Thyroid hormone affects both endothelial and vascular smooth muscle cells in rat arteries

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Abstract

Hypothyroidism impairs endothelium-dependent dilatations, while hyperthyroidism augments the production of endothelial nitric oxide. Thus, experiments were designed to determine if thyroid hormone causes endothelium-dependent responses, or alleviates diabetic endothelial dysfunction. Isometric tension was measured in rings with or without endothelium of arteries from normal and diabetic Sprague-Dawley rats. Release of 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_2 were measured by enzyme linked immunosorbent assay and protein levels [endothelial nitric oxide synthase (eNOS), cyclooxygenases (COX)] by immunoblotting. Triiodothyronine (T_3) caused concentration-dependent ($3x10^{-6}$ to $3x10^{-5}$M) relaxations in mesenteric (pEC_{50}, 4.96±0.19) and femoral (pEC_{50}, 4.57±0.35) arteries without endothelium. In femoral arteries of rats with diabetes, 5-methylamino-2-(2*S*,3*R*,5*R*,8*S*,9*S*)-3,5,9-trimethyl-2-(1-oxo-(1*H*-pyrrol-2-

-yl)propan-2-yl)-1,7-dioxaspiro-(5,5)undecan-8-yl)methyl)benzooxazole-4-carboxylic acid (A23187, $3x10^{-7}$ to 10^{-6}M) caused partly endothelium-dependent contractions. After chronic T_3-treatment with (10µg/kg/day; four weeks), the contractions to A23187 of preparations with and without endothelium were comparable, the thromboxane B_2-release was reduced (by 38.1±9.2%). The pEC_{50} of 9, 11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619, TP-receptor agonist) was increased in T_3-treated diabetic rats compared with controls (8.53±0.06 vs 7.94±0.09). The protein expression of eNOS increased (by 228%) but that of COX-1 decreased (by 35%) after chronic T_3 treatment. In human umbilical vein endothelial cells incubated for one week with T_3 (10^{-10} to 10^{-7}M) in the presence but not in the absence of interleukin-1β (1ng/ml), the expression of eNOS was increased compared to control. In conclusion, thyroid hormone acutely relaxes

mesenteric and femoral vascular smooth muscle, but given chronically reduces the release of endothelium-derived vasoconstrictor prostanoids while enhancing the responsiveness of TP receptors of vascular smooth muscle.

Key words

COX-1; endothelium-derived contracting factor(s); eNOS; type 1 diabetes; TP receptors

Chemical compounds studied in this article

3,5,3'-tri-iodothyronine (PubChem CID: 5920); A23187 (PubChem CID: 40486); acetylcholine (PubChem CID: 6060); indomethacin (PubChem CID: 3715); L-NAME (PubChem CID: 39386); phenylephrine (PubChem CID: 6041); U46619 (PubChem CID: 5311493)

Abbreviations

A23187,

5-methylamino-2-(2*S*,3*R*,5*R*,8*S*,9*S*)-3,5,9-trimethyl-2-(1-oxo-(1*H*-pyrrol-2-yl)propan-2-yl)-1,7-dioxa spiro-(5,5)undecan-8-yl)methyl)benzooxazole-4-carboxylic acid; COX, cyclooxygenase; EC, endothelial cell; EDCFs, endothelium-derived contracting factors; eNOS, endothelial nitric oxide synthase; HUVECs, human umbilical vein endothelial cells; L-NAME, Nω-nitro-L-arginine methyl ester; NO, nitric oxide; S18886, 3-[(6-amino-(4-chlorobenzensulphonyl)-2-methyl-5,6,7,8-tetrahydronapht]-1-yl)propionic acid; SD, Sprague-Dawley; SHR, spontaneously hypertensive rat; T_3 , 3, 5, 3'-tri-iodothyronine; TP receptor, thromboxane prostanoid receptor; U46619, 9, 11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$

1. Introduction

The endothelium, a thin layer of cells lining the interior surface of blood vessels, can control local vascular tone. It does so by releasing endothelium-derived relaxing factors **(Furchgott and Vanhoutte, 1989; Furchgott and Zawadzki, 1980)**, including nitric oxide (NO) and/or several other endothelium-derived hyperpolarizing substances **(Félétou and Vanhoutte, 2007, 2006; Vanhoutte 2004)**. In addition, in particular in arteries of obese, diabetic or hypertensive animals, the release of endothelium-derived contracting factors [EDCFs], causing activation of the underlying smooth muscle cells **(Tang and Vanhoutte, 2010; Vanhoutte, 2011; Wong and Vanhoutte, 2010c)**, contributes to endothelium-dependent changes in vascular diameter.

Several hormones can trigger endothelium-dependent responses. These include catecholamines acting on endothelial α2-adrenergic receptors **(Vanhoutte and Miller, 1989)**, vasopressin and oxytocin activating V1-vasopressinergic receptors **(Katusic et al., 1986, 1984)**, and insulin **(Liu et al., 2012)**. Hormones, in particular estrogens, also chronically modulate endothelium-dependent responses **(Chambliss and Shaul, 2002; Gisclard et al., 1988)**.

Hypothyroidism causes impaired endothelium-dependent dilatations **(Taddei et al., 2003)**. Furthermore, diabetic patients have a higher prevalence of thyroid disorders compared to the normal population **(Hage et al., 2011)**. Thyroid hormone is synthesized and stored in the follicular and the colloid cells of the thyroid gland and plays a role in differentiation, growth, and metabolism **(Yen, 2001)**. The hormone also affects the cardiovascular system. Thus, hyperthyroidism results in increased heart rate and atrial fibrillation, while hypothyroidism causes

opposite changes **(Ichiki, 2010)**. Although thyroid hormone can cause vasodilatation **(Carrillo-Sepúlveda et al., 2010; Ishikawa et al., 1989)**, its acute effect on endothelial cells is controversial, since both an absence of effect of 3, 5, 3'-tri-iodothyronine (T_3) on NO production **(Ojamaa et al., 1996)** and activation of endothelial NO synthase through the PI3K/Akt pathway **(Hiroi et al., 2006)** have been reported. Therefore, the present experiments were designed to determine in isolated arteries of normal rats whether or not the acute exposure to thyroid hormone causes or affects endothelium-dependent responses. Furthermore, clinical studies suggest impaired endothelium-dependent dilatations in hypothyroid patients **(Lekakis et al., 1997)** while hyperthyroidism causes excessive endothelial NO production **(Napoli et al., 2001)**. Such an increased production of NO should reduce the occurrence of endothelium-dependent contractions **(Tang et al., 2005a)**. Thus, the present study also investigated whether or not chronic treatment with thyroid hormone can alleviate the exaggerated EDCF-mediated responses that characterize the endothelial dysfunction resulting from type 1 diabetes **(Shi et al., 2007a)**.

