Thyroid hormone stimulates NO production via activation of the PI3K/Akt pathway in vascular myocytes

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Received 6 February 2009; revised 26 August 2009; accepted 1 September 2009; online publish-ahead-of-print 4 September 2009

Time for primary review: 17 days

Aims
Thyroid hormone (TH) rapidly relaxes vascular smooth muscle cells (VSMCs). However, the mechanisms involved in this effect remain unclear. We hypothesize that TH-induced rapid vascular relaxation is mediated by VSMC-derived nitric oxide (NO) production and is associated with the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signalling pathway.

Methods and results
NO levels were determined using a NO-specific fluorescent dye (DAF-2) and nitrite (NO2) levels. Expression of NO synthase (NOS) isoforms and proteins of the PI3K/Akt pathway was determined by both western blotting and immunocytochemistry. Myosin light chain (MLC) phosphorylation levels were also investigated by western blotting. Exposure of cultured VSMCs from rat thoracic aortas to triiodothyronine (T3) resulted in a significant decrease of MLC phosphorylation levels. T3 also induced a rapid increase in Akt phosphorylation and increased NO production in a dose-dependent manner (0.001–1 μM). VSMCs stimulated with T3 for 30 min showed an increase in the expression of all three NOS isoforms and augmented NO production, effects that were prevented by inhibitors of PI3K. Vascular reactivity studies showed that vessels treated with T3 displayed a decreased response to phenylephrine, which was reversed by NOS inhibition. These data suggest that T3 treatment induces greater generation of NO both in aorta and VSMCs and that this phenomenon is endothelium independent. In addition, these findings show for the first time that the PI3K/Akt signalling pathway is involved in T3-induced NO production by VSMCs, which occurs with expressive participation of inducible and neuronal NOS.

Conclusion
Our data strongly indicate that T3 causes NO-dependent rapid relaxation of VSMC and that this effect is mediated by the PI3K/Akt signalling pathway.

Keywords
Nitric oxide • Relaxation • Vascular smooth muscle cell • Thyroid hormone • PI3K/Akt pathway • Non-genomic actions

1. Introduction
Thyroid hormone (TH) exerts profound effects on the heart and peripheral circulatory system.1 In particular, hyperthyroidism induces a high-output state, with a marked decrease in peripheral vascular resistance. However, the mechanisms by which TH affects the vasculature are poorly understood. Increased capillary density as well as elevated expression of vascular endothelial growth factor has been reported in hyperthyroid rats and humans.3–4 Data obtained in rat aortic rings suggest that TH exerts part of its* Corresponding author. Tel: +55 11 3091 8049, Fax: +55 11 3091 7366, Email: mchaves@usp.br
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vascular effects through an endothelium-mediated mechanism. Consistent with these findings, endothelium-dependent vasodilation has been reported to be enhanced in hyperthyroid humans and rats. However, TH can also induce endothelium-independent relaxation. In vitro data suggest that smooth muscle cells, rather than the endothelium, are the primary target of TH. Considering that phosphorylation of myosin light chain (MLC), a major regulator of smooth muscle contraction, can be modulated by a variety of vasoactive hormones, we determined the effect of triiodothyronine (T3) on MLC phosphorylation as an indirect measurement of vascular relaxation.

