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Original Article

Comparative study of acute *in vitro* and short-term *in vivo* triiodothyronine treatments on the contractile activity of isolated rat thoracic aortas

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ABSTRACT We aimed to characterize the participation of rapid non-genomic and delayed non-genomic/genomic or genomic mechanisms in vasoactive effects to triiodothyronine (T3), emphasizing functional analysis of the involvement of these mechanisms in the genesis of nitric oxide (NO) of endothelial or muscular origin. Influences of *in vitro* and *in vivo* T3 treatments on contractile and relaxant responsiveness of isolated rat aortas were studied. *In vivo* T3-treatment was 500 $\mu g \cdot kg^{-1} \cdot d^{-1}$, subcutaneous injection, for 1 ($T3_{1d}$) and 3 ($T3_{3d}$) days. In experiments with endothelium-intact aortic rings contracted with phenylephrine, increasing concentrations of T3 did not alter contractility. Likewise, *in vitro* T3 did not modify relaxant responses induced by acetylcholine or sodium nitroprusside (SNP) nor contractile responses elicited by phenylephrine or angiotensin II in endothelium-intact aortas. Concentration-response curves (CRCs) to acetylcholine and SNP in endothelium-intact aortic rings from $T3_{1d}$ and $T3_{3d}$ rats were unmodified. $T3_{3d}$, but not $T3_{1d}$, treatment diminished CRCs to phenylephrine in endothelium-intact aortic rings. CRCs to phenylephrine remained significantly depressed in both endothelium-denuded and endothelium-intact, nitric oxide synthase inhibitor-treated, aortas of $T3_{3d}$ rats. In endothelium-denuded aortas of $T3_{3d}$ rats, CRCs to angiotensin II, and high K^+ contractures, were decreased. Thus, *in vitro* T3 neither modified phenylephrine-induced active tonus nor CRCs to relaxant and contractile agonists in endothelium-intact aortas, discarding rapid non-genomic actions of this hormone in smooth muscle and endothelial cells. Otherwise, $T3_{3d}$-treatment inhibited aortic smooth muscle capacity to contract, but not to relax, in an endothelium- and NO-independent manner. This effect may be mediated by delayed non-genomic/genomic or genomic mechanisms.

INTRODUCTION

Thyroid hormones (THs), 3,5,3',5' tetraiodo-L-thyronine (T4) and 3,5,3' triiodo-L-thyronine (T3), have important physiological and pathological effects on the cardiovascular system [1-4]. Excessive functional activity of the thyroid gland—hyperthyroid-ism—is characterized by an overstimulated cardiovascular state with increased heart rate, stroke volume, systolic blood pressure, pulse pressure, and decreased systemic vascular resistance (SVR) [1-4]. Conversely, a significant reduction in THs synthesis and release—hypothyroidism—results in decreased HR and stroke volume, increased SVR, diastolic hypertension, and lessened pulse

pressure [1-4].

THs exert their effects either per genomic mechanisms by binding to thyroid receptors (TR) inside the nucleus and regulating gene transcription, or through non-genomic mechanisms initiated outside cell nucleus at plasma membrane receptors and receptors located in the cytoplasm [5,6]. Several links have been recognized between non-genomic and genomic actions of THs (non-genomic/genomic mechanisms) [5,6].

Important reports indicate that THs can act directly on blood vessels causing smooth muscle depressor effects in an endothelium-dependent and -independent manner [7,8]. In isolated skeletal muscle resistance arteries of rats, T4 and T3 induced rapid onset (~20 min) vasodilator responses that were attenuated, but not abolished, with denudation or functional inhibition of the endothelium; implying that vasodilator responses to THs result of their combined effects on endothelium and smooth muscle [9]. In endothelium-denuded rat aortic rings, acute *in vitro* administration of T3 (30 min) reduced phenylephrine (PHE)-induced vasoconstriction, and this effect was reversed by nitric oxide (NO) synthesis inhibitor, L-NAME; which denotes that T3 inhibits aortic contractile responses inducing smooth muscle synthesis of NO [10]. Together, these data [9,10] exemplified the rapid non-genomic action of THs in isolated arteries. On the other hand, experimental hyperthyroidism induced by short-term T4 (3 days) and long-term T3 (6–12 weeks) treatments [11,12], provoked an increased relaxing effect of acetylcholine and a decreased contractile responses to norepinephrine in endothelium-intact rat aortic tissues; relaxant and contractile responses of isolated rat aortas were found modified by an increased endothelial vasodepressor function, elicited by THs effects pondered as genomic [12,13]. Contrariwise, abdominal aortas from long-term (6 weeks) hypothyroid rats were associated with diminished endothelium-dependent and -independent relaxations [14], indicating inhibition of vasomotor function through adaptations of both endothelial (ECs) and smooth muscle (SMCs) cells [14]. Noticeably, aortas from long-term (8–12 weeks) hypothyroid rats manifested a reduction in NO availability [15].

The following studies with ECs and SMCs cultures support the possibility of observing rapid non-genomic effects in isolated blood vessels. In SMCs isolated from rat aorta and cultured in a deformable matrix, T3 caused relaxation of these cells in 10 min, suggesting its direct action by a rapid non-genomic mechanism [16]. In other study, acute administration of T3 increased the generation of NO in SMCs of rat aorta, resulting in a significant decrease in myosin light chain phosphorylation levels as indirect evidence of contractility inhibition; prominently, T3 augmented the expression of neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) NO synthases, in 30 min, through the activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway [10]. Otherwise, T3 activated eNOS (within 10–20 min) through PI3K/Akt in ECs from the bovine aorta and human umbilical vein [17].