2. Materials and Methods

All experimental protocols were approved by The University of Hong Kong Committee on the Use of Live Animals for Teaching and Research.

2.1. Experimental animals

The experiments were performed on isolated arteries of male Sprague Dawley (SD) rats. The rats used to test the direct effect of thyroid hormone were eight weeks old (250-350g). To test the effect of the hormone on endothelial dysfunction, twelve weeks old rats (450-600g) were made

diabetic by the intraperitoneal administration of streptozotocin [30 mg/kg; dissolved in citric acid-trisodium citrate (0.2mM) buffer (pH 4.0-4.5)] given once per day for three consecutive days **(Shi et al., 2006)**. Seventy-two h after the last injection, tail blood samples were obtained, and the glucose concentration was measured using a one-touch glucometer (LifeScan Inc., Milpitas, CA, USA). Induction of diabetes was considered successful when the fasting glucose level was higher than 16.6mM. The diabetic rats were divided randomly into two groups, one receiving vehicle alone and the other a daily intraperitoneal injection of T_3 (10μg/kg/day). T_3 was selected over thyroxine, because it mediates the effects of the latter *in vivo* and is more widely used in experimental animals **(Carrillo-Sepúlveda et al., 2010; Dillmann, 1982; Hiroi et al., 2006; Szkudelski et al., 2003)**. Doses of 3μg/kg (considered physiological) and 30 or 50μg/kg (considered pharmacological) of T_3 have been administrated daily to diabetic rats by others investigators **(Dillmann, 1982; Szkudelski et al., 2003)**. Since 3μg/kg has no obvious metabolic effect, preliminary experiments were performed using 10μg/kg, 50μg/kg and 100μg/kg of T_3, however, the latter two doses were severely toxic in diabetic rats, prompting the use of 10μg/kg/day for further studies.

The rats were housed in the laboratory animal unit of The University of Hong Kong, and fed with normal chow. Water was provided *ad libitum*. The diabetic rats were studied four weeks after the last streptozotocin injection. On the day of the experiment, the non-fasting glucose level was measured again. After three h fasting, the rats were anesthetized with sodium pentobarbitone (70mg/kg, intraperitoneally) and euthanized. Blood samples were collected from the inferior vena cava for measuring the T_3 level in serum.

2.2. Tissue preparation

The thoracic aorta, mesenteric arteries or femoral arteries were dissected free and placed in ice-cold modified Krebs-Ringer solution of the following composition (mM): NaCl 120, KCL 4.76, $CaCl_2$ 2.5, $MgSO_4$ 1.18, $NaHCO_3$ 25.0, NaH_2PO_4 1.18 and calcium disodium ethylenediaminetetraacetic acid 0.026, glucose 5.5 (control solution). The blood vessels were cut into rings (3-4mm length in aorta, 1.5-2mm length in mesenteric and femoral arteries). In some preparations, the endothelial cell layer was removed by the injection of 1ml of Triton (0.5%, diluted in control solution) in the artery prior to cutting it into rings.

2.3. Isometric tension measurement

The preparations were suspended in organ chambers, which contained warmed (37°C), aerated (95% O_2, 5% CO_2) control solution (5ml). They were connected to a force transducer and a bio-signal acquisition system (PowerLab, ADInstruments, Sydney, Australia) to record isometric tension. The rings were stretched to an optimal tension (2.5g in aorta, 2g in mesenteric and 1g in femoral arteries; determined in preliminary experiments; data not shown) and allowed to equilibrate for 90 min. They were then exposed twice to 60mM KCL to obtain a reference contraction.

To study the acute vascular effects of T_3 in normal aorta, mesenteric or femoral arteries, U46619 (3×10^{-8}M) was added to the chamber and when a stable contraction level had been reached, cumulative concentrations (10^{-7} to 3×10^{-5}M) of T_3 were administered. In some experiments, the rings were first incubated with pharmacological inhibitors **(Table 1)**. The concentrations of the inhibitors tested were selected from earlier work in the laboratory or from the literature **(Table**

1).

To examine whether or not the hormone has indirect effects, 10^{-7}M T_3 (a concentration that has no direct effect) was added to the organ chamber; after an incubation period of 30 min, cumulative concentrations of phenylephrine (10^{-9} to 10^{-5}M) were given to quiescent rings or cumulative concentrations of acetylcholine (10^{-9} to 10^{-6}M) were added during contractions to phenylephrine (10^{-6}M).

To study the effect of chronic treatment with thyroid hormone on endothelium-dependent relaxations, cumulative concentrations of acetylcholine (10^{-9} to 10^{-6}M) were added during contractions to phenylephrine (10^{-6}M) to rings of the aorta and mesenteric arteries from both T_3 and vehicle treated diabetic rats.

To study the acute effect of thyroid hormone on endothelium-dependent contractions, rings of femoral arteries from diabetic rats were incubated with L-NAME (3×10^{-4}M, 30 min) plus T_3 (10^{-7}M), and exposed to cumulative concentrations of the calcium ionophore A23187 (10^{-8} to 10^{-6}M) .

To study the effect of chronic treatment with thyroid hormone on endothelium-dependent contractions, rings of femoral arteries from both T_3 and vehicle treated group were incubated with L-NAME (3×10^{-4}M, 30 min) or L-NAME (3×10^{-4}M, 30 min) plus indomethacin [cyclooxygenase (COX) inhibitor, 5×10^{-6}M, 30 min) or S18886 [thromboxane prostanoid (TP) receptor antagonist, 10^{-7}M, 30 min], and exposed to cumulative concentrations of the calcium ionophore A23187 (10^{-8} to 10^{-6}M). The responses to cumulative concentrations of phenylephrine or U46619 of femoral arteries without endothelium of T_3 and vehicle treated rats were also determined.