Recently, nitric oxide (NO) production by vascular smooth muscle cells (VSMCs) has been considered an important mechanism for overproduction of NO in the vascular wall and might be of importance for the local control of vascular function. In addition, increased vascular NO production has been reported in rats in hyperthyroid states or after treatment with T3 for 3–4 months. Although this response has been associated with augmented endothelium-dependent vasodilation, greater neuronal NO synthase (nNOS) and endothelial NOS (eNOS) expression was found in the VSMCs, suggesting that changes in NO production occur not only in the endothelial cells but also in other layers of the vascular wall, contributing to the augmented vasodilation. Accordingly, currently available data show that only nNOS and eNOS have been identified as targets for the actions of TH. Certainly, the modulation of nNOS and eNOS expression by TH involves several stimuli, such as TH concentration and chronic increased blood flow/shear stress. In addition to the well-described nuclear effects on the cardiovascular system, various effects of TH are the result of non-genomic mechanisms involving extranuclear sites of action and occur under conditions in which transcription and translation have been inhibited. Accordingly, in patients and in animal models, significant decreases in systemic and coronary vascular resistance have been reported within 30 min of T3 administration. In this context, it is unknown whether acute administration of TH also stimulates VSMC to generate NO. In addition, other hormones with potent vascular effects promote acute vasodilation by non-genomic mechanisms or more specifically by stimulating NO formation through phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) in VSMCs. Obviously, the cellular/molecular basis of TH signalling in the vasculature is highly complicated. However, understanding these mechanisms is an essential step to predict both the physiological and potential therapeutic effects of TH in patients with cardiovascular disease.

In the present study, we hypothesized that TH acutely stimulates relaxation of VSMCs by increasing production of NO via activation of the PI3K/Akt signalling system. The effects of T3 on NO production, NOS isoform expression and activation of the PI3K/Akt pathway in VSMCs were determined.

2. Methods

2.1 Animals

Adult, male Wistar rats weighing 200–250 g were housed in a temperature-controlled environment (22 ± 1°C; 60% humidity) and maintained on a 12/12 h light/dark cycle throughout the study period. All experimental procedures were performed in accordance with the guidelines for Ethical Principles in Animal Research set forth by the Brazilian College of Animal Experimentation. The study design was approved by the Ethics Committee for Animal Research of the Institute of Biomedical Sciences, University of São Paulo.

2.2 Cell isolation and culture

VSMCs were isolated from rat thoracic aortas by explant, as previously described. Cultures were maintained in Dulbecco Modified Eagle’s Medium (DMEM) (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY, USA). Only third-passage cells were used. Immunoreactivity assays were used to characterize the VSMCs and to test the absence of other cell types in the cultures. The cells expressed α-smooth muscle actin and calponin, contractile proteins that are indicative of VSMCs. After maximum confluence and 24 h removal of serum, cells were incubated with T3 (0.1 μM) or with T3 plus either wortmannin (100 nM) or LY294002 (LY, 2.5 μM), both of which are selective and chemically unrelated inhibitors widely used to study the involvement of PI3K/Akt in different cellular events.

2.3 Contractile state assay

Prior to their use in experiments to assess contractile state, cells were serum-starved for 24 h, after which they were incubated with 100 nM of angiotensin II (Ang II), which is known to induce contraction of VSMCs in the culture medium was measured as nitrite (NO2−) accumulation in the culture media as previously described. We analysed the chemiluminescence reaction between ozone and the NO generated by the reduction of the sample with vanadium chloride in acid at 95°C using an NO analyser (Model 208A; Sievers Instruments Inc., Boulder, CO, USA) according to the manufacturer protocols. NO2− levels were corrected for total protein content of VSMC extracts determined by the Bradford method. The rates of NO2− accumulation were expressed as micromole per gram of protein.

2.4 Measurement of nitrite levels in culture media

After the addition of 0.1 μM T3 for 30 min, release of NO from VSMCs in the culture medium was measured as nitrite (NO2−) accumulation in the culture media as previously described. We analysed the chemiluminescence reaction between ozone and the NO generated by the reduction of the sample with vanadium chloride in acid at 95°C using an NO analyser (Model 208A; Sievers Instruments Inc., Boulder, CO, USA) according to the manufacturer protocols. NO2− levels were corrected for total protein content of VSMC extracts determined by the Bradford method. The rates of NO2− accumulation were expressed as micromole per gram of protein.

