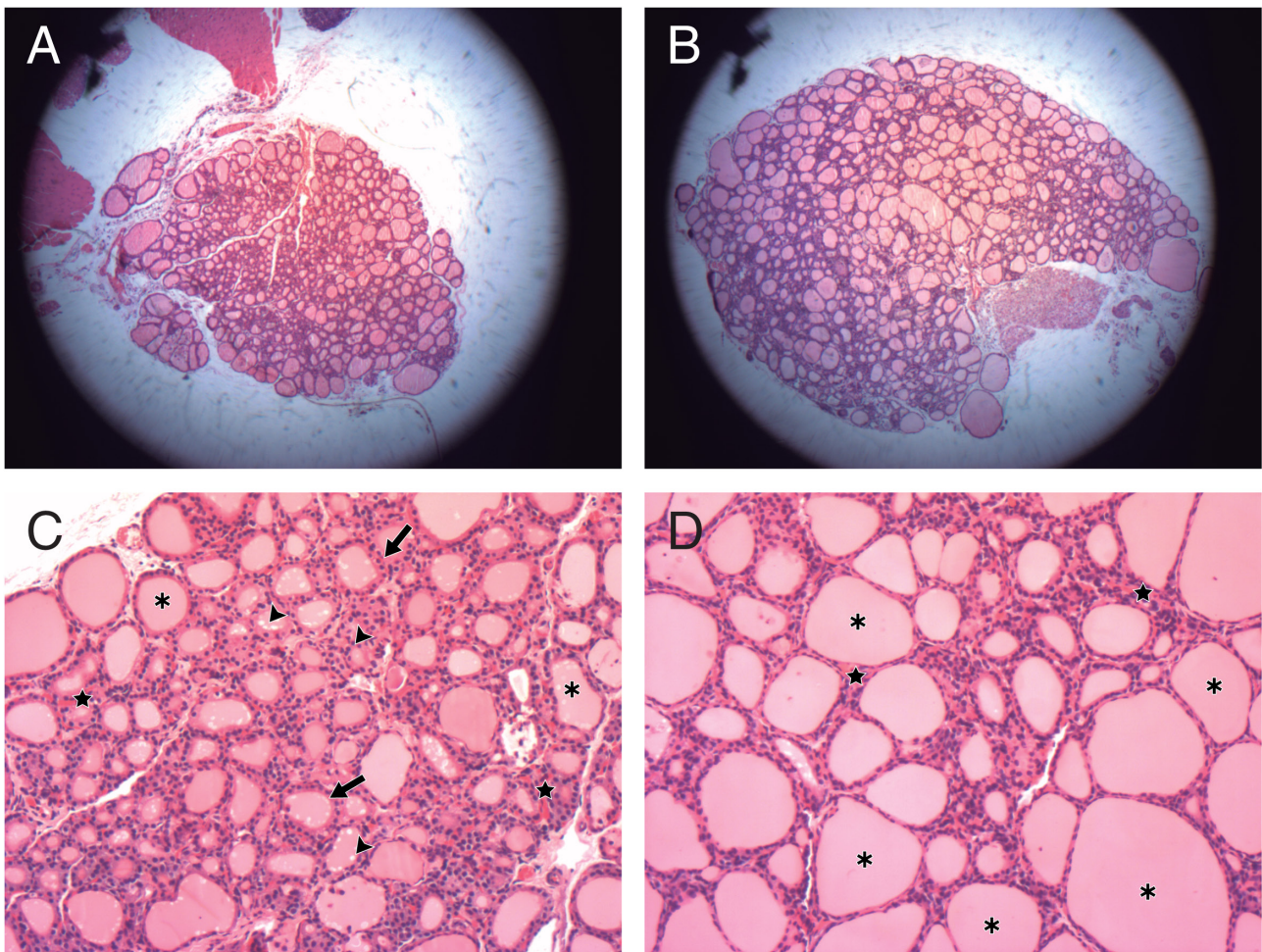
Group	Body weight (g)	Absolute thyroid weight (mg)	Relative thyroid weight (mg/100 g BW)
Control	218.6±3.4	17.3±0.9	7.8±0.4
BPA	228.0±3.1*	16.9±0.8	7.7±0.3

Female Wistar rats were treated with vehicle (Control, corn oil:isopropanol 20% v/v) or bisphenol A (BPA, 40 mg/kg BW), orally by gavage, daily for 15 days (n=16 per group, \*P<0.05 vs control group).