2.4. Serum levels of thyroid hormone

The serum T_3 levels from vehicle and T_3 treated diabetic rats were measured with an enzyme linked immunosorbent assay using commercially available kits [NO. 1700 for total T_3; Alpha Diagnostic International (San Antonio, TX, USA)]. The levels of T_3 are expressed as nanograms per deciliter of serum.

2.5. Release of 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_2

Rings of femoral artery from both T_3 and vehicle treated diabetic rats were suspended in organ chambers containing warm and aerated control solution. After 90 min of equilibration and 30 min of incubation with L-NAME (3×10^{-4}M), a single dose (10^{-6}M) of A23187 was added. After ten min, 0.5ml of the bath solution was sampled for the measurement of the release of 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_2 using enzyme linked immunosorbent assay kits [Cayman Chemical Company (Ann Arbor, MI, USA)]. These samples were assayed in triplicates. The release of prostanoids is expressed in picograms per millimeter length of the ring **(Wong et al., 2008; Wong et al., 2010b)**.

2.6. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in Ham's Kaighn's Modification F12K medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), containing 1% penicillin/streptomycin (100U/ml). Heparin (0.1 mg/ml) and endothelial cell growth factor (0.05mg/ml) were added in the medium. The cells were incubated at 37°C in an

atmosphere containing 5% CO_2 and 95% room air. To study the acute or chronic effect of thyroid hormone on the expression of endothelial nitric oxide synthase (eNOS), COX-1 and COX-2 in HUVECs, different concentrations (10^{-10} to 10^{-7}M) of T_3 were administered for 30 min, 24 h or seven days. In some experiments, cells were also exposed to interleukin (IL)-1β (1ng/ml).

2.7. Protein extraction and Western blotting

Femoral arteries (cut into small pieces) or cultured cells were homogenized in lysis buffer with the usual inhibitors **(Li et al., 2011)**. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and detected with the appropriate antibodies (1:1000 eNOS monoclonal, 1:300 COX-1 monoclonal, 1:300 COX-2 polyclonal, 1:200 TP receptor polyclonal, 1:3000 β-actin monoclonal). Then, the blots were treated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:3000), incubated with Amersham™ ECL™ Western Blotting Detection Reagent (GE Healthcare, Boston, MA, USA) and subsequently exposed to X-ray film (Fuji Super RX medical X-ray film; Fuji Photo Film, Dusseldorf, Germany). ImageJ software (National Institutes of Health, MD, USA) was used to analyze the optical densities of the immunoreactive bands. The presence of protein was normalized to that of β-actin.

2.8. Chemicals

3,5,3'-tri-iodothyronine, A23187 [5-methylamino-2-(2*S*,3*R*,5*R*,8*S*,9*S*)-3,5,9-trimethyl-2-(1-oxo-(1*H*-pyrrol-2-yl)propan-2-yl)-1,7-dioxaspiro-(5,5)undecan-8-yl)methyl)benzooxazole-4-carboxylic acid], acetylcholine, $BaCl_2$,

charybdotoxin, glibenclamide, iberiotoxin, L-NAME [Nω-nitro-L-arginine methyl ester], nifedipine, ODQ [1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one], ouabain, phenylephrine, indomethacin and thapsigargin were purchased from Sigma Chemical (St. Louis, MO, USA). U46619 [9, 11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$] was purchased from Biomol (Plymouth Meeting, PA, USA). 1400W [N-(3-(Aminomethyl)benzyl)acetamidine] was purchased from Enzo Life science (Farmingdale, NY, USA). Rp-8-Br-cAMPs [8-bromoadenosine-3',5'-cyclic monophosphorothioate, Rp-isomer] was purchased from Biolog Life Science (Bremen, Germany). Calhex 231 [4-Chloro-N-[(1S,2S)-2-[[(1R)-1-(1-naphthalenyl)ethyl]amino]cyclohexyl]-benzamide hydrochloride] was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). [D-$Trp^{7,9,10}$]-Substance P was purchased from Tocris Bioscience (Bristol, UK). The eNOS monoclonal antibody was purchased from BD Transduction Laboratories (San Jose, CA, USA). KT5823 [2,3,9,10,11,12-hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S,12R-epoxy-1H-diindolo[1, 2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester], COX-1 monoclonal, COX-2 polyclonal, TP receptor polyclonal antibodies and anti-β-actin were purchased from Cayman Chemical. S18886, 3-[(6-amino-(4-chlorobenzensulphonyl)-2-methyl-5,6,7,8-tetrahydronapht]-1-yl)propionic acid was a kind gift of the Institut de Recherches Servier (Suresnes, France). T_3 was dissolved in 50mM NaOH per 1mg, U46619 was prepared in absolute ethanol. A23187 and ODQ were dissolved in absolute DMSO (0.1% in the organ chamber). All other chemicals were prepared in deionized water. Concentrations are expressed as final molar concentrations.

2.9. Data analysis

Relaxations are expressed as percentage of the contractions to either phenylephrine or U46619, and contractions are expressed as a percentage of the reference response to 60mM KCL obtained at the beginning of the experiment. The pEC_{50} is defined as the negative logarithm to base 10 of the EC_{50}, which is the concentration of an agonist required to produce 50% of the maximal possible effect. Data are presented as means ± S.E.M.; n refers to the number of rats or cell cultures. Statistical analysis was done by one or two-way analysis of variance. The results were analyzed with and graphed by Prism version 5 (GraphPad Software Inc. San Diego, CA). Differences were considered to be statistically significant when P was less than 0.05.