SVR is decreased in experimental and clinical hyperthyroidism, and it is increased in THs deficiency [1-4]. However, the direct effects of THs on blood vessels have not been convincingly explained. The present work was designed to help characterize the participation of rapid non-genomic, and delayed genomic or non-genomic/genomic, mechanisms in THs-elicited vasodepressor effects; emphasizing the functional analysis of the involvement of these mechanisms in the genesis of NO of endothelial or muscular origin. We aimed to elucidate whether acute *in vitro* and short-term *in vivo* T3 treatments inhibit the development of active tonus in rat thoracic aortas by increasing the synthesis of NO in endothelium and/or smooth muscle.

METHODS

All experimental procedures were approved by the Animal Care and Use Committee of our Institution and complied with the Mexican Federal Regulations for Animal Experimentation and Care (NOM-062-ZOO-1999; Ministry of Agriculture, Mexico City, Mexico). Male Wistar rats (250–300 g body mass, 10–12 weeks old) were housed under controlled conditions (22°C ± 2°C, 60% ± 5% humidity, and 12 h light/dark cycle); normal chow and tap water were given *ad libitum*.

Short-term *in vivo* T3 treatment

Based on previous research [11], experimental hyperthyroidism was induced by subcutaneous injection of T3 (500 μg·kg^{-1}·d^{-1}) diluted in alkaline saline solution (0.5 mM NaOH in 0.9% NaCl) for 1 (T3$_{1d}$) and 3 (T3$_{3d}$) days. Control animals were injected daily with the vehicle at the same volume (1 ml·kg^{-1}) for 1 (V$_{1d}$) and 3 (V$_{3d}$) days. Experiments were performed 24 h after the last injection.

Assessment of the hyperthyroid state

Blood samples were collected without anticoagulant from abdominal aortas, centrifuged at *1,000 g* for 10 min at 4°C, and stored at –20°C until assay. Levels of free T3 (fT3) and thyroid-stimulating hormone (TSH) were determined in the serum of control and T3-treated rats using enzyme-linked immunosorbent assay (ELISA) kits (fT3; Monobind, Inc., Lake Forest, CA, USA; TSH; ALPCO, Salem, NH, USA) according to manufacturer specifications.

Besides, left ventricular dry mass (LVdM) to tibia length (TL) ratio, LVdM/TL (mg/mm), was used as an index of cardiac hypertrophy, regularly observed in hyperthyroid disorder [1-3]. Body mass was recorded every day.

Tension measurements

Rats were anesthetized with pentobarbital sodium (50 mg·kg^{-1}, intraperitoneal) and exsanguinated. Thoracic aortas were removed, cleaned of fat and connective tissue, and cut into ring segments (4–5 mm in length). In some preparations, the endothelium was damaged by gently abrading the intimae of aortic rings with the tip of small forceps. The isolated arteries were placed in 10 ml tissue chambers filled with Krebs-bicarbonate solution (KBS) of the following composition (mM): NaCl 118, KCl 4.7, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, CaCl$_2$ 2.5, NaHCO$_3$ 25, and dextrose 11.7. High K$^+$ (40 and 80 mM) depolarizing solutions were prepared with equimolar replacement of NaCl with KCl. The medium was gassed continuously (95% O$_2$, 5% CO$_2$) and maintained at pH 7.4°C and 37°C. Each tissue sample was placed under an initial resting tension of a 2 g mass and equilibrated for 60 min prior to the execution of the experimental protocols. Contractions were measured isometrically and recorded on a computer with the AcqKnowledge software (MP100WSW; Biopac Systems, Inc., Santa Barbara, CA, USA). Functional endothelium was determined by the presence of at least 80% relaxation in response to acetylcholine (1 μM) after pre-constricting the tissues with PHE (1 μM). Otherwise, successful endothelial denudation was confirmed by the presence of small (less than 10%) relaxations, or absence of relaxations, in response to acetylcholine.

Acute *in vitro* T3 treatment

Cumulative concentration-response curves (CRCs) for T3 in aortic tissues pre-contracted with PHE

Aortic rings with endothelium received PHE (1 μM) and contractions were let to stabilize for 15 min. Next, increasing concentrations of T3 (1 nM–10 μM) were added to the tissues, every 30 min; temporal controls received the corresponding concentration of the vehicle.

CRCs to relaxant and contractile agonists in *in vitro* T3-treated aortic tissues

After the stabilization period, T3 (0.01, 01, and 1 μM) or vehicle (vehicle concentration needed to dissolve T3 1 μM) was administered to endothelium-intact aortic rings for 30 or 120 min. Then, aortic rings were stimulated with PHE (1 μM), contractions were stabilized (15 min) and CRCs to acetylcholine (endothelium-dependent relaxing agonist) or sodium nitroprusside (SNP; endothelium-independent relaxing drug, a NO donor) were generated.

In other series of experiments, aortic rings with endothelium were independently treated with T3 or vehicle as explained before; subsequently, CRCs to PHE (selective α_1-adrenergic receptors agonist) or angiotensin II (ANG II; non-selective agonist of ANG II type 1 and type 2 receptors) were developed.

Short-term *in vivo* T3 treatment

CRCs to relaxant agonists in aortic tissues from short-term *in vivo* T3-treated rats

In T3$_{1d}$ and V$_{1d}$ rats, CRCs to acetylcholine and SNP were obtained in endothelium-intact aortic rings pre-contracted with PHE (1 μM). Since the contractions induced by PHE were significantly diminished in aortic rings of T3$_{3d}$ rats (see Results), the CRCs to acetylcholine and SNP were tested at the same level of pre-contraction obtained by appropriately selected concentrations of PHE in endothelium-intact aortic tissues from T3$_{3d}$ and V$_{3d}$ rats.