2.5 NO production in VSMC

Production of NO was determined using the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2, Sigma, St Louis, MO, USA), as described previously. VSMCs were loaded with DAF-2 for 30 min at 37°C and then treated with T3 for 30 min. In some experiments, a PI3K/Akt inhibitor (wortmannin or LY) was added 30 min before loading cells with DAF-2. In addition, to try to address which type of NOS isoform could be participating of this effect, specific NOS inhibitors were also used: N-[3-(aminomethyl)benzyl] acetamide—1400 W (100 μM), an iNOS blocker, and (4S)-(−)-N-(4-amino-5-[aminoethyll]aminopentyl)-N′-nitroguanidine (AAAN) (10 μM), a nNOS blocker, for 30 min. Digital images were collected using a fluorescence microscope (Axioskop; Zeiss, Göttingen, Germany). The images were analysed using image software (KS-300 Software; Zeiss). The intensity of the fluorescence is proportional to the amount of NO present.
2.6 Immunocytochemistry

Immunocytochemistry was performed on fixed cells using a primary murine antibody directed against α-actin (Sigma) and calponin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in order to characterize the VSMC cultures. eNOS, iNOS, nNOS, Akt, and p-Akt (Santa Cruz Biotechnology) were also measured in VSMC cultures stimulated with T3 for 30 min. For subsequent detection, the cells were incubated with a Cy3-conjugated or FITC-conjugated IgG secondary antibody (Jackson ImmunoResearch, Hamburg, Germany). Nuclei were visualized with the fluorescent dye DAPI (Sigma). Negative controls were performed by omission of the primary antibodies. Samples were mounted on slides and coverslipped, after which the labelled cells were examined using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Images were obtained using the Q-FISH program (Leica).

2.7 Western blotting

Quiescent VSMCs were pretreated with PI3K inhibitors for 30 min before T3 stimulation. After 30 min of T3 administration, total VSMC lysate was subjected to western blotting as previously described. Equal protein loading was confirmed by staining with Ponceau-S dye. The primary antibodies used in these experiments were Akt, p-Akt, MLC, and p-MLC (Cell Signaling Technology, Beverly, MA, USA), eNOS, iNOS, and nNOS (BD Biosciences, San Jose, CA, USA). Membranes also were probed with a mouse monoclonal α-actinin specific antibody (Santa Cruz Biotechnology) as an internal control. Bound proteins were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Inc., Uppsala, Sweden).

2.8 Vascular functional study

After euthanasia, thoracic aortas were removed and cleaned from fat tissue in an ice-cold physiological salt solution, containing (mM): NaCl, 130; NaHCO3, 14.9; KCl, 4.7; KH2PO4, 1.18; MgSO4·7H2O, 1.18; CaCl2·2H2O, 1.56; EDTA, 0.026; glucose, 5.5. In all experiments, the endothelium was mechanically removed by gently rolling the lumen of the vessel on a thin metallic wire. We certify the absence of endothelium by pre-contracting the aorta ring preparations with phenylephrine (PE) for 10 min and after achievement of a stable contraction, the rings were stimulated with acetylcholine (ACh), an endothelium-dependent vasodilator. The absence of any relaxation in response to ACh is functional evidence of endothelium removal. Arterial segments were quickly cut into 4 mm rings and incubated in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and antibiotics, and incubated with vehicle (NaOH 0.1 M) or T3 0.1 μM for 1 h. After incubation, vessels were carefully mounted as rings preparations in standard organ chambers for isometric tension recordings by a PowerLab 8/SP data acquisition system (ADInstruments Pty Ltd., Castle Hill, Australia). Concentration-response curves to PE (0.1 nM to 10 μM) were performed in the presence and absence of N-nitro-L-arginine methyl ester (L-NAME; 100 μM).

![Figure 1](#) Expression of differentiation markers in cultured rat aortic VSMCs at passage 3. (A) Expression of α-actin gene (left blot) and protein (right blot). Bottom: VSMCs labelled for α-actin (red: Rhodamine), showing stress fibre organization. (B) Expression of calponin gene (left blot) and protein (right blot). Bottom: VSMCs labelled for calponin (green: FITC). (C) VSMCs showing negative immunoreactivity for von Willebrand factor VIII (1) and CD31 (PECAM-1) (3) (magnification ×200), excluding endothelial cell contamination. Rat aorta endothelial cells showing immunostaining positive for anti-von Willebrand factor VIII (red: Rhodamine) (2) and for CD31 (PECAM-1) (red: Rhodamine) (4). Nuclei stained in blue (DAPI) or in green (Sytox S7020-Invitrogen). Representative results from three-independent experiments are shown. All immunofluorescence micrographs were imaged by fluorescence microscopy (LSM510: Carl Zeiss).
2.9 Statistical analysis

