



Effect of thimerosal on thyroid hormones metabolism in rats

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Abstract

Mercury seems to exert an inhibitory effect on deiodinases, but there are few studies using Thimerosal (TM) as the mercury source. We aimed to elucidate the effect of TM on thyroid hormones peripheral metabolism. Adult Wistar female rats received 0.25 µg or 250 µg TM/100 g BW, IM, twice a week, for a month. We evaluated serum total T₃ and T₄, D1 activity using ¹²⁵I-rT₃ as tracer, and D2 activity using ¹²⁵I-T₄. NADPH oxidase activity was measured by Amplex-red/HRP method and mRNA levels by real time PCR. Serum T₄ was increased and T₃ decreased by the greatest dose of TM. Even though D1 activity in pituitary and kidney was reduced by the highest dose of TM, hepatic D1 activity and D1 mRNA levels remained unchanged. D2 activity was also significantly decreased by the highest dose of TM in all CNS samples tested, except cerebellum, but D2 mRNA was unaltered. mRNA levels of the tested NADPH oxidases were not affected by TM and NADPH oxidase activity was either unaltered or decreased. Our results indicate that TM might directly interact with deiodinases, inhibiting their activity probably by binding to their selenium catalytic site, without changes in enzyme expression.

Key Words

- ▶ thimerosal
- ▶ thyroid function
- ▶ iodothyronine deiodinases

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Introduction

For many years, mercury has been used in a wide variety of human activities and has been identified as a factor exerting a range of harmful neurological and cognitive effects in humans and experimental animals, and is implicated in the etiology of a number of neuropsychiatric disorders. There are three categories of mercury compounds: organic, inorganic, and elemental (1). The major environmental organic compounds of mercury include ethylmercury (Et-Hg) and methylmercury (Met-Hg) (2). At present, humans are mostly exposed to mercury through the consumption of mercury-contaminated fish, the administration of vaccines containing thimerosal (ethyl-mercury thiosalicylate, used as preservative in some vaccines administered to mothers and infants), metallic mercury from dental

amalgam fillings, and cinnabar, a naturally occurring mineral consisting primarily of mercury sulphide, used in the traditional Chinese medicine (1, 3).

Thyroid hormones (TH) play an important role in the development and differentiation of several tissues. In the central nervous system, thyroid hormones are critical for the survival, proliferation, migration, and arborization of neurons (4). As aforementioned, mercury produces important deleterious effects on central nervous system. In the brain, most nuclear triiodothyronine (T₃) is locally produced from the prohormone thyroxine (T₄) by type 2 iodothyronine deiodinase (D2) (5). Since deiodinases regulate the metabolism of thyroid hormones, they are key determinants of TH concentration in the serum and in the different tissues (6).



Mercury seems to exert an inhibitory effect on iodothyronine deiodinases activity. Mori and coworkers showed that MeHg inhibited D2 activity in both mouse neuroblastoma cell line (7) and rat pituitary tumor cell line (8). In mice, maternal exposure to methylmercury was able to inhibit D3 activity in the brain of newborns, but D2 activity was not affected (9). Sulkowski and coworkers have recently reported a decrease in cerebellar D2 activity following exposure to thimerosal during perinatal period in SHR rats (2).

The mechanisms by which mercury inhibits deiodinase activity have not been clarified. Deiodinases contain selenocysteine in the catalytic site (10). This amino acid is essential for the deiodination reaction and can be a target for MeHg, preventing normal enzymatic activity. The activity can also be changed by the reduction of the cofactor glutathione, a reducing agent that can be decreased by mercury. Moreover, increased reactive oxygen species (ROS) production, eventually caused by mercury, could lead to the oxidation of both thiol group and selenocysteine, thus reducing deiodinase activity (11, 12).