Contractile stimuli in aortic tissues of short-term *in vivo* T3-treated rats

In annular segments with endothelium of thoracic aortas from T3$_{1d}$, T3$_{3d}$, V$_{1d}$, and V$_{3d}$ rats, CRCs to PHE were built. Since the CRCs to PHE were inhibited in endothelium-intact aortic rings of T3$_{3d}$ rats compared to their respective temporal controls (see Results), to inquire about the endothelium participation in this T3-elicited vasodepressor effect, PHE-induced CRCs were developed in endothelium-denuded aortic rings of T3$_{3d}$ and V$_{3d}$ rats. Furthermore, CRCs to PHE were constructed in aortic rings with endothelium of T3$_{3d}$ and V$_{3d}$ rats, 30 min after their incubation with the inhibitor of NOS, N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM). These studies indicated that the observed effect of T3$_{3d}$-treatment on PHE-induced contractions in aortic rings was endothelium-independent (see Results and Discussion); thus, the following experiments with ANG II and high K$^+$ were developed in endothelium-denuded aortic tissues.

In endothelium-denuded aortic rings from T3$_{3d}$ and V$_{3d}$ rats, CRCs to ANG II were constructed. Besides, high K$^+$ depolarizing solutions were used to circumvent G protein-coupled receptors (GPCR). Endothelium-denuded aortic rings of T3$_{3d}$ and V$_{3d}$ rats were constricted with high K$^+$ (40 and 80 mM) solutions, and contractions were stabilized for 30 min.

For all purposes, each aortic ring was subjected to only one of the described procedures.

Chemicals

3,5,3' triiodo-L-tironine, L-phenylephrine hydrochloride, acetylcholine chloride, sodium nitroprusside, angiotensin II, L-NAME (Sigma Chemical Company, St. Louis, MO, USA). T3 was dissolved as already explained. The other drugs were dissolved in distilled water, and subsequent dilutions were made using assay buffer.

Statistical analysis

Results are reported as means ± standard error of the means for the number of rats or samples (number) obtained from 4–8 different rats. Comparisons between two independent groups

were made using an unpaired Student t-test, and between multiple groups using one-way analysis of variance (ANOVA). Two-way ANOVA was used to compare two or more CRCs. Where one-way or two-way ANOVA showed significant differences, the results were analyzed further using the Tukey or Sidak *post-hoc* test, respectively (Prism version 6.0; Graph Pad Software, San Diego, CA, USA). In all comparisons, a value of p < 0.05 was considered statistically significant.

RESULTS

PHE-induced pre-contractions in aortic tissues are not altered by T3 *in vitro* application

In endothelium-intact aortic rings contracted with PHE (1 μM), increasing cumulative concentrations of T3 (1 nM–10 μM) did not cause modifications of contractile tone compared to temporal controls (Fig. 1).

Relaxant and contractile responses to selective agonists in aortic tissues stayed unchanged by *in vitro* T3-treatments

Acetylcholine and SNP in a concentration-dependent manner

produced relaxations of endothelium-intact aortic rings contracted with PHE. *In vitro* treatment with T3 (0.01, 0.1, and 1 μM) for 30 min or 2 h did not alter the CRCs to either acetylcholine or SNP compared to their respective controls receiving vehicle (Fig. 2).

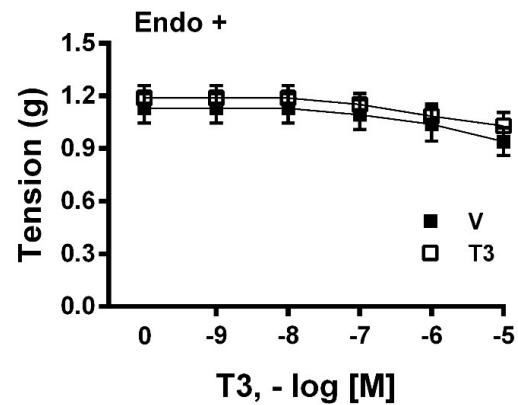Compared to temporal controls, CRCs elicited by either PHE

Fig. 1. Triiodothyronine (T3) applied *in vitro* did not influence previously established aortic active tonus. Cumulative administration of increasing concentrations of T3, every 30 min, in endothelium intact (Endo +) aortic rings contracted with phenylephrine (1 μM). Temporal controls received the corresponding concentration of vehicle (V). Values are presented as means ± standard error of the means (n = 16). p = not significant (two-way ANOVA).
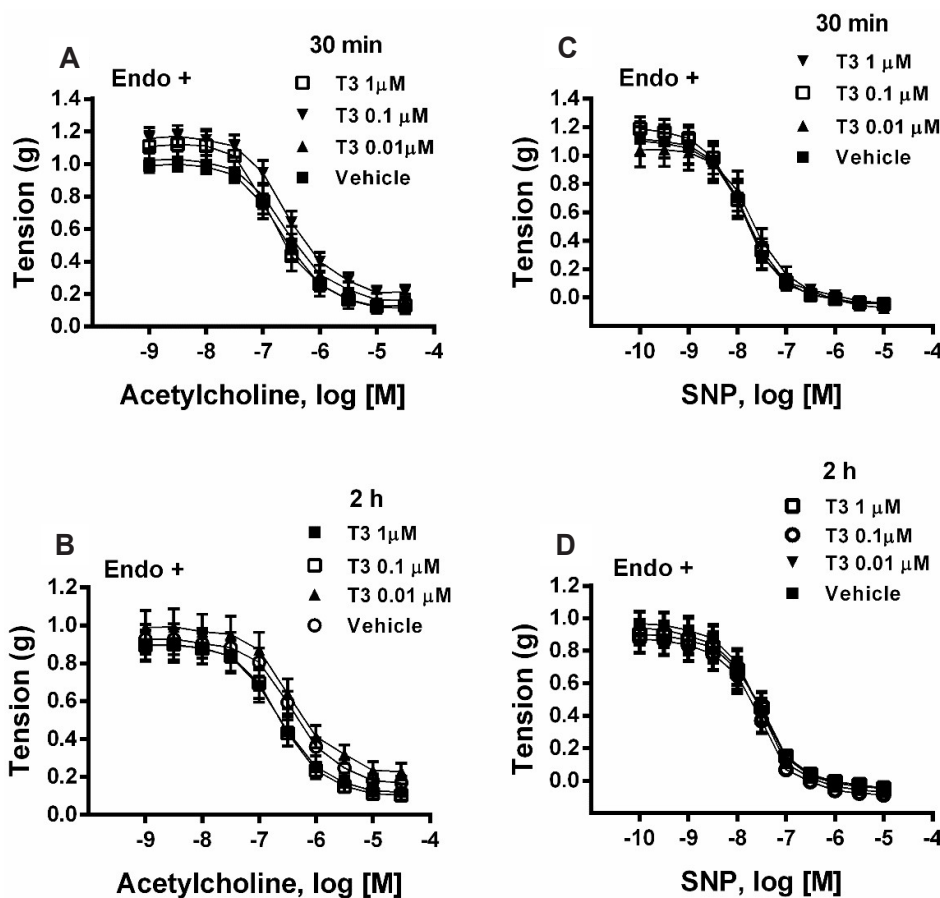
Fig. 2. Lack of effect of 3,5,3' triiodo-L-thyronine (T3) *in vitro* treatments on the relaxing responses of agonists in aortic tissues. (A, B) Relaxing responses to acetylcholine and (C, D) sodium nitroprusside (SNP) in endothelium-intact aortic rings (Endo +) contracted with phenylephrine (1 μM), treated previously with T3 (0.01, 0.1, and 1 μM) or vehicle (V) during 30 min or 2 h. Vehicle concentration corresponds to that required to dissolve T3 1 μM. Relaxing responses were calculated as decreases of phenylephrine-induced tension (grams). Values are presented as means ± standard error of the means (n = 15–16). p = not significant (two-way ANOVA).