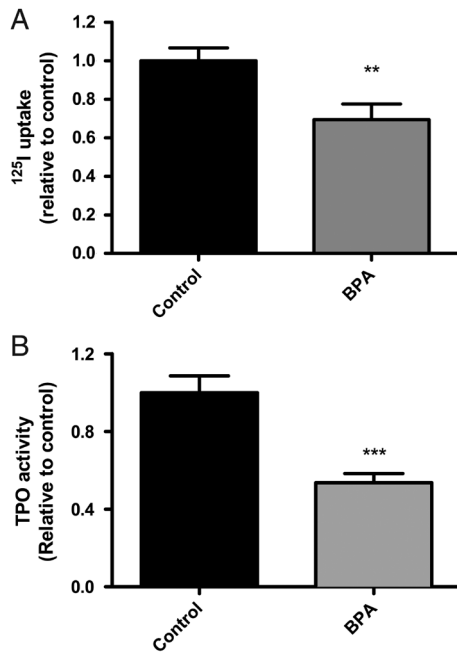
**Thyroid iodide uptake, TPO activity and H<sub>2</sub>O<sub>2</sub> generation in rats**

Since iodide is an essential element for TH biosynthesis (12), we have evaluated the effect of BPA on *in vivo* thyroid radioiodide uptake. In fact, BPA treatment significantly decreased thyroid iodide uptake (Fig. 2A).

TH synthesis requires not only iodide uptake, but also iodide oxidation and organification, reactions catalyzed by thyroperoxidase, in the presence of H<sub>2</sub>O<sub>2</sub> (38). We have thus evaluated TPO activity and H<sub>2</sub>O<sub>2</sub> generation. BPA led to a significant reduction of TPO activity (Fig. 2B), similarly to that found for NIS. Therefore, two fundamental steps for TH biosynthesis



**Figure 1** Photomicrographs of thyroid gland histological sections of rats exposed to BPA. Hematoxylin and eosin staining. Female Wistar rats were treated with vehicle (Control, corn oil:isopropanol 20%) or bisphenol A (BPA, 40 mg/kg BW), orally by gavage, daily for 15 days. (A) Control: lobe of the thyroid showing follicles of different size and activity status (normo, hypo and hyperactive). (B) BPA: lobe showing large number of hypoactive follicles. (C) Control: note the predominantly follicular structures, circled by a simple epithelium (arrow), containing eosinophilic material (colloid, asterisk), areas of colloid reabsorption (arrowhead) and interfollicular rich vascularized stroma (star). (D) BPA: note many hypoactive follicles with squamous epithelium (asterisk) and interfollicular connective tissue (star). Magnification of A and B=20x and C and D=200x. N=3 per group.