While the effects of Met-Hg have been widely studied, little is known about the effects of thimerosal on biological systems. Most of the information about the thimerosal in literature is based on extrapolations of the knowledge regarding methylmercury molecule. However, especially in developing countries, the population is exposed to numerous medications containing thimerosal, which could lead to irreparable damage to health. Thus, this work aimed to understand how thimerosal could interfere with the peripheral metabolism of thyroid hormones, with a special focus on the central nervous system.

Materials and methods

Animals

In all experiments, adult (2 months old) female Wistar rats were kept in a controlled temperature (22–25°C) and 12h/12h light/dark cycle (lights on 07:00–19:00h) animal room. 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: IBCCF196-07/16), 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. Each animal received 0.25 or 250 µg of thimerosal per 100g body weight, intramuscular, dissolved in saline solution twice a week for a month. Control animals received intramuscular injections of saline solution. The lower dose was chosen based on the one that a one-year-old child receives in a vaccine dose (13). The higher one was chosen based on concentrations previously used in vaccines for children in Denmark during the period of 1970–1992 (14). Cerebellum, hypothalamus, cerebral cortex, hippocampus, pituitary, liver and kidney were excised after treatment, immediately frozen in liquid nitrogen, and stored at –80°C for mRNA extraction and deiodinase and H₂O₂ generating activities measurement. Blood was collected and serum was stored at –20°C for thyroid hormone measurements.

Serum T₃ and T₄ were determined by specific Coated Tube RIA kits (Diagnostic Systems Laboratories Inc., TX, USA). Intra- and interassay coefficients of variation for T₃ were 5.0–6.5% and 4.2–6.0%, respectively, and sensitivity was 4.3 ng/dL. For T₄, intra- and interassay coefficients of variation were 2.9–5.1% and 7.1–7.4% respectively, and sensitivity was 0.4 µg/dL. All procedures were performed following the fabricant recommendations.

D1 activity was determined using methods previously published (15). Briefly, whole pituitary, 15 mg of liver and 25 mg of kidney were homogenized in 150mM sodium phosphate buffer containing 1mM EDTA, 250mM sucrose, and 10mM DTT (pH 6.9). Homogenates were incubated in duplicate for 1h at 37°C with 1 µM rT₃, freshly purified tracer ¹²⁵I-labeled rT₃ (Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA), and 10mM DTT in 0.1M potassium phosphate buffer containing 1mM EDTA, pH 6.9. Total reaction volume was 300 µL. In our assay conditions, only D1 activity is measured since deiodinases activities were completely blocked in the presence of 100mM PTU, a specific D1 inhibitor. D1 final activity was expressed as the difference between the sample activity and its respective background. D2 activity was also determined by methods previously published (16). In short, 30mg from each tissue analyzed were homogenized in 0.1M sodium phosphate buffer containing 1mM EDTA, 0.25M sucrose, and 20mM dithiothreitol, pH 6.9. Homogenates (30 µg protein) were incubated, in duplicate, for 3h at 37°C with 1nM T₄, freshly purified tracer ¹²⁵I-labeled T₄ (PerkinElmer Life Sciences), 1mM PTU, and 20mM DTT in 0.1M sodium phosphate buffer containing 1mM EDTA, pH 6.9. Total reaction volume was 300 µL. In our assays conditions, only D2 activity was measured since deiodinase activities were completely blocked in the presence of T₄ in excess

Table 1 Primers used for real time PCR analysis.

	Forward	Reverse
D1	5' GTGATACAGGAAGGCAGGATC 3'	5' GAACGAAGGTCAAAGCTAAAGG 3'
D2	5' CTCCTAGACGCCTACAAACAG 3'	5' TGCTTCAGGATTGGACACG 3'
β-Actin	5' CACTTTCTACAATGAGCTGCG 3'	5' CTGGATGGCTACGTACATGG 3'

(100 nM). D2 final activity was expressed as the difference as the difference between the sample activity and its respective background. Protein was measured by Bradford method (17), after incubation of homogenates with NaOH (2.5 M) for 30 min at room temperature.