or ANG II in aortic rings with endothelium were not modified in the presence of T3 (0.01, 0.1, and 1 μM) administered beforehand during 30 min or 2 h (Fig. 3).

Short-term *in vivo* T3-treatment efficiently induced hyperthyroidism in rats

Serum fT3 was increased and TSH decreased, both significantly, in $T3_{1d}$ and $T3_{3d}$ compared respectively with V_{1d} and V_{3d} rats (Table 1). These data indicate the hyperthyroid state of T3-treated rats. Serum concentrations of fT3 augmented in $T3_{3d}$ concerning $T3_{1d}$ rats (Table 1). Thus, a rise in intensity of the hyperthyroid state on $T3_{3d}$ rats is appreciated. However, TSH concentrations did not vary in a significant way between $T3_{1d}$ and $T3_{3d}$ rats (Table 1). The body mass of $T3_{3d}$ rats was significantly lesser than that of V_{3d} rats (Table 1). $T3_{1d}$ rats did not alter their body mass compared to V_{1d} rats. The stimulatory effects of THs on metabolic activity can explain the loss of body mass [18]. Contrasted with their respec-

tive time-based controls, $T3_{3d}$—but not $T3_{1d}$—treatment led to cardiac hypertrophy determined by an increase in heart dry mass to tibia length ratio (Table 1). Overall, these findings confirm the hyperthyroid status of animals submitted to treatment with T3 [1,3,18].

Relaxant responses to selective agonists remained unaltered in aortic tissues of short-term *in vivo* T3-treated rats

Acetylcholine caused concentration-dependent relaxant responses in endothelium-intact aortic rings from $T3_{1d}$ and V_{1d} rats, or $T3_{3d}$ and V_{3d} rats, which did not differ between them, respectively (Fig. 4). Likewise, the concentration-dependent relaxations induced by SNP in aortic rings with endothelium of $T3_{1d}$ and V_{1d} rats, or $T3_{3d}$ and V_{3d} rats, were in that order analogous (Fig. 4). When compared, the CRCs to acetylcholine and SNP were initiated under isotonic pre-contractions (Fig. 4).
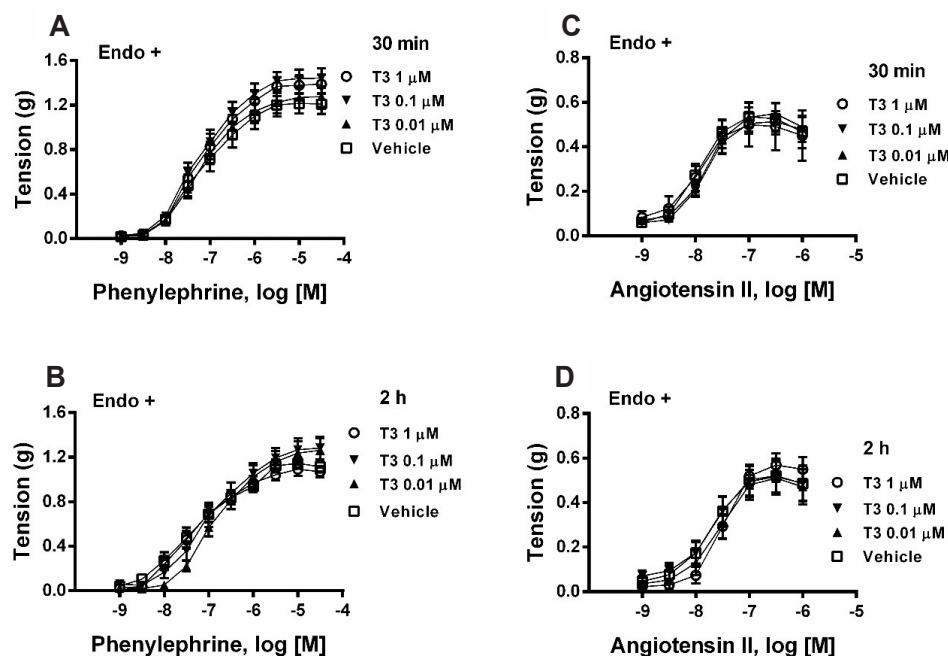

Fig. 3. Lack of effect of *in vitro* 3,5,3′ triiodo-L-thyronine (T3) treatments on the contractile responses to agonists in aortic segments. (A, B) Cumulative concentration-response curves to Phenylephrine and (C, D) Angiotensin II in annular segments of aortas with endothelium (Endo +) treated previously with T3 (0.01, 0.1, and 1 μM) or vehicle (V) for 30 min and 2 h. Vehicle concentration corresponded to that required to dissolve T3 1 μM. The contractile responses represent grams of developed tension. Values are presented as means ± standard error of the means (n = 16). p = not significant (two-way ANOVA).