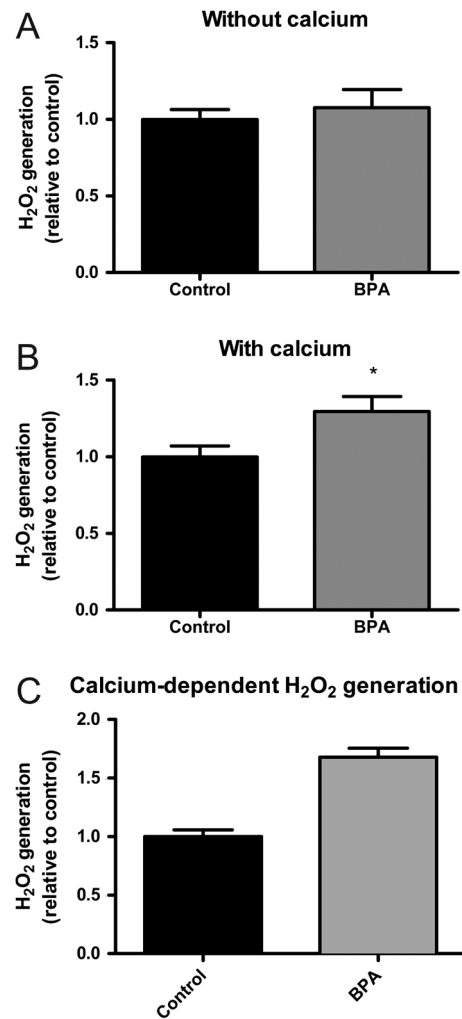
**Figure 2**  
Effect of BPA exposure on thyroid radioiodide uptake and thyroperoxidase (TPO) activity. Female Wistar rats were treated with vehicle (Control, corn oil:isopropanol 20%) or bisphenol A (BPA, 40 mg/kg BW), orally by gavage, daily for 15 days. (A) Radioiodide was administered to rats (i.p.) and 15 min later they were killed, thyroids were removed, weighed and the radioactivity was measured. (B) Thyroids were processed and iodide oxidation TPO activity was measured as described in 'Methods' section. Results are expressed as relative to control (control,  $n=10$  and BPA,  $n=9$ ; \*\* $P<0.01$  vs control and \*\*\* $P<0.001$  vs control).

were affected by BPA treatment: iodide uptake and TPO activity.

Thyroid  $\text{H}_2\text{O}_2$  generation in the absence of calcium remained unchanged (Fig. 3A), while thyroid  $\text{H}_2\text{O}_2$  generation in the presence of calcium was increased in BPA-treated group (Fig. 3B). Thus, calcium-dependent  $\text{H}_2\text{O}_2$  generation was significantly increased in the thyroid of rats treated with BPA (Fig. 3C). Since  $\text{H}_2\text{O}_2$  is a ROS, the increment in  $\text{H}_2\text{O}_2$  levels could lead to an enhancement of oxidative stress in the gland, especially because TPO activity, which consumes  $\text{H}_2\text{O}_2$ , is reduced.

### Serum total T4 and T3 and pituitary $\beta$ -Tsh mRNA

Since thyroid iodide uptake and thyroperoxidase activity were reduced by BPA treatment, we next evaluated TH levels. Surprisingly, we have found an increment in T4 levels in the serum of BPA-exposed rats, while T3 remained unchanged (Table 3). Thus, despite the reduction of TPO activity found in the dosage *in vitro*, it is possible that, *in vivo*, the greater availability of the TPO cofactor,  $\text{H}_2\text{O}_2$ ,



**Figure 3**  
Effect of BPA exposure on thyroid  $\text{H}_2\text{O}_2$  generation. Female Wistar rats were treated with vehicle (Control, corn oil:isopropanol 20%) or bisphenol A (BPA, 40 mg/kg BW), orally by gavage, daily for 15 days. Thyroid  $\text{H}_2\text{O}_2$  generation was measured by Amplex red method, as described in methods. Activity was measured in the absence (A) and in the presence (B) of calcium, since dual oxidase is a calcium-dependent NADPH oxidase and calcium-dependent  $\text{H}_2\text{O}_2$  generation (C) was obtained subtracting the activity in the presence of calcium by that obtained in the absence of calcium. Results are expressed as relative to control (control,  $n=10$  and BPA,  $n=9$ ; \* $P<0.05$  vs control and \*\*\* $P<0.001$  vs control).

has led to the increased T4 synthesis. In accordance to the increased serum T4, we have found a significant reduction of *Tsh* beta chain mRNA levels in the pituitary of the rats treated with BPA (Table 3).