3. Results

3.1. Normal SD rats

3.1.1. Vascular responsiveness

3.1.1.1 Direct effects

Cumulative concentrations (10^{-7} to 3×10^{-5}M) of T_3 did not cause significant changes in tension in aortic rings with or without endothelium of normal rats. In mesenteric arteries, T_3, from $3x10^{-7}$ to 3×10^{-5}M, caused significant and comparable decreases in tension in preparations with and without endothelium contracted with U46619 (3×10^{-8}M). Similar results were obtained in femoral arteries **(Fig. 1)**. The T_3-induced relaxations in U46619 pre-contracted mesenteric arteries without endothelium were not affected significantly by inhibitors of NOS [L-NAME (3×10^{-4}M) and 1400W (10^{-5}M)], of potassium channels [iberiotoxin (10^{-7}M), charybdotoxin (10^{-7}M), $BaCl_2$ (10^{-6}M) or glibenclamide (10^{-5}M)], of Na^+-K^+-ATPase [ouabain (5×10^{-7}M)], of L-type calcium channels

[nifedipine (10^{-7}M)], of the calcium sensing receptor [calhex 231 (3×10^{-6} M)], of G-proteins [pertussis toxin (400ng/ml) or [D-Trp^{7,9,10}]-Substance P (10^{-5}M)], of sarco/endoplasmic reticulum calcium transport ATPase [thapsigargin (10^{-6}M)], of soluble guanylyl cyclase [ODQ (10^{-5}M)] or of protein kinases [Rp-8-Br-cAMPs (10^{-4}M) or KT5823 (10^{-6}M)] **(Table 1)**.

3.1.1.2. Indirect effects

In the aorta or mesenteric and femoral arteries with endothelium, 30 min of incubation with T_3 (10^{-7}M) did not significantly affect phenylephrine-induced contractions or acetylcholine-induced relaxations **(Fig. 2)**.

3.2. Diabetic rats

3.2.1. General conditions

Four weeks after the last streptozotocin injection, the body weight was significantly lower and the blood glucose level significantly increased in both T_3 and vehicle treated diabetic rats; there were no significant differences between the two groups as concerns those two parameters **(Table 2)**. After four weeks of chronic T_3 treatment, the total T_3 serum level was significantly greater than that in the vehicle treated diabetic group **(Table 2)**.

3.2.2. Vascular responsiveness

3.2.2.1. Endothelium-dependent relaxations

In the aorta and mesenteric arteries of vehicle treated diabetic rats, there was no impairment of relaxations to the endothelium-dependent vasodilator acetylcholine when compared with the

age-matched normal SD rats **(Fig. 3)**. Chronic treatment with T_3 did not affect the reference contractions to 60mM KCL in aortic [3.22±0.15 and 3.15±0.19 gram in rings from vehicle and hormone-treated rats, respectively], mesenteric [2.31±0.04 and 2.42±0.13 gram, respectively] preparations and did not affect acetylcholine-induced relaxations significantly either **(Fig. 3)**.

3.2.2.2. Endothelium-dependent contractions

These experiments were performed in preparations incubated with the NOS inhibitor L-NAME (3×10^{-4}M), in order to optimize endothelium-dependent contractions **(Auch-Schwelk et al., 1992; Tang et al., 2005a**). In the femoral artery of vehicle treated diabetic rats , A23187 (10^{-8} to 10^{-6}M) caused concentration-dependent contractions which were significantly larger in preparations with than in those without endothelium; the difference between the two types of preparations was reduced significantly by incubation with indomethacin (5×10^{-6}M) or S18886 (10^{-7}M) **(Fig. 4 and Fig. 5, *Left*)**. Thirty min of incubation with T_3 (10^{-7}M) did not significantly affect A23187-induced contractions in rings with endothelium **(Fig. 4)**. After four weeks of chronic treatment with T_3, there was no significant difference in the response to the ionophore between preparations with and without endothelium. The A23187 induced contractions in rings without endothelium were significantly larger in arteries from rats treated chronically with T_3 than in those from vehicle-treated animals; the augmented contraction to the ionophore in the former was abolished by incubation with indomethacin or S18886 **(Fig. 5, *Right*)**. Chronic treatment with T_3 did not affect the reference contractions to 60mM KCL in femoral preparations with and without endothelium.

3.2.2.3. Endothelium-independent contractions

In femoral artery rings without endothelium, both phenylephrine (10^{-9} to 10^{-5}M) and U46619 (10^{-9} to 10^{-7}M) evoked concentration-dependent contractions. There was no significant difference in phenylephrine-induced contraction between preparations of T_3 and vehicle treated diabetic rats **(Fig. 6, *Left*)**. However, the concentration-response curve to the TP-agonist was shifted significantly to the left **(Fig. 6, *Right*)** in arteries from T_3 treated animals **(Table 3)**.

3.2.3. Release of prostanoids

A23187 evoked the release of 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_2, in femoral arteries of both vehicle and T_3 treated diabetic rats. The release of the two prostanoids was significantly less in rings without than in those with endothelium. The production of 6-keto prostaglandin $F_{1\alpha}$ was comparable in preparations with endothelium of T_3 and vehicle treated diabetic rats **(Fig. 7, *Left*)**. A significantly lower release of thromboxane B_2 (by 38.1±9.2%) was observed in the rings with, but not in those without endothelium, of T_3 compared to vehicle treated animals **(Fig. 7, *Right*)**.

3.2.4. Protein expression

3.2.4.1. Arteries

Four weeks after the injection of streptozotocin, the protein level of eNOS and COX-1 were significantly greater in preparations with than in those without endothelium of both T_3 and vehicle treated diabetic rats. The expression of eNOS (140kDa) was significantly increased by 227.5±24.1% **(Fig. 8, *A*)** but that of COX-1 (72kDa) was significantly reduced by 34.9±7.4% **(Fig. 8, *B*)** in preparations with endothelium of T_3 treated diabetic rats. The expression level of COX-2

(72kDa) was similar among the different groups **(Fig. 8, *C*)**. In femoral artery rings without endothelium, the protein levels of TP receptors (55kDa) were not significantly different between T_3 and vehicle treated diabetic rats **(Fig. 8, *D*)**.

3.2.4.2. Cell culture

Acute (30 min) or more prolonged (one or seven days) exposure to T_3 (10^{-10} to 10^{-7}M), did not significantly affect the protein levels of eNOS, or COX-1 in quiescent HUVECs (data not shown). COX-2 was not detectable in either vehicle or T_3 treated unstimulated HUVECs (data not shown).