Table 1. Validation of short-term hyperthyroidism

Treatment	fT3 (pg/ml)	TSH (ng/ml)	BM (g)	LVdM/LT (mg/mm)
V_{1d}	2.54 ± 0.18	1.79 ± 0.18	286.82 ± 3.52	4.16 ± 0.24
$T3_{1d}$	6.38 ± 0.49[a]	0.28 ± 0.07[a]	282.74 ± 3.57	4.69 ± 0.33
V_{3d}	2.11 ± 0.27	1.83 ± 0.11	292.45 ± 3.75	4.33 ± 0.30
$T3_{3d}$	9.91 ± 0.72[b,c]	0.18 ± 0.02[b]	275.56 ± 3.60[d]	5.66 ± 0.25[e]

Values are presented as means ± standard error of the means. Serum levels of free 3,5,3′ triiodo-L-thyronine (fT3) and thyroid stimulating hormone (TSH). Body mass (BM) values. Left ventricular dry mass to tibia length (LVdM/TL) ratio as an indicator of cardiac hypertrophy. Short-term hyperthyroidism in rats was induced by daily subcutaneous injection of T3 (500 μg·kg⁻¹) for 1 ($T3_{1d}$) and 3 ($T3_{3d}$) days. Control animals were injected every day with the vehicle (1 ml·kg⁻¹·d⁻¹) for 1 (V_{1d}) and 3 (V_{3d}) days. [a]p < 0.05 *vs.* V_{1d}, [b]p < 0.05 *vs.* V_{3d}, [c]p < 0.05 *vs.* $T3_{1d}$, n = 6 rats per group (one-way ANOVA with Tukey *post-hoc* test). [d]p < 0.05 *vs.* V_{3d}, n = 30 rats per group (unpaired Student t-test). [e]p < 0.05 *vs.* V_{3d}; n = 8 hearts per group (one-way ANOVA with Tukey *post-hoc* test).
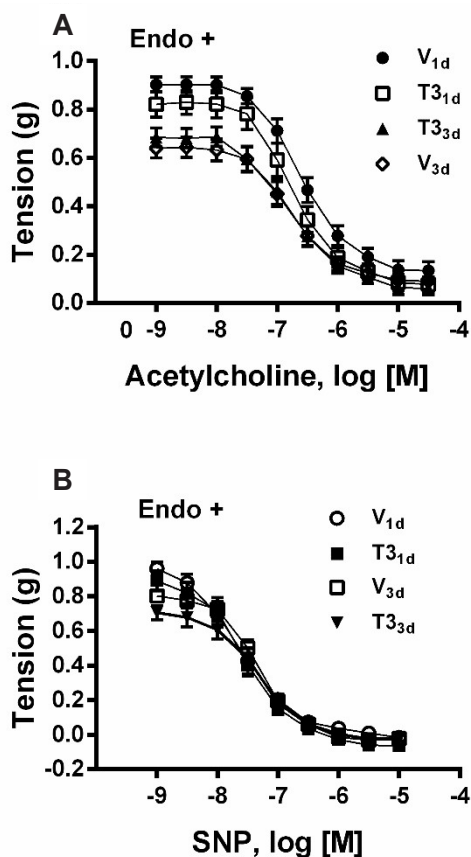
Fig. 4. Short-term *in vivo* 3,5,3' triiodo-L-thyronine (T3) treatment did not affect relaxant responses of agonists in aortic tissues. (A) Cumulative concentration-relaxing response curves to acetylcholine and (B) sodium nitroprusside (SNP) in endothelium-intact (Endo +) thoracic aortic rings isolated from rats treated subcutaneously with T3 (500 μg·kg⁻¹·d⁻¹) or vehicle (V; 1 ml·kg⁻¹·d⁻¹) for 1 and 3 days (T3₁d, T3₃d and V₁d, V₃d, respectively). Relaxing responses were calculated as decreases of phenylephrine-induced tension. Values are presented as means ± standard error of the means (n = 14–16). p = not significant (two-way ANOVA).
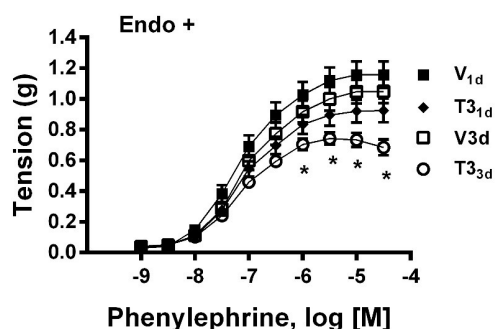