### PCCL3 cell viability

Since the changes in proteins involved in TH synthesis observed *in vivo* could be due to a direct effect of BPA or

**Table 3** Effect of BPA treatment on serum total T4 and T3 concentrations and mRNA levels of TSH beta chain in pituitary of female rats.

Group	Total T4 (µg/dL)	Total T3 (ng/dL)	Pituitary mRNA levels of β-TSH (relative to control)
Control	3.6 ± 0.2	50.7 ± 3.4	1.0 ± 0.2
BPA	4.8 ± 0.3*	59.0 ± 5.3	0.5 ± 0.1*

Female Wistar rats were treated with vehicle (Control, corn oil:isopropanol 20% v/v) or bisphenol A (BPA, 40 mg/kg BW), orally by gavage, daily for 15 days (n=10 per group for serum total T4 and total T3 and n=4 per group for pituitary β-TSH mRNA levels, \*P<0.05 vs control group).

could be related to hormonal changes caused by BPA, we next evaluated the direct effect of BPA on thyrocytes, using the non-tumor rat thyroid cell line PCCL3 as a model. However, since BPA could be toxic for PCCL3, we have first evaluated the effect of BPA on cell viability. In fact, at the concentration 10<sup>-3</sup> mol/L, BPA seems to be toxic for PCCL3, significantly reducing its viability (Fig. 4A). Surprisingly, 10<sup>-7</sup> mol/L BPA significantly increased cell viability, suggesting that this compound could increase cell proliferation, reduce cell death or both. Since 10<sup>-9</sup> mol/L BPA did not affect cell viability and since this concentration is in the range of BPA concentration found

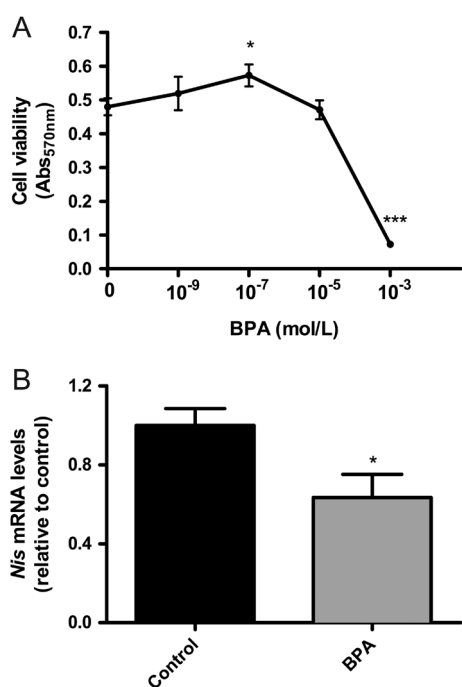
in human serum (33, 34), we decided to use 10<sup>-9</sup> mol/L BPA for the next experiments.

### Nis mRNA levels in PCCL3

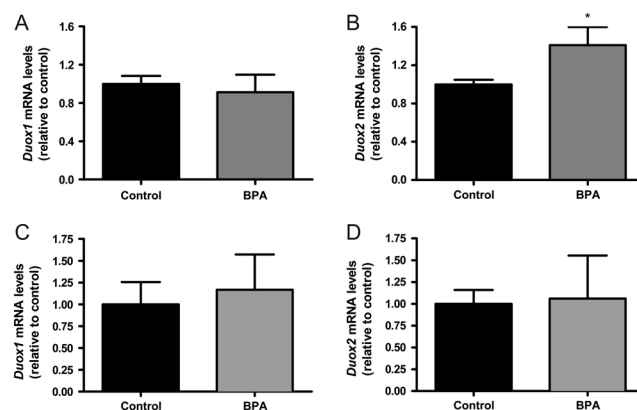
Since *in vivo* data have shown an inhibitory effect of BPA on thyroid iodide uptake, which is mediated by NIS, we have next evaluated *Nis* mRNA levels in PCCL3 cells treated with BPA. We have found a reduction of *Nis* mRNA levels in BPA-treated cells (Fig. 4B). Thus, BPA seems to act by transcriptional mechanism to regulate *Nis* mRNA levels.