Incubation of HUVECs with IL-1β (1ng/ml; for one or seven days) induced a weak protein expression of COX-2, which was not different between vehicle and T_3 treated cells. When combined with IL-1β, incubation with T_3 (10^{-10} to 10^{-7}M, one day) did not significantly affect the protein levels of eNOS or those of either COX-1 or COX-2 (data not shown); however, co-incubation with IL-1β for seven days resulted in a significant, concentration-dependent increase in the protein content of eNOS without changes in those of COX-1 or COX-2 **(Fig. 9)**.

4. Discussion

The present study determined the direct effect of thyroid hormone in arteries of different size of normal rats. The hormone did not relax the aorta, a conduit artery, but did so in smaller femoral and mesenteric arteries, implying that its direct vasodilator effect may be more important in resistance vessels. To judge from the similar relaxations observed in presence and absence of endothelium, the effect of the hormone is endothelium-independent, a conclusion consistent

with previous studies **(Ishikawa et al., 1989; Park et al., 1997)**. This direct vasodilator effect of T_3 in smaller arteries may help to explain why systemic vascular resistance is closely linked to the thyroid status in patients **(Klein and Ojamaa, 2001)**. The present study explored, using accepted pharmacological inhibitors, a variety of possible explanations for the direct vasodilator properties of thyroid hormone. However, the actual underlying mechanism remained elusive. One unexplored possibility is acute inhibition of the transmembrane conductance regulator chloride channel, although the lack of effect of glibenclamide makes this unlikely **(Cai, 2013; Fong, 2013)**.

To investigate whether or not T_3 modifies endothelial dysfunction, rats with streptozotocin-induced diabetes were exposed chronically to the hormone. Streptozotocin destroys β cells of the Langerhans islets, reducing insulin release **(Junod et al., 1969)**. The lower body weight and the higher blood glucose levels demonstrate the successful development of diabetes. The present experiments do not confirm in mice with type 1 diabetes the attenuation of hyperglycemia, observed in db/db mice - with chronic T_3 treatment, attributed to improved insulin signaling rather than increased production of the hormone **(Lin and Sun, 2011)**.

Diabetic endothelial dysfunction **(De Vriese et al., 2000; Shi et al., 2007a; Stehouwer et al., 1997)**, is characterized in type 1 diabetes by occurrence of endothelium-dependent contractions **(Shi et al., 2007a)**. The present study confirms the absence of impaired endothelium-dependent relaxations to acetylcholine in arteries of rats at an early stage of diabetes **(Shi et al., 2006; Shi and Vanhoutte, 2009)**. Impairment of acetylcholine-induced relaxations can occur in arteries such as used in the present study after longer exposure to **(Cameron and Cotter, 1992; Fukao et**

al., 1997; Pieper and Peltier, 1995; Shimizu et al., 1993), or with higher doses of **(Palmer et al., 1998; Pieper et al., 1997; Taylor et al., 1994)** streptozotocin, or in skeletal muscle **(Hill and Ege, 1994)** and basilar **(Mayhan and Patel, 1998)** arteries. The present absence of effect of chronic T_3 treatment on relaxations to acetylcholine contrasts with the augmentation observed in the aorta and femoral artery of normoglycemic animals **(Deng et al., 2010)**. In the latter study, the relaxations to acetylcholine were also studied during phenylephrine contractions, but the response to the α_1-adrenergic agonist was reduced by hyperthyroidism, which was not the case in our experiments. Functional antagonists such as endothelium-derived or exogenous nitric oxide are more effective in causing relaxation against weaker pre-contraction levels **(Flavahan and Vanhoutte, 1988)**.

Acetylcholine induces endothelium-dependent contractions in aorta of spontaneously hypertensive (SHR) and normotensive rats **(Li et al., 2011; Tang et al., 2005b)**. However, it does not evoke such responses in femoral arteries of SD rats whereas the calcium ionophore A23187 does **(Shi et al., 2007a; Shi et al., 2007b)**. Streptozotocin-induced diabetes exacerbates such EDCF-mediated responses **(Shi et al., 2007a)**. Therefore, the present study focused on A23187 responses in the femoral artery**.** The results confirm that it elicits endothelium-dependent contractions of this preparation from diabetic rats **(Shi et al., 2007a).** This endothelium-dependent contraction was abolished by indomethacin [a nonspecific cyclooxygenases (COX) inhibitor] or S18886 [a specific TP receptor antagonist], confirming that A23187 releases COX-derived EDCF **(Lüscher and Vanhoutte, 1986; Tang et al., 2007; Wong et al., 2010a)** and that these factors activate TP receptors of the underlying vascular smooth muscle

(Auch-Schwelk et al., 1990; Tang and Vanhoutte, 2009). In the SHR aorta, A23187 releases both prostacyclin and thromboxane A_2 as EDCF, and the latter is the most potent TP receptor agonist **(Gluais et al., 2005; Gluais et al., 2006)**. The present results confirm that A23187 augments the endothelial production of 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_2. After four weeks of T_3 treatment, no difference in contraction to A23187 was observed between preparations with and without endothelium, implying that the hormone reduces EDCF release. This interpretation is strengthened by the lesser endothelial production of thromboxane B_2 in arteries of diabetic rats. T_3, through changes in gene transcription, increases the number of calcium-activated ATPase pump units in the sarcoplasmic reticulum and thus causes more efficient pumping by the calcium-activated ATPase **(Dillmann, 1990)**. If this were to occur in the endothelium of diabetic rats, increased activity of calcium-activated ATPase may counteract the effect of A23187 on the COX-1 axis, reducing EDCF release.

The difference of COX-1 level between preparations with and without endothelium in T_3 and vehicle treated diabetic rats, is in line with the importance of endothelial COX-1 for the occurrence of endothelium-dependent contractions **(Tang and Vanhoutte, 2010)**. The absence of difference in COX-2 level confirms that COX-1 plays the prominent role in endothelial dysfunction in rodents **(Ge et al., 1995; Shi et al., 2008; Tang et al., 2005b)**. However, a lower COX-1 level was observed in T_3 treated diabetic rats, which also fits with the lower production of thromboxane B_2 by the endothelial cells of arteries chronically exposed to the hormone.