H₂O₂ generation was quantified in the particulate fractions obtained from cerebellum, hypothalamus, cerebral cortex, hippocampus, pituitary, liver and kidney by the Amplex red/horseradish peroxidase assay (Molecular Probes, Invitrogen). In order to obtain the microsomal fraction, the homogenates from the tissues were centrifuged at 3000g for 15 min at 4°C. Then, the supernatants were centrifuged at 100,000g for 35 min at 4°C and the pellets were suspended in 0.5 mL of 50 mM sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose, 2 mM MgCl₂, 5 mg/mL aprotinin and 34.8 mg/mL phenylmethanesulfonyl fluoride (PMSF) and stored at –20°C until the analyses were performed. For H₂O₂ generation measure, the microsomal fraction was incubated in 150 mM sodium phosphate buffer (pH 7.4) containing SOD (100 U/mL; Sigma), horseradish peroxidase (0.5 U/mL, Roche), Amplex red (50 mM; Molecular Probes) and 1 mM EGTA, in the presence or absence of 1 mM NADPH. The fluorescence was immediately measured in a microplate reader (Victor X4; PerkinElmer) at 30°C, using wavelength excitation at 530 nm and emission at 595 nm (18). Specific NADPH oxidase activity was calculated by the differences between the activities in the presence and absence of NADPH and the specific enzymatic activity was expressed as nanomoles H₂O₂ per hour per milligram of protein (nmol/h/mg). Protein concentration was determined by the Bradford assay (17).

Total RNA was extracted from the cerebellum, hypothalamus, cerebral cortex, hippocampus, pituitary, liver and kidney using the RNeasy Plus Mini Kit (Qiagen), following the manufacturer's instructions. After DNase treatment, reverse transcription of 1 µg RNA was followed by real time polymerase chain reaction (PCR), as previously described (19). β-Actin was used as an internal control. The specific oligonucleotides were purchased from Applied Biosystems (Table 1).

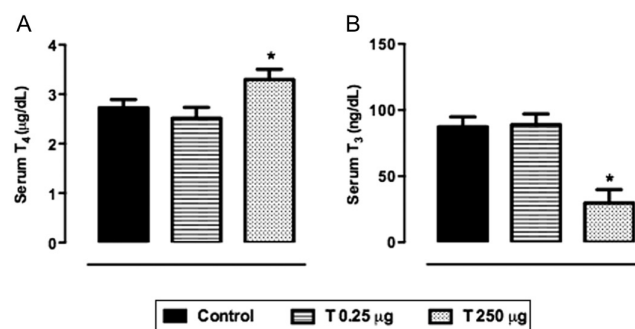
Results are expressed as the mean ± s.e.m. and were analyzed by one-way ANOVA, followed by Bonferroni's

multiple comparison test. To analyze H₂O₂ generation, a non-parametric data, Kruskal–Wallis test followed by Dunn's multi-comparison test was used. All analyses were performed using the Graphpad Prism software (version 5, Graphpad Software). A value of $P \leq 0.05$ was considered statistically significant.