### H<sub>2</sub>O<sub>2</sub> generation and *Duox* and *Duoxa* mRNA levels in PCCL3

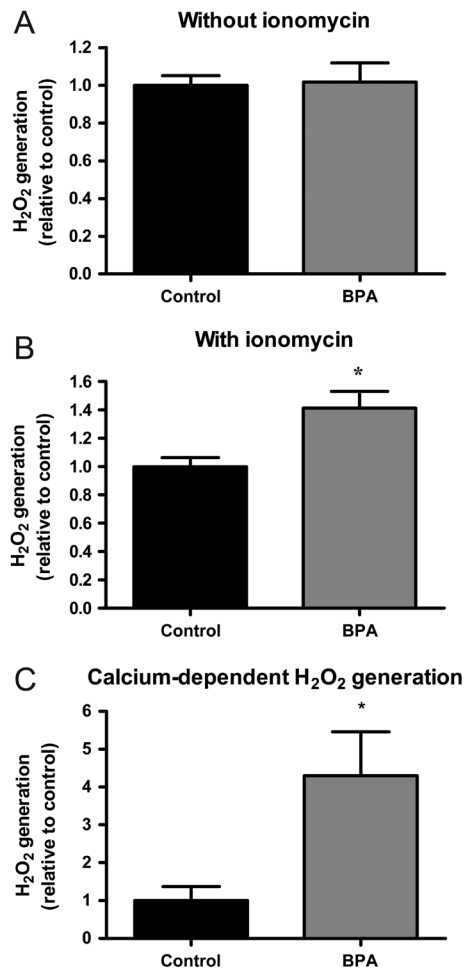
Since *in vivo* data found herein showed an increment of thyroid H<sub>2</sub>O<sub>2</sub> generation and since it has been shown that ROS have an inhibitory effect on NIS (39), we have then evaluated the effect of BPA on H<sub>2</sub>O<sub>2</sub> generation and on *Duox1*, *Duox2*, *Duoxa1* and *Duoxa2* mRNA levels. DUOX1 and DUOX2 are calcium-dependent NADPH oxidases responsible for H<sub>2</sub>O<sub>2</sub> generation associated to TH



**Figure 4** Effect of BPA on cell viability and sodium-iodide symporter (*Nis*) mRNA levels in PCCL3 cells. (A) Rat thyroid cell line PCCL3 was incubated with vehicle (control, 0.1% ethanol) or different BPA concentrations (10<sup>-9</sup>, 10<sup>-7</sup>, 10<sup>-5</sup> and 10<sup>-3</sup> mol/L BPA) and 24h later cell viability was assayed by MTT, as described in methods (n=12 per experimental condition; \*P<0.05 vs control and \*\*\*P<0.001 vs control). (B) PCCL3 cells were incubated with vehicle (control, 0.1% ethanol) or 10<sup>-9</sup> mol/L BPA and *Nis* mRNA levels were evaluated by qRT-PCR, as described in methods. Results are expressed as relative to control (control, n=6; BPA, n=4; \*P<0.05 vs control).