All results are expressed as mean ± SD. Contractions were recorded as changes in the displacement (mN) from baseline. Concentration–response curves were fitted using a non-linear interactive fitting program (Graph Pad Prism 4.0; GraphPad Software Inc.), and two pharmacological parameters were obtained: the maximal effect generated by the agonist (or $E_{\text{max}}$) and $-\log EC_{50}$ (or $pD_2$). Statistical analyses were performed using one-way ANOVA or Student t-test. Post hoc comparisons were performed using Newman–Keuls test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1 Contractile proteins in VSMC cultures

Two to three days after plating, we observed an outgrowth of spindle-shaped cells, originating from the outer surface of the rat thoracic aorta explants. Confluence was reached within 2 weeks, and post-confluent cells exhibited the ‘hill and valley’ growth pattern typical of cultured VSMCs. Since expression of contractile proteins is an essential parameter for characterizing VSMC phenotype, expression of $\alpha$-smooth muscle actin and smooth muscle calponin was determined (Figure 1A and B). In addition, VSMCs presented negative immunoreactivity for von Willebrand factor and CD31 (Platelet Endothelial Cell Adhesion Molecule-1), which are specific proteins of endothelial cells, thereby excluding contamination by them (Figure 1C).

3.2 Acute T3 effect on state of MLC phosphorylation

Although reduction of systemic vascular resistance (SVR) by T3 has been attributed to chronic action of TH, acute effects of T3 in the vascular tone have also been described. Under certain circumstances, smooth muscle contraction has been shown to depend on MLC phosphorylation. The Ser-19 residue of MLC has been shown to be the primary residue phosphorylated by MLC kinase, with Thr-18 as a secondary site. VSMCs were pre-incubated with 100 nM Ang II for 10 min, which resulted in a 3.1-fold increase (over basal) in MCL phosphorylation. Treatment with T3 for 10, 20, 30, and 60 min reduced levels of phosphorylated MLC down to 20, 35, 52, and 65% of the levels observed after incubation with Ang II only (Figure 2). Cells incubated only with Ang II during 60 min maintained MLC phosphorylation levels, discarding any inhibitory effect of Ang II by itself and showing that the effect observed was due to T3 treatment.

3.3 T3 acutely stimulates NO production

To test whether T3 is able to induce NO production in cultured VSMCs, levels of nitrite ($NO_2^-$), the stable metabolite of NO, were determined in supernatants of cultured VSMCs treated with 0.1 $\mu$M of T3 for 30 min. Supernatants of untreated VSMCs were used as controls. As shown in Figure 3, T3 increased $NO_2^-$ content in VSMCs ($1.00 ± 0.09$ vs. $0.54 ± 0.016$, in T3 and vehicle-treated VSMCs, respectively). NO production was also determined by the NO-specific fluorescent dye (DAF-2). Under control (unstimulated) conditions, we detected a small amount of specific fluorescence, shown by the positive and negative controls (Figure 4A). Treatment of VSMCs with T3 for 30 min produced a significant increase in fluorescence at all concentrations.
3.4 Role of the PI3K/Akt pathway in T3-induced NO production