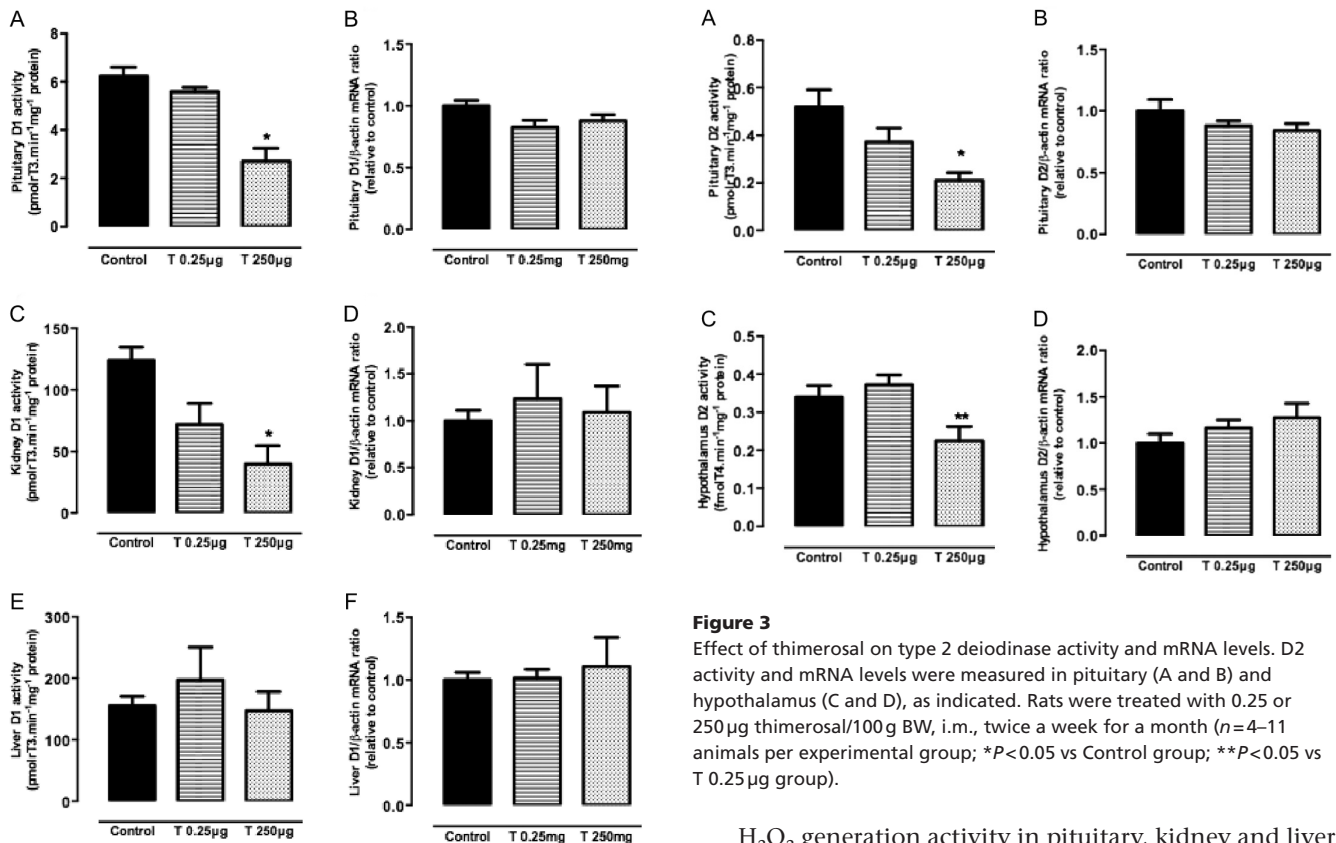
Results

In fact only the highest dose of thimerosal affected serum levels of thyroid hormones. While the total serum T₄ was increased (C: 2.73 ± 0.165; T 0.25: 2.52 ± 0.220; T 250: 3.30 ± 0.208 µg/dL) (Fig. 1A), the total serum T₃ was decreased (C: 87.23 ± 7.517; T 0.25: 88.87 ± 8.098; T 250: 29.66 ± 10.11 ng/dL) (Fig. 1B). This already suggested the decrease in peripheral T₄ deiodination that we found (see below).