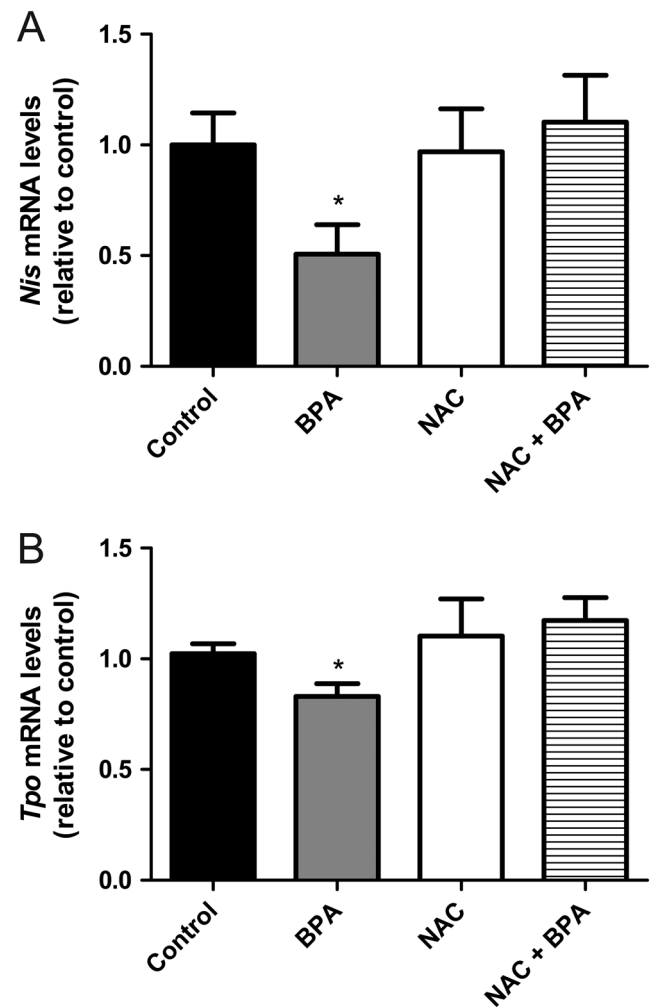
**Figure 5** Effect of BPA on *Duox1*, *Duox2*, *Duoxa1* and *Duoxa2* mRNA levels in PCCL3 cells. PCCL3 cells were incubated with vehicle (control, 0.1% ethanol) or 10<sup>-9</sup> mol/L BPA and 24h later mRNA levels of (A) *Duox1*; (B) *Duox2*; (C) *Duoxa1* and (D) *Duoxa2* were evaluated by qRT-PCR, as described in 'Methods' section. Results are expressed as relative to control (control, n=6; BPA, n=4; \*P<0.05 vs control).



**Figure 6** Effect of BPA on extracellular H<sub>2</sub>O<sub>2</sub> generation in PCCL3 cells. PCCL3 cells were incubated with vehicle (control, 0.1% ethanol) or 10<sup>-9</sup> mol/L BPA and 24 h later, extracellular H<sub>2</sub>O<sub>2</sub> generation was measured by Amplex red method, as described in methods. Activity was measured in the absence (A) and in the presence (B) of ionomycin, a calcium ionophore, since dual oxidase is a calcium-dependent NADPH oxidase, and calcium-dependent H<sub>2</sub>O<sub>2</sub> generation (C) was obtained subtracting the activity in the presence of calcium by that obtained in the absence of calcium. Results are expressed as relative to control (control, n=9; BPA, n=9; \*P<0.05 vs control).

biosynthesis (15, 16) and DUOX1 and DUOX2 are the maturation factors necessary for full DUOX1 and DUOX2 activities, respectively (40). In fact, mutations in *Duox2* have been associated to dyshormonogenesis and goiter (16, 41). *Duox2* mRNA levels showed a significant increase in BPA-treated cells, (Fig. 5B), while *Duox1* mRNA levels remained unchanged (Fig. 5A). Moreover, mRNA levels of both *Duoxa1* (Fig. 5C) and *Duoxa2* (Fig. 5D) were not altered by BPA treatment.

H<sub>2</sub>O<sub>2</sub> generation in the absence of ionomycin remained unchanged (Fig. 6A), while H<sub>2</sub>O<sub>2</sub> generation in the presence of ionomycin was increased by BPA (Fig. 6B).