Since it has been demonstrated that the PI3K/Akt pathway plays a relevant physiological role in NO production by VSMCs submitted to various stimuli, we determined Akt phosphorylation in Ser$^{473}$ and Thr$^{308}$ residues, since both are crucial for the activity of the enzyme.$^{33}$ Treatment with T3-increased Akt phosphorylation in Ser$^{473}$ after 10–30 min, whereas phosphorylation in Thr$^{308}$ occurred after 20–30 min (Figure 5A). In addition, activation of Akt in VSMCs was also assessed by fluorescence microscopy. As shown in Figure 5B, after 30 min of stimulation with T3, total Akt was translocated to the plasma membrane, a step that is absolutely crucial for Akt activation, whereas phosphorylated Akt Ser$^{473}$ migrated from the cytoplasm to the nuclei. We also determined whether PI3K/Akt signalling is involved in NO production by VSMCs. As shown in Figure 6, the stimulatory effect of T3 on NO production was greatly attenuated by treating VSMCs with wortmannin (100 nM) or LY (2.5 μM), selective and chemically unrelated inhibitors of PI3K/Akt pathways.$^{23}$ These findings suggest that T3-induced NO production by VSMCs involves stimulation of the PI3K/Akt signalling cascade.

Figure 4 T3-induced NO production in VSMCs. Cells were serum-starved and loaded with DAF-2 followed by sequential treatment with graded concentrations of T3. (A) Photomicrographs showing changes in green fluorescence images indicative of NO production. C, control; SNP, sodium nitroprusside. (B) Relative changes in fluorescence intensity were quantified. Data are expressed as mean ± SD ($n = 6$). *$P < 0.05$ vs. control.
Figure 5 Akt activation by T3 in VSMCs. (A) Cells were serum-starved and then stimulated with 0.1 μM T3 for 5, 10, 20, 30, and 60 min. Akt phosphorylation levels in two residues (serine and threonine) are represented as mean ± SD. (n = 6); *p < 0.05 vs. control group. (B) Immuno-fluorescence images indicating expression of total Akt (above) and phosphorylated Akt Ser-473 (below) in VSMCs. Left: images of control VSMCs (non-stimulated). Right: images of VSMCs after stimulation with T3 for 30 min. Images are representative of five individual experiments.
3.5 Participation of NOS isoforms in T3-induced NO production

To investigate the potential mechanism by which T3 increases NO production, expression of eNOS, nNOS, and iNOS was determined by immunofluorescence. Under controlled conditions, we detected basal fluorescence to nNOS and eNOS in the VSMC cytoplasm, whereas iNOS was absent (Figure 7A). After stimulation of VSMCs with T3, all isoforms were detected in the cytoplasm (Figure 7A). To supplement these findings, we also determine the effects of T3 on expression of NOS isoforms using western blotting. T3 induced a significant increase in the expression of eNOS, nNOS, and iNOS in the VSMCs. T3-induced expression of NOS isoforms was partially blocked by LY and wortmannin, indicating that this effect is mediated by PI3K/Akt signalling (Figure 7B).

Since the expression of all NOS isoforms was increased after stimulation with T3, we evaluated the contribution of each isoform in the NO production by using specific inhibitors. The increase of NO production induced by T3 was completely abolished in cells pretreated with iNOS inhibitor (1400 W—100 μM) or with nNOS inhibitor (AAAN—10 μM). T3-induced NO production was completely abolished in cells pretreated with 1400 W plus AAAN, suggesting that the eNOS is not involved in this process (Figure 7C).

3.6 T3 decreases Ang II-induced MLC phosphorylation by stimulating NO production

The effect of T3 on Ang II-induced phosphorylation of MLC was determined in the presence of the NOS inhibitor, L-NAME. This inhibitor partially abolished the inhibitory effect of T3 on Ang II-mediated MLC phosphorylation (Figure 8). These findings underscore a role for NO as a mediator of T3-induced endothelium-independent relaxation.

3.7 T3 modulates PE-induced contraction in endothelium-denuded aortic rings by NO generation

To further confirm our hypothesis that T3 induces rapid relaxation of VSMC via NO production, concentration–effect curves to PE were performed in endothelium-denuded aortic rings that were either pre-incubated in T3 or in vehicle. PE produced concentration-dependent contraction in all tested aortic rings. As shown in Figure 9 aortas incubated with T3 displayed decreased contraction to PE compared with control aortas (E_max 26.30 ± 0.61 vs. 17.35 ± 0.54 (mN), P < 0.05, respectively). L-NAME treatment slightly increased PE-induced contractile responses in vehicle-treated aortas (28.80 ± 0.48 mN), but produced a greater displacement in PE responses in T3-treated aortas (22.43 ± 0.73 mN) (Table 1). No differences were observed in the pD2 among the groups. All together, the results suggest that T3 treatment induces greater generation of NO and this phenomenon is endothelium-independent. These data support the results obtained in VSMC cultures, where increased production of NO was observed after T3 treatment.