Only in highest thimerosal dose decreased D1 activity in pituitary (C: 6.24 ± 0.374; T 0.25: 5.59 ± 0.181; T 250: 2.71 ± 0.541 pmol rT₃/min/mg protein – Fig. 2A) and kidney (C: 124.0 ± 10.48; T 0.25: 71.8 ± 17.21; T 250: 39.7 ± 14.62 pmol rT₃/min/mg protein – Fig. 2C), without affecting D1 activity in liver (Fig. 2E), although serum T₃ is significantly decreased. D1 mRNA levels were unaffected in all tissues studied (Fig. 2B, D and F). Moreover, in all the tested tissues, thimerosal was able to directly inhibit D1 activity *in vitro* (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

**Figure 1**

Effect of thimerosal on serum total T₄ (A) and T₃ (B) levels. Rats were treated with 0.25 or 250 µg thimerosal/100 g BW, i.m., twice a week for a month ($n \geq 10$ per experimental group; * $P < 0.05$ vs Control and T 0.25 µg groups).

**Figure 2**

Effect of thimerosal on type 1 deiodinase activity and mRNA levels. D1 activity and mRNA levels were measured in pituitary (A and B), kidney (C and D) and liver (E and F), as indicated. Rats were treated with 0.25 or 250 µg thimerosal/100 g BW, i.m., twice a week for a month ($n=4-6$ animals per experimental group; * $P<0.05$ vs Control group).

D2 activity and mRNA expression in pituitary and hypothalamus are shown in Fig. 3. The highest dose of thimerosal treatment significantly decreased D2 activity in the pituitary (C: 0.52 ± 0.071 ; T 0.25: 0.37 ± 0.059 ; T 250: 0.21 ± 0.033 fmol T₄/min/mg protein) (Fig. 3A) as well as in hypothalamus (C: 0.34 ± 0.030 ; T 0.25: 0.37 ± 0.025 ; T 250: 0.22 ± 0.038 fmol T₄/min/mg protein) (Fig. 3C). D2 mRNA expression were unaffected in both tissues (Fig. 3B and D).

The greater doses of thimerosal also decreased D2 enzymatic activity in the hippocampus (C: 0.25 ± 0.035 ; T 0.25: 0.25 ± 0.030 ; T 250: 0.15 ± 0.014 fmol T₄/min/mg protein) (Fig. 4A) and in cerebral cortex (C: 0.17 ± 0.053 ; T 0.25: 0.17 ± 0.054 ; T 250: 0.06 ± 0.013 fmol T₄/min/mg protein) (Fig. 4C), although the later did not reach statistical significance. Neither cerebellar D2 activity, nor D2 mRNA in hippocampus, cerebral cortex or cerebellum were changed by thimerosal treatment (Fig. 4B, D, E and F). Moreover, in all tested tissues, thimerosal was able to directly inhibit D2 activity *in vitro* (Supplementary Fig. 2).

Figure 3

Effect of thimerosal on type 2 deiodinase activity and mRNA levels. D2 activity and mRNA levels were measured in pituitary (A and B) and hypothalamus (C and D), as indicated. Rats were treated with 0.25 or 250 µg thimerosal/100 g BW, i.m., twice a week for a month ($n=4-11$ animals per experimental group; * $P<0.05$ vs Control group; ** $P<0.05$ vs T 0.25 µg group).

H₂O₂ generation activity in pituitary, kidney and liver is shown in Fig. 5. Pituitary H₂O₂ generation was decreased by both doses of thimerosal (C: 0.39 ± 0.019 ; T 0.25: 0.36 ± 0.008 ; T 250: 0.29 ± 0.015 nmol H₂O₂/h/mg protein). In the kidney H₂O₂ generation activity was decreased only at the highest dose (C: 0.39 ± 0.020 ; T 0.25: 0.34 ± 0.014 ; T 250: 0.15 ± 0.022 nmol H₂O₂/h/mg protein) (Fig. 5A and B), however the liver H₂O₂ generation remained unaffected by thimerosal treatment (Fig. 5C).