Chronic inflammation is involved in the pathogenesis of type 1 diabetes, and contributes to the

inhibition of growth and function of the β cells of the pancreas **(Eizirik et al., 2009; Wellen and Hotamisligil, 2005)**. Conversely, diabetes-induced increases in oxidative stress augment the release of pro-inflammatory cytokines sustaining the inflammatory state **(Zhang et al. 2003)**. In addition, the inflammatory mediators may accelerate the onset of endothelial dysfunction in type 1 diabetes **(Goldberg, 2009; Stehouwer et al., 1997)**. Hence, to mimic the chronic impact of diabetes on endothelial cells, cultures of HUVECs were exposed to IL-1β, a cytokine involved in the pathology of the disease **(Liu et al., 2012; Mandrup-Poulsen et al., 2010; Reimers, 1998)**. In the present study, prolonged exposure to the hormone on a background of IL-1β stimulation, caused the expected expression of COX-2 **(Uracz et al., 2002)**, but also increased the protein level of eNOS, a finding in line with the present observations in arteries of T_3-treated diabetic rats. Thus, the inflammatory response that accompanies diabetes **(Wellen and Hotamisligil, 2005)** may be required for the expression of a chronic augmentation in eNOS caused by thyroid hormone. The resulting increase in NO bioavailability would explain the curtailment of endothelium-dependent contractions **(Tang et al., 2005a)**. Taken in conjunction, the present findings permit to conclude that chronic treatment with T_3 of rats with type 1 diabetes attenuates the release of EDCF by augmenting total eNOS and reducing the presence and/or activity of cyclooxygenase in their endothelial cells. Since acute exposure to T_3 did not affect endothelium-dependent contractions to A23187, the effect of the long-term treatment with the hormone is genomic in nature.

The absence of difference in the response to phenylephrine between femoral arteries of the T_3 and vehicle treated groups, contrasts with the reduction observed in normoglycemic rats (**Deng**

et al., 2010). We have no explanation for this discrepancy. However the findings obtained under our experimental conditions imply that T_3 has no effect on α-adrenoceptors and does not chronically increase the intrinsic responsiveness of vascular smooth muscle in arteries of diabetic rats. By contrast, since no changes in the protein presence of TP receptors were obvious, the augmented contractions and the shift to the left of the concentration-response curve to the prototypical TP agonist U44169 **(Gluais et al., 2005)** imply that chronic treatment with T_3 increases the sensitivity of these receptors. Hence, chronic T_3 treatment in diabetic rats also augmented contractions to A23187 in preparations without endothelium. Since this augmented contraction was abolished by indomethacin and S18886 (implying that A23187 triggers the COX-mediated pathway in vascular smooth muscle), it must be due to activation of TP receptors and is explained by the hyperresponsiveness of the TP receptors. The present experiments do not permit to speculate further on the molecular mechanism underlying this phenomenon. In terms of relevance for the *in vivo* situation, any increase in TP receptor responsiveness would be offset by the reduced release in endothelium-derived vasoconstrictor prostanoids.

In summary, the present study demonstrates that, administered *in vitro* in pharmacological high concentrations, T_3 acutely relaxes mesenteric and femoral vascular smooth muscle of normal rats but does so in an endothelium-independent manner. The mechanism underlying this direct relaxing effect remains elusive, as is its importance *in vivo*. In type 1 diabetic rats, while chronic *in vivo* T_3 treatment with pathophysiological relevant doses can reduce the *ex vivo* release of EDCF (an interpretation confirmed by the measurement of prostanoid release and explained by the reduced expression of COX-1 and the increased presence of eNOS), it increases the sensitivity of

TP receptors of vascular smooth muscle **(Fig. 10)**. Thus, thyroid hormone can affect both endothelial and vascular smooth muscle cells in rat arteries.

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Disclosures

The authors declare no conflicts of interest, financial or otherwise.

Author contributions

Designed the experiments: Yin Cai, Eva H.C. Tang, Paul M. Vanhoutte. Performed the experiments: Yin Cai, Michael M. Manio. Analyzed the data: Yin Cai, George P.H. Leung, Aimin Xu, Eva H.C. Tang, Paul M. Vanhoutte. Wrote the paper: Yin Cai, Eva H.C. Tang, Paul M. Vanhoutte.

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Legends

Fig. 1

Effects of increasing concentrations of T_3 (five min/dose) on contractions of rat arteries with [+EC] or without [-EC] endothelium during sustained contractions to U46619 (3×10^{-8}M). Data expressed as percentage of the pre-contraction to U46619 [100%: aorta, 3.64±0.23g; mesenteric arteries, 2.38±0.25g; femoral arteries, 2.04±0.17g] and shown as means ± S.E.M.; n=4. *Statistically significant difference between preparations of vehicle and T_3 groups ($P< 0.05$).

Fig. 2

Effect of incubation with T_3 (10^{-7}M, 30 min) on responses of rat arteries with endothelium. *Left*: contractions of quiescent preparations to increasing concentrations of phenylephrine. Data expressed as percentage of the pre-contraction to KCL [100%: aorta, 3.52±0.14g; mesenteric arteries, 2.27±0.32g; femoral arteries, 2.24±0.35g] and shown as means ± S.E.M.; *Right*: relaxations to increasing concentrations of acetylcholine during sustained contractions to phenylephrine (10^{-6}M). Data expressed as percentage of the pre-contraction to phenylephrine [100%: aorta, 3.45±0.19g; mesenteric arteries, 2.12±0.34g; femoral arteries, 2.15±0.26g] and shown as means ± S.E.M.; n=4.

Fig. 3

Relaxations to cumulative concentrations of acetylcholine during sustained contractions to phenylephrine (10^{-6}M), in aorta and mesenteric arteries with endothelium from normal rats, vehicle and T_3 treated diabetic rats. Data expressed as percentage of the pre-contraction to phenylephrine [100%: vehicle treated diabetic rats, aorta, 3.36±0.27g, mesenteric arteries, 2.08±0.20g; T_3 treated diabetic rats, aorta, 3.22±0.41g, mesenteric arteries, 2.29±0.25g] and

shown as means ± S.E.M.; n= 4.

Fig. 4

Responses, in the presence of L-NAME (3×10^{-4}M) or L-NAME (3×10^{-4} M) plus T_3 (10^{-7}M, 30 min), to cumulative concentrations of A23187, in femoral arteries with (+EC) or without (-EC) from diabetic rats. Data expressed as percentage of the pre-contraction to KCL [100%: with endothelium, 2.03±0.32g; without endothelium, 1.98±0.28g] and shown as means ± S.E.M.; n= 5.