**Figure 7** Effect of BPA and NAC on *Nis* and *Tpo* mRNA levels in PCCL3 cells. PCCL3 cells were incubated with vehicle (control, 0.1% ethanol) or 10<sup>-9</sup> mol/L BPA, with or without 1 mmol/L NAC, and 24 h later mRNA levels of (A) *Nis* and (B) *Tpo* were evaluated by qRT-PCR, as described in methods. Results are expressed as relative to control (NIS: control, n=5; BPA, n=5; NAC, n=6; NAC+BPA, n=6; TPO: control, n=6; BPA, n=5; NAC, n=6; NAC+BPA, n=6; \*P<0.05 vs control).

Calcium-dependent extracellular H<sub>2</sub>O<sub>2</sub> generation was significantly increased in BPA-treated cells (Fig. 6C), thus suggesting that the stimulatory effect of BPA on thyrocyte H<sub>2</sub>O<sub>2</sub> generation occurs not only *in vivo* but also *in vitro*, what could be deleterious to the thyroid gland since H<sub>2</sub>O<sub>2</sub> is a ROS.

### Effect of BPA on *Nis* and *Tpo* mRNA levels is reversed by NAC

Since BPA seems to have an inhibitory effect on NIS and TPO, besides increasing ROS production, we have next evaluated the involvement of oxidative stress in the



effect of BPA. As shown in Fig. 7A, co-incubation of BPA-treated cells with N-acetylcysteine (NAC), an antioxidant, prevented the inhibitory effect of BPA on *Nis* expression. In a similar way, NAC also prevented the reduction of *Tpo* mRNA levels by BPA (Fig. 7B). Thus, our result suggests that the inhibitory effect of BPA on *Nis* and *Tpo* expression might involve increased oxidative stress.

## Discussion

BPA has been shown to be a widespread environmental contaminant and can be found in the serum and urine of both wildlife (42) and humans (43). This exposition affects many organs and systems, including reproductive system (44), nervous system (45) and thyroid (46). Despite that, data regarding the impact of BPA on thyroid redox homeostasis and its consequences on proteins involved in TH synthesis remains elusive.

TH synthesis requires hydrogen peroxide generation, since  $H_2O_2$  is the essential cofactor for TPO (47). *In vivo*, DUOX is the enzyme responsible for  $H_2O_2$  generation associated to TH biosynthesis (16). Since DUOX is a calcium-dependent NADPH oxidase (48), herein we have evaluated thyroid  $H_2O_2$  generation both in the presence and in the absence of calcium. In fact, thyroid calcium-dependent  $H_2O_2$  generation was increased in BPA-treated group, suggesting that the gland could be exposed to an increased oxidative stress. The reduction in TPO activity reinforces the idea of an unbalance in redox homeostasis, since TPO is an important consumer of the  $H_2O_2$  generated by DUOX (49). Moreover, TPO has been shown to be sensitive to the oxidative stress induced in conditions of high DUOX activity (31). Besides, there is a negative correlation between DUOX and TPO in human thyroid nodular lesions and in experimental model of type 1 diabetes mellitus (18, 50). Therefore, increased  $H_2O_2$  generation in the thyroid of rats exposed to BPA, due to a higher DUOX activity, might have induced a decrease in TPO activity through the oxidation of this enzyme.

In zebrafish embryo-larvae, *tpo* mRNA levels remained unchanged after BPA exposition (51), and TPO activity from rat thyroid microsomes was shown to be unaffected by incubation with BPA *in vitro* (52). On the other hand, in the rat thyroid cell line FRTL-5, BPA reduced *Tpo* mRNA levels (52). Therefore, besides the oxidation of TPO protein due to increased ROS production, BPA treatment could also reduce TPO activity due to the downregulation of *Tpo* gene expression.

Despite the reduced *in vitro* TPO activity found in rats treated with BPA, T4 levels were not reduced in this group, in fact T4 was increased. The greater availability of TPO cofactor  $H_2O_2$  in the thyroid gland of BPA-treated animals might have contributed to the increment in serum T4 levels in this group.