Fig. 5. Delayed depression of Phenylephrine-induced contractions in aortic tissues from short-term thyroid hormone-treated rats. Cumulative concentration-contractile response curves to phenylephrine, in endothelium-intact (Endo +) thoracic aortic rings isolated from rats treated subcutaneously with 3,5,3' triiodo-L-thyronine (T3; 500 μg·kg⁻¹·d⁻¹) or vehicle (V; 1 ml·kg⁻¹·d⁻¹) for 1 and 3 days (T3₁d, T3₃d and V₁d, V₃d, respectively). Contractile responses are expressed as grams (g) of developed force. Values are presented as means ± SEM (n = 16). *p < 0.05 T3₃d *vs.* V₃d (two-way ANOVA with Sidak's *post-hoc* test).
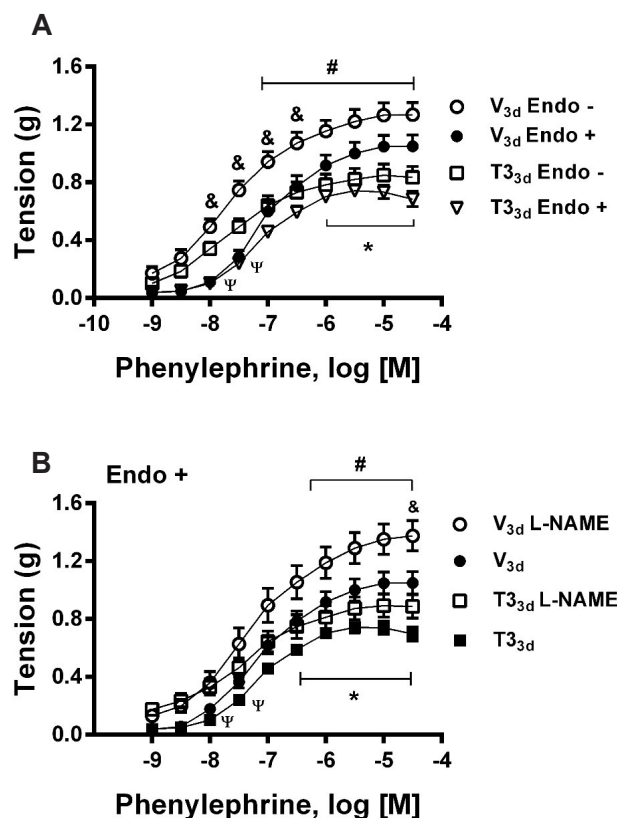

Fig. 6. Endothelium-independent inhibition of contractile responses to Phenylephrine in aortic tissues from 3,5,3' triiodo-L-thyronine (T3) *in vivo* treated rats. (A) Cumulative concentration-response curves (CRCs) to phenylephrine in annular segments of aortas with (Endo +) and without (Endo –) endothelium. (B) CRCs to phenylephrine in endothelium-intact (Endo +) thoracic aortic rings in the presence or absence of Nᴳ-Nitro-L-arginine methyl ester (L-NAME, 100 μM). Aortic tissues were obtained from rats treated with subcutaneous injections of T3 (500 μg·kg⁻¹·d⁻¹) or vehicle (1 ml·kg⁻¹·d⁻¹) for 3 days (T3₃d and V₃d, respectively). All data are expressed as grams (g) of developed force. Values are presented as means ± standard error of the means. (A) (n = 16) *p < 0.05 T3₃d (Endo +) *vs.* V₃d (Endo +); #p < 0.05 T3₃d (Endo –) *vs.* V₃d (Endo –); ᵠp < 0.05 T3₃d (Endo +) *vs.* T3₃d (Endo –); &p < 0.05 V₃d (Endo +) *vs.* V₃d (Endo –). Two-way ANOVA with Sidak's *post-hoc* test. (B) (n = 14–16) *p < 0.05 T3₃d *vs.* V₃d; #p < 0.05 T3₃d L-NAME *vs.* V₃d L-NAME; ᵠp < 0.05 T3₃d *vs.* T3₃d L-NAME; &p < 0.05 V₃d *vs.* V₃d L-NAME. Two-way ANOVA with Sidak's *post-hoc* test.

Depression of contractile responses to PHE, ANG II, and high K⁺ in aortic tissues from short-term *in vivo* T3-treated rats

When associated, endothelium-intact aortic rings of T3₁d, V₁d, and V₃d rats showed not statistically different CRCs to PHE (Fig. 5); nevertheless, the CRCs to PHE of T3₁d rats displayed a tendency toward a reduced maximal response (20%). PHE-induced CRCs in aortic segments with endothelium of T3₃d rats were significantly depressed (35%) in comparison to their respective V₃d controls (Figs. 5 and 6A). Furthermore, *in vivo* T3-treatment for 3 days,

decreased the CRCs to PHE (34%) in endothelium-denuded aortic rings compared to their corresponding V_{3d} controls (Fig. 6A). Equally, CRCs to PHE were observed statistically depressed (36%) in endothelium-intact, L-NAME-treated, aortas of $T3_{3d}$ rats (Fig. 6B).

Once the CRCs for PHE in aortic rings with and without endothelium from either $T3_{3d}$ or V_{3d} rats were respectively compared (Fig. 6A), the contractile activity in aortic rings without endothelium was detected higher than in tissues with endothelium in the two groups of rats. However, the contractile responses to PHE in endothelium-denuded aortas continued largely depressed (34%) in $T3_{3d}$ compared to V_{3d} rats (Fig. 6A). In the same way, treatment with L-NAME significantly increased the contractions caused by PHE in endothelium-intact aortic tissues of $T3_{3d}$ and V_{3d} rats (Fig. 6B); nevertheless, in the L-NAME presence, the CRCs to PHE in aortic rings from $T3_{3d}$ compared to V_{3d} rats, remained clearly inhibited (36%) (Fig. 6B). In this way, the data indicate that the endothelium and, particularly, NO seem not to participate in the depression of contractile responses to PHE in aortic rings of $T3_{3d}$ rats (see Discussion).