Fig. 5

Responses, in the presence of L-NAME (3×10^{-4} M), to cumulative concentrations of A23187, with or without indomethacin (5×10^{-6} M) or S18886 (10^{-7}M), in femoral arteries with (+EC) or without (-EC) from vehicle (*Left*) and T_3 (*Right*) treated diabetic rats. Data expressed as percentage of the pre-contraction to KCL [100%: vehicle treated diabetic rats, with endothelium, 2.03±0.32g; without endothelium, 1.98±0.28g; T_3 treated diabetic rats, with endothelium, 2.05±0.18g; without endothelium, 2.12±0.33g] and shown as means ± S.E.M.; n=10 to 15. *Statistically significant difference between preparations with and without endothelium ($P<0.05$). $^{\#}$Statistically significant difference between preparations in the presence and absence of indomethacin or S18886 ($P< 0.05$).

Fig. 6

Responses to cumulative concentrations of phenylephrine (*Left*) and U46619 (*Right*) in rings of femoral arteries without endothelium from T_3 and vehicle treated diabetic rats. Data expressed as percentage of the pre-contraction to KCL [100%: vehicle treated diabetic rats, 1.98±0.28g; T_3 treated diabetic rats, 2.12±0.33g] and shown as means ± S.E.M.; n=5 to 10. *Statistically

significant difference between arteries of vehicle and T_3 treated diabetic rats (P< 0.05).

Fig. 7

Release of 6-keto prostaglandin $F_{1\alpha}$ (*Left*) and thromboxane B_2 (*Right*) in response to A23187 (10^{-6}M) in femoral arterial rings with (+EC) and without (-EC) endothelium from T_3 and vehicle treated diabetic rats. Data shown as means ± S.E.M.; n=8 to 12. *Statistically significant difference between preparations with and without endothelium (P< 0.05); #Statistically significant difference between preparations of vehicle and T_3 treated diabetic rats (P< 0.05).

Fig. 8

Western blotting analysis of the protein expression of eNOS (A), COX-1 (B), COX-2 (C) and TP receptor (D) in femoral arterial rings from vehicle and T_3 treated diabetic rats. Data shown as means ± S.E.M.; n=3. *Statistically significant difference between preparations with and without endothelium (P< 0.05); #statistically significant difference between arteries of vehicle and T_3 treated diabetic rats (P< 0.05). All the samples were derived at the same time and processed in parallel.

Fig. 9

Western blotting analysis of the protein expression of eNOS, COX-1 and COX-2 in HUVECs stimulated with T_3 (10^{-10} to 10^{-7}M) for one week in the presence of IL-1β (1ng/ml). Data shown as means ± S.E.M.; n=3. *Statistically significant difference between preparations of vehicle and T_3 treated groups.

Fig. 10

In arteries of diabetic rat, A23187 elicits endothelium-dependent contractions, which can be abolished by indomethacin or S18886. After chronic T_3 treatment, the release of EDCF is reduced,

which could be due to the increased expression of eNOS and/or the reduced presence of COX-1. T_3 treatment increases the sensitivity of TP receptors of the vascular smooth muscle, which lead to the comparable contractions to A23187 in preparations with and without endothelium. The presence of indomethacin or S18886 can block A23187 induced contractions in preparations without endothelium.

Fig. 1

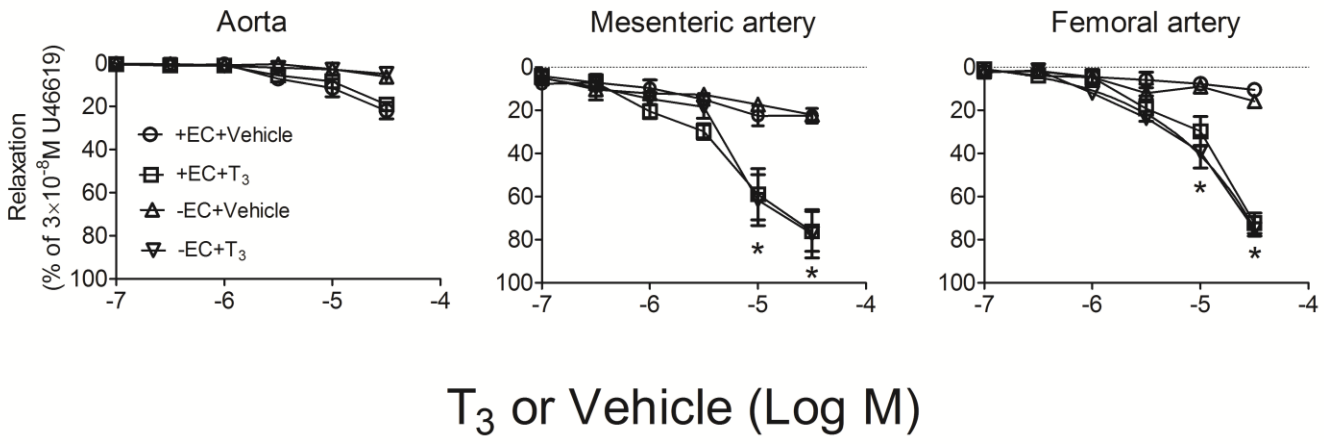

Aorta
Mesenteric artery
Femoral artery
Relaxation
(% of 3×10-8M U46619)
0
20
40
60
80
100
-7
-6
-5
-4
+EC+Vehicle
+EC+T3
-EC+Vehicle
-EC+T3
*
T3 or Vehicle (Log M)