The H₂O₂ generation activities in hypothalamus (C: 0.25 ± 0.016 ; T 0.25: 0.27 ± 0.016 ; T 250: 0.20 ± 0.012 nmol H₂O₂/h/mg protein) and cerebral cortex (C: 0.30 ± 0.016 ; T 0.25: 0.34 ± 0.025 ; T 250: 0.12 ± 0.025 nmol H₂O₂/h/mg protein) were decreased by thimerosal but only at the greatest dose (Fig. 6A and B) without affecting hippocampal and cerebellar H₂O₂ generation activity (Fig. 6C and D).

Discussion

Thimerosal (TM) has been widely used as preservative in several drug products, including vaccines. However, it has been identified as a factor exerting a range of harmful neurological and cognitive effects in humans and has been implicated in the etiology of many neuropsychiatric disorders in children (20), even though these data were

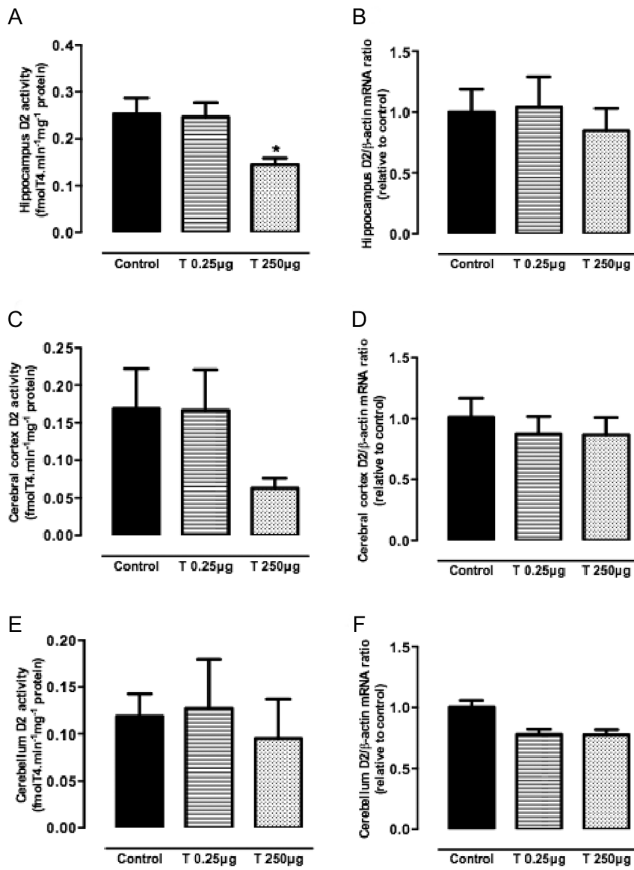


Figure 4 Effect of thimerosal on type 2 deiodinase activity and mRNA levels. D2 activity and mRNA levels were measured in hippocampus (A and B), cerebral cortex (C and D) and cerebellum (E and F), as indicated. Rats were treated with 0.25 or 250 µg thimerosal/100 g BW, i.m., twice a week for a month ($n=3-10$ animals per experimental group; * $P<0.05$ vs Control group).

not confirmed in infant rhesus macaques following the recommended paediatric vaccination procedure (21). Although the use of mercury compounds, such as TM in antiseptic medicines is decreasing, it is still used as preservative in some vaccines mainly in developing countries. Herein, we compared two different TM doses, the lower (0.25 µg/100 g BW) was based on the 2001 US immunization schedule (13) and the higher (250 µg/100 g BW) was based on concentrations previously used in vaccines for children in Denmark during the period of 1970–1992 (14). Since the lower dose did not produce any relevant effect on the vast majority of the parameters evaluated, our data suggests that only the higher dose could possibly be associated with neuropsychiatric disorders related to changes in thyroid hormones metabolism in children.