To investigate the selectivity of the depression of contractile responses to PHE in aortic rings from $T3_{3d}$ rats, experiments were carried out with ANG II (acting on a different family of receptors) and high K^+ depolarizing solutions (as a receptor-independent stimulus). In endothelium-denuded aortic rings, the CRCs to ANG II were significantly diminished (40%) after $T3_{3d}$-treatment concerning time-matched control tissues (Fig. 7A). Additionally, high K^+ (40 and 80 mM) contractile responses in endothelium-denuded aortic preparations of $T3_{3d}$ rats, were inhibited (35% and 32%, respectively) when compared with the corresponding responses in V_{3d} tissues (Fig. 7B).

DISCUSSION

Taken together, the data indicate that T3, administered *in vitro*, neither modified the active tone previously established by PHE nor the effects of both relaxant and contractile agonists in rat thoracic aortas with endothelium. However, short-term *in vivo* T3 administration significantly reduced contractile tone development in isolated thoracic aortas, with a latency greater than one day. $T3_{3d}$-treatment equally decreased the CRCs to PHE in endothelium-intact, endothelium-denuded, and endothelium-intact NOS inhibitor-treated, aortic rings. Moreover, $T3_{3d}$-treatment depressed the contractile responses of ANG II and high K^+ independently of the endothelial function and, in contrast, the agonist-induced endothelium-dependent and -independent relaxations were not modified in rat aortas. Hence, short-term thyrotoxicosis induced by T3 in rats lessened the ability of the aortic smooth muscle to contract, but not to relax, in an endothelium- and NO-independent way. The temporal course of occurrence of this depressant effect suggests that it is mediated by genomic or through non-genomic/genomic mechanisms. Otherwise, T3 does not
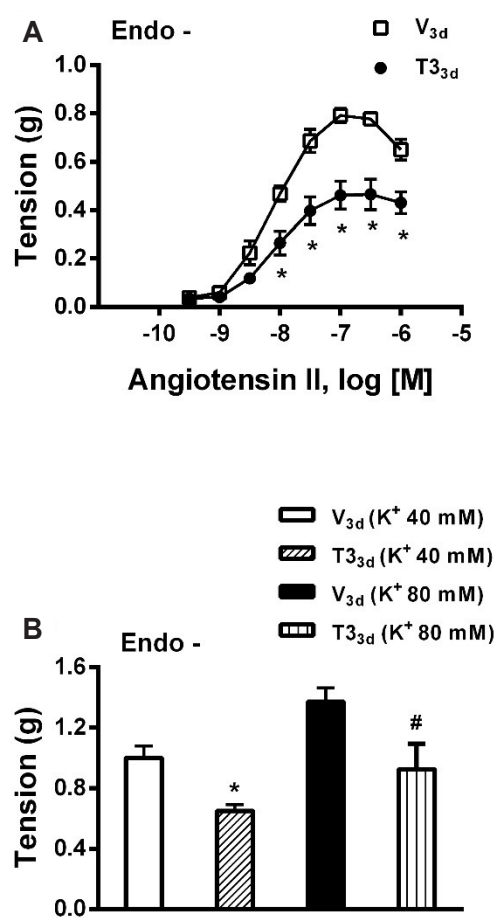

Fig. 7. Endothelium-independent inhibition of contractile responses to angiotensin II and high K^+ in aortic tissues from thyroid hormone-treated rats. (A) Cumulative concentration-response curves to angiotensin II in endothelium-denuded (Endo –) thoracic aortic rings. (B) Contractile responses to high K^+ (40 and 80 mM) in endothelium-denuded (Endo –) thoracic aortic rings. Aortic tissues were obtained from rats treated with subcutaneous injections of 3,5,3' triiodo-L-thyronine (500 $\mu g \cdot kg^{-1} \cdot d^{-1}$) or vehicle (1 $ml \cdot kg^{-1} \cdot d^{-1}$) for 3 days ($T3_{3d}$ and V_{3d}, respectively). All data are expressed as grams (g) of developed force. Values are presented as means ± standard error of the means. (A) (n = 15) *p < 0.05 *vs.* V_{3d} (two-way ANOVA with Sidak's *post-hoc* test). (B) (n = 13–15) *p < 0.05 *vs.* V_{3d} (K^+ 40 mM); #p < 0.05 *vs.* V_{3d} (K^+ 80 mM) (unpaired Student t-test).

modify aortic contractility by a rapid non-genomic mechanism, as shown by its lack of effect when applied acutely *in vitro*.

Influences of acute *in vitro* and short-term *in vivo* T3 treatments on the responses of isolated rat thoracic aortas to contractile and relaxant stimuli were studied comparatively. A fundamental consideration was that there have been described in small and large blood vessels, rapid (seconds to minutes) non-genomic effects of THs contrasting with their genomic or non-genomic/genomic effects of delayed presentation (from several hours on) [5]. The latest effects are initiated in TR located in the plasma membrane or cytoplasm, eventually, associated with gene transcription [5,6]. Admittedly, some studies have reported a direct vasodepres-

sor action of THs that may be interpreted as rapid non-genomic. Park *et al.* [9] showed that T3 and T4 cause rapid onset vasodilator responses (20 min) acting on both the endothelium and smooth muscle of isolated skeletal muscle resistance arteries of rats. Besides, Carrillo-Sepúlveda and colleagues [10] reported that in 30 min, T3 inhibited contractile responses to PHE in endothelium-denuded rat thoracic aortas by inducing smooth muscle synthesis of NO. In endothelium-intact rat thoracic aortas, however, we found that increasing cumulative concentrations of T3 (every 30 minutes up to 2 and a half hours) did not provoke alterations in contractile tone induced by PHE, as would be expected if T3 were inducing NO-dependent relaxation by a rapid non-genomic action in endothelial or smooth muscle cells. Additionally, *in vitro* T3 treatment with concentrations (0.01, 0.1, and 1 μM for 30 min or two hours) that include those reported as inducing vasodepressor effects by a rapid non-genomic mechanism [9,10] did not modify the relaxant responses produced by acetylcholine (whose vasodepressor effect depends on endothelium-derived NO) and SNP (which breaks down releasing NO) in endothelium-intact aortic rings contracted with PHE. In this way, synthesis and release of endothelial NO induced by agonist or the relaxant action of NO in the smooth muscle do not seem to be affected in aortas of rats treated acutely *in vitro* with T3. Moreover, contractions triggered by PHE and ANG II in aortic rings with endothelium remained unchanged in the presence of T3 (0.01, 0.1, and 1 μM) administered previously for 30 min and 2 h. In rat aorta, basal or induced NO release plays a key role in the endothelium-dependent depression of responses caused by contractile agonists [19-23]. Hence, the data showed that neither endothelium nor NO, particularly, depressed the contractile responses obtained with either PHE or ANG II in aortic rings treated acutely *in vitro* with T3, as would be expected if synthesis and release of NO had been increased in aortic walls.