BPA has been shown to increase oxidative stress in some tissues, such as testis (53), liver (54) and heart (55). However, to our knowledge, this is the first report describing the effect of BPA increasing thyroid  $H_2O_2$  generation. It was previously shown that the thyroid gland is exposed to a higher risk of oxidative DNA damage when compared to other tissues, due to the necessity of  $H_2O_2$  generation during TH biosynthesis (56). Therefore, the increment in ROS generation due to BPA exposure could predispose the gland to oxidative stress and thus to thyroid diseases.

NIS has been shown to be sensitive to ROS (57, 58, 59). Since BPA induced an increment in  $H_2O_2$  generation, we have also evaluated NIS-mediated thyroid iodide uptake. In fact, *in vivo* thyroid iodide uptake was reduced by BPA treatment, which could be related to an increased oxidative stress or could be related to a direct effect of BPA regulating NIS expression. Then, we have evaluated BPA effect on *Nis* mRNA levels. In fact, the treatment of the rat thyroid cell line PCCL3 cells with  $10^{-9}$ M BPA led to a significant reduction of *Nis* expression.

In zebrafish embryo-larvae, the treatment with BPA did not affect *nis* mRNA levels (51), while in rat thyroid cell line FRTL5, BPA was shown to reduce iodide uptake, besides reducing *Nis* expression (52). Therefore, at least in rats, BPA seems to be able to reduce both NIS expression and function, what is in accordance to our data. Since BPA is a xenoestrogen (60), it is possible that the effect of BPA downregulating NIS is due to its estrogenic activity, since estrogen has been shown to downregulate NIS in FRTL-5 cells (61). On the other hand, the possible estrogenic effect of BPA regulating NIS expression does not exclude the possibility that the increased ROS production could also affect NIS expression and function, so we have also evaluated  $H_2O_2$  generation in PCCL3 exposed to BPA.

We have observed that, similarly to that found *in vivo*, BPA treatment has also increased calcium-dependent extracellular  $H_2O_2$  generation *in vitro*. This effect could be related to the increased *Duox2* expression found herein, which has been shown to be the DUOX isoform essential to hormonogenesis, since mutation in this enzyme leads to hypothyroidism (41, 62). On the other hand, it is known that the main isoform responsible

for H<sub>2</sub>O<sub>2</sub> generation in PCCL3 cells is DUOX1 (63). Another possibility is the regulation of DUOX activity at post-transcriptional level by BPA. Both DUOX1 and DUOX2 have been described to be regulated by cascades of phosphorylation (64). Even though BPA can activate nuclear estrogen receptor, this endocrine disruptor is known to be more potent acting by non-nuclear estrogen receptors (65). Therefore, BPA could act at membrane estrogen receptors, activating phosphorylation cascades and thus activating DUOX and increasing H<sub>2</sub>O<sub>2</sub> generation in PCCL3. In fact, estrogen has been shown to increase ROS production in PCCL3 cells, which express both nuclear and membrane estrogen receptors (66). However, future studies are needed to elucidate the mechanism of BPA action regulating ROS production by thyrocytes.

The effect of BPA downregulating *Nis* and *Tpo* requires increased oxidative stress, since NAC was able to prevent the reduction of *Nis* and *Tpo* mRNA levels induced by BPA in PCCL3. NAC has previously been shown to prevent oxidative damage induced by BPA in Sertoli cells (67) and rat brain (68). Thus, BPA seem to cause a redox imbalance in the thyrocytes, disrupting the normal functioning of the thyroid gland.

## Conclusion

The present study showed that the exposition of thyrocytes to the endocrine disruptor bisphenol A can increase ROS production, both *in vivo* and *in vitro*. Moreover, BPA decreased thyroid iodide uptake and thyroperoxidase activity, two essential steps for TH synthesis. This effect might be related to an increased oxidative stress since NAC could prevent the reduction of *Tpo* and *Nis* mRNA levels induced by BPA in PCCL3. The enhancement of ROS production by thyrocytes related to the exposition to BPA could lead to oxidative damage of the gland, thus predisposing individuals exposed to BPA to thyroid diseases.

### 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.

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