4. Discussion

TH causes a decrease in SVR and, in contrast, SVR is elevated in TH deficiency or hypothyroidism, a condition that can be rapidly
reversed after TH replacement. However, the precise mechanism by which TH regulates vascular tone and SVR is poorly understood. Although there is evidence that T3 exerts a direct vasodilator effect through different endothelial mechanisms, some studies suggest that the smooth muscle layer also constitutes an important target for the vasorelaxant effects of T3. Therefore, the present study was designed to further explore the complex mechanisms by which T3 directly relaxes VSMCs. We used primary cultures of third-passage rat aortic VSMCs as experimental model. Under these conditions, the contractile phenotype is preserved, which is consistent with the findings of a previous study that showed phenotypic changes in the culture of VSMCs submitted to multiple passages.

Given that the primary mechanisms for the contraction of smooth muscle are the phosphorylation of MLC by MLC kinase and the inhibition of MLC phosphatase and that Ang II is known as a potent agent that promotes phosphorylation of MLC, we tested the effect of T3 on the Ang II-induced MLC phosphorylation. Our results revealed that T3 significantly decreased the Ang II-induced MLC phosphorylation, showing an important role of T3 as a regulator of constriction mechanism in VSMCs.

Figure 7  T3-induced NO production by nNOS and iNOS. (A) Immunofluorescence images indicating expression of NOS isoforms in unstimulated VSMCs (above) and in VSMCs stimulated with T3 (below). eNOS-positive immunoreaction (red), nNOS-positive immunoreactions (red), iNOS-positive immunoreaction (green), and nuclei (blue-DAPI). Images are representative of five individual experiments. (B) Some cells were pretreated with wortmannin (W; 100 nM) and then stimulated with T3 (0.1 μM) for 30 min. C+ (positive control). (C) Cells were pretreated with 1400 W (100 μM) or AAAN (10 μM) or 1400 W plus AAAN for 30 min and then stimulated with T3 (0.1 μM) for 30 min. Left panel: changes in green fluorescence images are indicative of NO production. Right panel: relative changes in fluorescence intensity were quantified. Data are expressed as mean ± SD (n = 6). *P < 0.05 vs. control; #P < 0.05 vs. T3.
The vasoconstrictive action of Ang II is based on its ability to elevate the calcium in the smooth muscle layer. However, NO has been found to reduce Ang II-induced vasoconstriction in VSMCs by activating calcium clearing mechanisms, such as the sarcoplasmic Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, as well as by reducing intracellular calcium transients. Our data also provide evidence that T3 reduces Ang II-induced phosphorylation of MLC through NO-dependent mechanisms. In this sense, although previous reports have not indicated the presence of eNOS or nNOS in VSMCs, data from the present study show the expression of these proteins in the cytoplasm of VSMCs under basal conditions.

In addition, our data showed that T3 significantly increased the expression of three NOS isoforms in these cells, but only nNOS and iNOS play a significant role on NO production by VSMCs. In fact, this is the first study showing a direct effect of TH in iNOS, since currently available data show that only nNOS and eNOS were previously identified as targets for the actions of TH. Furthermore, some studies have evidenced that in VSMCs the NO is mainly produced by iNOS, a Ca\textsuperscript{2+}-independent enzyme that is regulated at transcriptional and post-transcriptional levels. Considering that the amount of NO produced by iNOS is 1000 times greater than that produced by eNOS and nNOS, it is conceivable that iNOS has a greater contribution than nNOS for T3-induced NO production in VSMCs.