On the other hand, it has been reported that endothelial NO-dependent vasodilation elicited by acetylcholine is increased, and contractile responses induced by norepinephrine are depressed in an endothelium-dependent manner, in aortic tissues of hyperthyroid rats by short-term T4 administration (500 $\mu g \cdot kg^{-1} \cdot d^{-1}$, 3 days) [11] and long-term T3 treatment (300 $\mu g \cdot kg^{-1} \cdot 2d^{-1}$, 6–12 weeks) [12]. Thus, both relaxing and contractile responses of rat aorta have been found modified by an increased vasodepressor function of the endothelium, caused by THs actions considered genomic [12,13]. Herein, it is important to point out that these studies, like most works with isolated vessels from hyperthyroid animals [8], were defined by a unique period of treatment; that is, the temporal evolution of functional changes in vascular responsiveness was not characterized. In our study, *in vivo* treatment with T3 (500 $\mu g \cdot kg^{-1} \cdot d^{-1}$) for 3 days, but not for 1 day, significantly decreased the contractile responses induced by PHE in aortic rings with and without endothelium, correlated with their respective controls from vehicle-treated rats. The absence of endothelium and L-NAME treatment (100 μM) in the presence of

endothelium, respectively, enhanced the contractile responses to PHE in aortic rings of T3$_{3d}$ and V$_{3d}$ rats. However, the contractile responses to PHE remained mostly depressed in the aortas without endothelium, and with endothelium treated with L-NAME, of T3$_{3d}$ rats when compared properly to those obtained from V$_{3d}$ rats. If depression of contractile responses to PHE in aortic tissues of T3$_{3d}$ rats were fundamentally endothelium-dependent and mediated by NO, endothelium denudation and inhibition of NO synthesis should result in a left shift (potentiation) of CRCs to PHE significantly greater in these tissues than in controls tissues; depression of contractile responses to PHE ought to be practically reverted to the values obtained in the rings without endothelium of control V$_{3d}$ animals. As mentioned, this was not the case.

An additional important finding was that contractile responses to ANG II and high K$^+$ in endothelium-denuded thoracic aortas under T3$_{3d}$-treatment were also attenuated. Accordingly, CRCs to PHE in endothelium-denuded aortic tissues of T3$_{3d}$ rats were not selectively depressed since CRCs to ANG II and contractile responses to high K$^+$ (a GPCR-independent stimulus), were significantly inhibited compared to their suitable controls of V$_{3d}$ rats. It is well known that PHE and ANG II, acting on different GPCR, mainly use the Gq/11-phospholipase C signaling pathway to stimulate vascular smooth muscle contraction via the release of sarcoplasmic reticulum stored Ca^{2+}, extracellular Ca^{2+} influx (through voltage-dependent, receptor-operated, and store-operated channels), and concomitant Ca^{2+} sensitization [24-28]. On the other hand, extracellular Ca^{2+} entry through voltage-dependent Ca^{2+} channels is considered the main cause of contractions induced by elevated K$^+$ in vascular smooth muscle [29]; nonetheless, high K$^+$ also causes Ca^{2+} sensitization [30]. So, given the complexity of the signal transduction pathways involved in contractile activity caused by PHE, ANG II, and high K$^+$, it is necessary to develop additional research to solve what actions common (or not) to these stimuli allow explaining the seemingly non-selective depression of contractile activity.

Deepening on the analysis of effects of short-term *in vivo* T3 treatments in endothelium and smooth muscle of rat aortas, we studied comparatively relaxant effects of acetylcholine and SNP on PHE pre-contracted aortic rings. Because PHE-induced contractions were significantly suppressed in aortic tissues of T3$_{3d}$ rats compared with their time-based controls, the relaxations of Ach and SNP were tested under isotonic conditions (obtained by fittingly selected concentrations of PHE). Results indicate that T3$_{1d}$ and T3$_{3d}$ treatments did not modify CRCs to acetylcholine and SNP in endothelium-intact thoracic aortas. Thus, data put forward that acetylcholine-induced endothelial NO release and smooth muscle sensitivity to NO released by SNP, were not modified in aortas of T3$_{1d}$ and T3$_{3d}$ rats compared to their corresponding temporal controls.

Absence of agreement of our data with other publications requires further investigation; nonetheless, distinct researchers, using diverse isolated blood vessels, have found that responses to

either contractile or relaxant agonists can be reduced, unchanged or enhanced in hyperthyroid animals, without expounding convincing explanations for these inconsistencies [8].

In summary, acute *in vitro* T3 administration did not alter smooth muscle contractility in rat thoracic aortas; however, short-term *in vivo* T3 administration depressed contractile activity independently of the endothelium, and NO either of endothelial or smooth muscle origin, in isolated thoracic aortas. The time course of onset of this depressant effect suggests that it is mediated by slow development genomic or non-genomic/genomic mechanisms and excludes the participation of rapid non-genomic mechanisms. It remains to elucidate the mechanism by which short-term *in vivo* treatment with thyroid hormone causes depression of contractile response in rat thoracic aorta.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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