Thyroid hormones (TH) play a critical role during brain development and differentiation in humans and

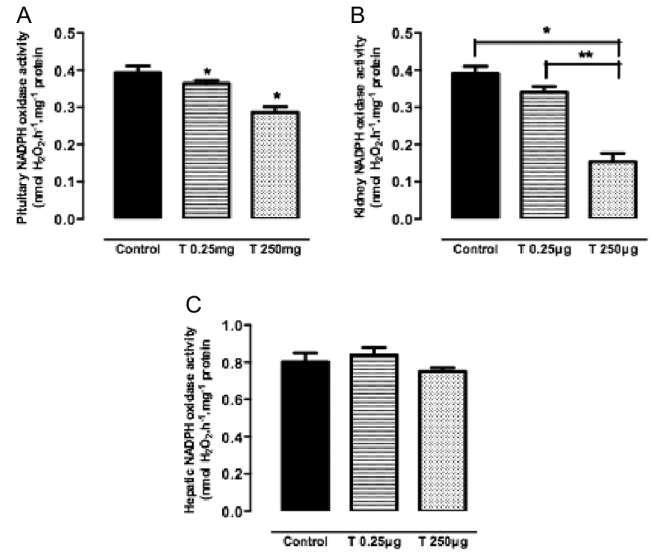


Figure 5 Effect of thimerosal on H₂O₂ generation. NADPH oxidase activity was measured in pituitary (A), kidney (B) and liver (C), as indicated. Rats were treated with 0.25 or 250 µg thimerosal/100 g BW, i.m., twice a week for a month ($n=5-7$ animals per experimental group; * $P<0.05$ vs Control group; ** $P<0.05$ vs T 0.25 µg).

animal models (22). Much of the information known about thyroid hormones and brain development has derived from rodent experiments. Deiodinases regulate

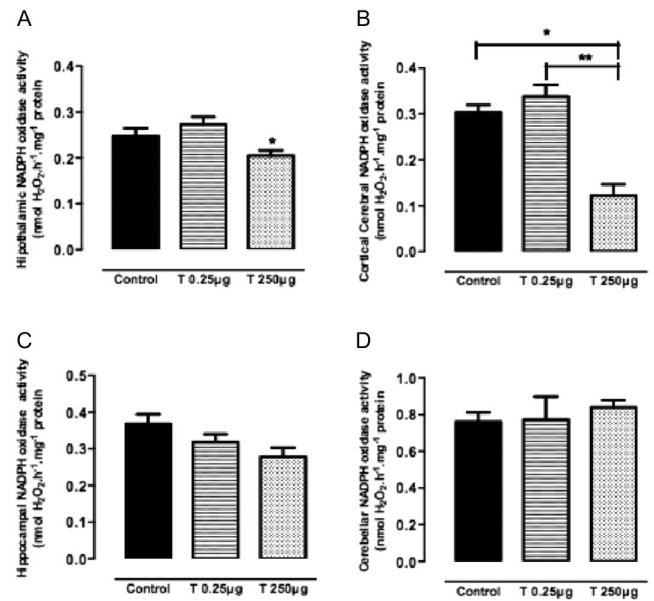


Figure 6 Effect of thimerosal on H₂O₂ generation. NADPH oxidase activity was measured in hypothalamus (A), cerebral cortex (B), hippocampus (C) and cerebellum (D), as indicated. Rats were treated with 0.25 or 250 µg thimerosal/100 g BW, i.m., twice a week for a month ($n=3-6$ animals per experimental group; * $P<0.05$ vs Control group; ** $P<0.05$ vs T 0.25 µg).

the metabolism of thyroid hormones and thus, regulate the concentration of these hormones in the different tissues (6). D1 and D2 are selenocysteine-containing enzymes that catalyze the deiodination of the prohormone thyroxine (T_4) into the active hormone triiodothyronine (T_3), both in central and peripheral tissues. In the brain, the major part of nuclear and intracellular T_3 is locally produced from T_4 by D2. Perinatal exposure to thimerosal can exert neurotoxic effects on the developing cerebellum and is associated with a decrease in D2 activity, which could result in local brain T_3 deficiency. Our data in adult female Wistar rats show that TM treatment (at the highest dose) was able to inhibit D1 activity in pituitary and kidney, probably by directly binding to the selenium of the catalytic site, without affecting D1 mRNA expression in these tissues. Since the kidney is an important site for thyroid hormones metabolism, this inhibition might be involved in the decreased serum T_3 levels associated with enhanced serum T_4 levels detected after TM treatment in the present work. No statistically significant effect was detected in liver, neither in D1 activity nor mRNA levels, as could be expected by the decreased serum T_3 , what reinforces the idea that TM might directly act as an inhibitor of D1 in both pituitary and kidney.