Regarding to the signalling pathway responsible to deflagrate this process, we demonstrated that PI3K/Akt inhibitors markedly reversed T3-induced increase in NO isoforms, suggesting that the PI3K/Akt pathway is involved in T3-induced NO production. Akt phosphorylation has been shown to induce concomitant activation of the transcription factor nuclear factor-κB, a pivotal regulator of iNOS expression. In a previous elegant study by Hiroi et al., the authors have already demonstrated that the activation of PI3K/Akt pathways can mediate in endothelial cells some acute effects of TH. In that work, in contrast to observed for us in VSMCs, the authors showed that T3 rapidly activates eNOS and that this effect is not dependent of TH responsive elements (TREs).

The classic genomic action of T3 occurs through its binding to nuclear thyroid hormone receptor (TR), which binds to TREs in the promoter regions of target genes, thereby activating or repressing their transcription. Recently, mRNAs for TR isoforms, TR-alpha and TR-beta, were identified in human aortic and

### Table 1

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<th>$E_{\text{max}}$</th>
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<tr>
<td>Control</td>
<td>26.30 ± 0.61</td>
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<tr>
<td>Control + L-NAME</td>
<td>28.80 ± 0.48*</td>
<td>8.20 ± 0.05</td>
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<tr>
<td>T3</td>
<td>17.35 ± 0.54*</td>
<td>8.01 ± 0.10</td>
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<tr>
<td>T3 + L-NAME</td>
<td>22.43 ± 0.73#</td>
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Values are mean ± SEM for $n = 4$ experiments in each group. $E_{\text{max}}$ values are represented as mN and $pD_2$ values are $-\log EC_{50}$ (concentration that produces 50% of the maximum response).

* $p < 0.05$ vs. control.

# $p < 0.05$ vs. T3.
coronary smooth muscle cells,43 suggesting that a direct genomic action of TH may play a significant role in vascular smooth muscle. In addition, the binding of T3 to TR-alpha located in endothelial cells can activate PI3/Akt pathways that contribute to some of the acute vasodilatory effects of TH.35

On the other hand, some recent studies have showed that rapid or non-genomic actions of TH are involved to the binding of the hormone to alphaVbeta3 integrin present in plasma membrane or to receptors located in the cytosol.17 These non-genomic or TRE-independent actions of TH have been recently recognized at the molecular level and the ubiquitous distribution of TRs suggests that this non-genomic action, which is mediated by different signaling cascades, may modulate diverse cellular functions cooperatively with genomic actions.

Herein we found accumulating evidence of important non-genomic action of T3 in which the hormone rapidly induces NO synthesis in VSMCs through activation of the PI3K/Akt signaling pathway, which has been shown to mediate the more rapid effects of T3.44–46 We can conclude that PI3K is critically important to promoting VSMCs through activation of the PI3K/Akt signaling pathway, which action of T3 in which the hormone rapidly induces NO synthesis in VSMCs. A previous study demonstrated that hyperthyroidism reduces both vasoconstriction and blood pressure elevation induced by catecholamines, which also involves NO generation.47 All together, our data suggest a potential mechanism that partially explains those results: T3 treatment induces increased production of NO, which is associated with activation of the PI3K/Akt signaling pathway, increased NOS isoforms, resulting in a significant decrease of MLC phosphorylation levels and decreased vascular response to contractile stimuli.

In summary, T3 acutely stimulates endothelium-independent vascular relaxation. This vasoilator effect is mediated by increased production of NO, which occurs rapidly by the activation of iNOS and nNOS and this mechanism is involved to the PI3K/Akt signaling pathway.

Conflict of interest: none declared.

Funding
This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Foundation for the Support of Research in the State of São Paulo) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, National Council for Scientific and Technological Development).

References
30. Diniz GP, Carneiro-Ramos MS, Barreto-Chaves ML. Angiotensin type 1 (AT1) and type 2 (AT2) receptors mediate the increase in TGF-beta1 in thyroid hormone-induced cardiac hypertrophy. J Cell Sci 2007; 120:856–862.