D2 activity and mRNA levels were evaluated in pituitary, hippocampus, cerebellum, cerebral cortex and hypothalamus of adult female rats. We detected a decrease in D2 activity induced by TM at the highest dose of treatment in most tissues studied, except in cerebellum. On the other hand, no changes were detected in D2 mRNA levels in the studied tissues. In fact, Carneiro and coworkers evaluated the deposition and metabolism of mercury species in mice after exposure to TM (23). The authors showed marked mercury species accumulation in tissues such as brain and kidney, where we have detected decreased D2 and D1 activities respectively. Since we have found that TM was able to inhibit D1 and D2 activities both *in vivo* and *in vitro*, without changes in the mRNA levels of the enzymes, we think that this might be due to a direct interaction of TM with the protein(s) involved in the deiodination activity. It is tempting to suggest that TM might interact with the selenocysteine of the active site of deiodinases, or with the *in vivo* enzyme cofactor, even though the cofactor is in excess in the *in vitro* assays.

The affinity of mercury to thiol groups ($-SH$) makes peptides and proteins vulnerable to its inhibition, especially when sulfhydryl groups are in the active site of the enzyme. Thioredoxin system plays a key role in many physiological processes. It has been demonstrated that inhibition of thioredoxin system is one of the main

mechanisms of Hg^{2+} and MeHg toxicity. This system is responsible for maintaining the general reduced state in cells and represents a potential biomarker of mercury toxicity (24). Thus, the reduced capacity of this system due to TM could be involved in the inhibitory effect on deiodinases, which require a reducing factor (probably glutathione) to catalyze deiodination reaction.

The effect of mercurial compounds on immune system is still controversial. Haase and coworkers demonstrated that ethylmercury and Hg^{+2} induce the formation of neutrophils extracellular traps (NETs) by human neutrophils granulocytes (25). This effect was triggered by ROS production in a NADPH oxidase independent way, since a NADPH oxidase inhibitor, did not abolish this effect. These results are in agreement with the present work, since we did not find any increment in NADPH oxidase activity. Increased oxidative stress was previously associated with the inhibition of D1 (26), but herein we did not detect any association between increased H_2O_2 generation and D1 activity. In fact, we have shown that at the highest dose (250 $\mu g/100 g$ BW), the treatment with thimerosal induced a decrease in H_2O_2 generation in pituitary, hippocampus, cerebral cortex and hypothalamus. Interestingly, D2 activity was also reduced in these same tissues. On the other hand, tissues in which H_2O_2 generation remained unchanged, such as cerebellum and liver, deiodinase activities were not affected too, thus suggesting a common cause for deiodinase and NADPH oxidase inhibition. The decrease in H_2O_2 generation in TM-treated rats does not seem to be due to a transcriptional effect of TM, since the mRNA levels of the ROS-generation enzymes, remained unaffected after thimerosal treatment in all tissues studied. In summary, our results indicate that TM (higher dose) inhibit D1 and D2 activities in some tissues, probably by binding to their selenium catalytic site, without altering their mRNA expression. NADPH oxidases are not involved in the diminished deiodinase activities, since the activity of these enzymes remained either unaltered or decreased. Our data contribute to increase the knowledge about the safety use of lower amounts of thimerosal and reinforces that low concentrations might be used in children vaccines.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EC-17-0220>.

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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