Fig. 2

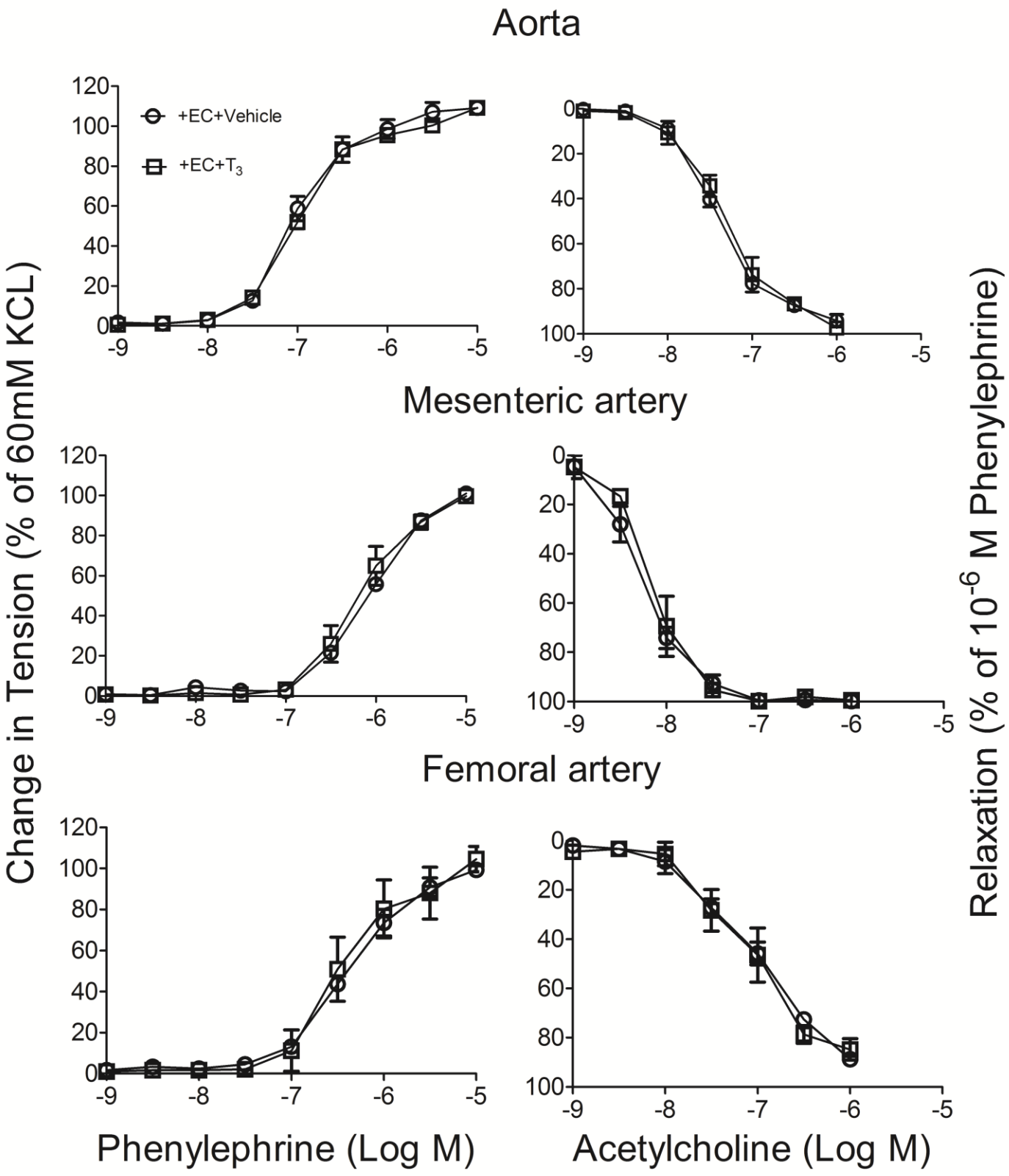

Aorta
+EC+Vehicle
+EC+T3
Mesenteric artery
Femoral artery
Change in Tension (% of 60mM KCL)
Relaxation (% of 10-6 M Phenylephrine)
Phenylephrine (Log M)
Acetylcholine (Log M)

Fig. 3

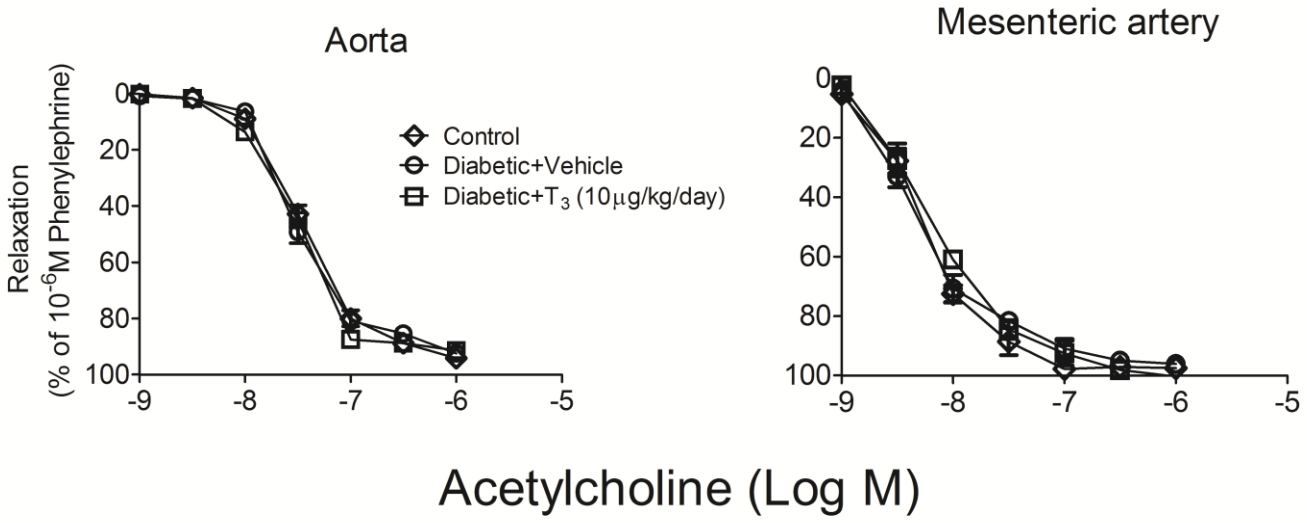

Aorta
Mesenteric artery
Relaxation
(% of 10-6M Phenylephrine)
0
20
40
60
80
100
-9
-8
-7
-6
-5
Control
Diabetic+Vehicle
Diabetic+T3 (10μg/kg/day)
Acetylcholine (Log M)

Fig. 4

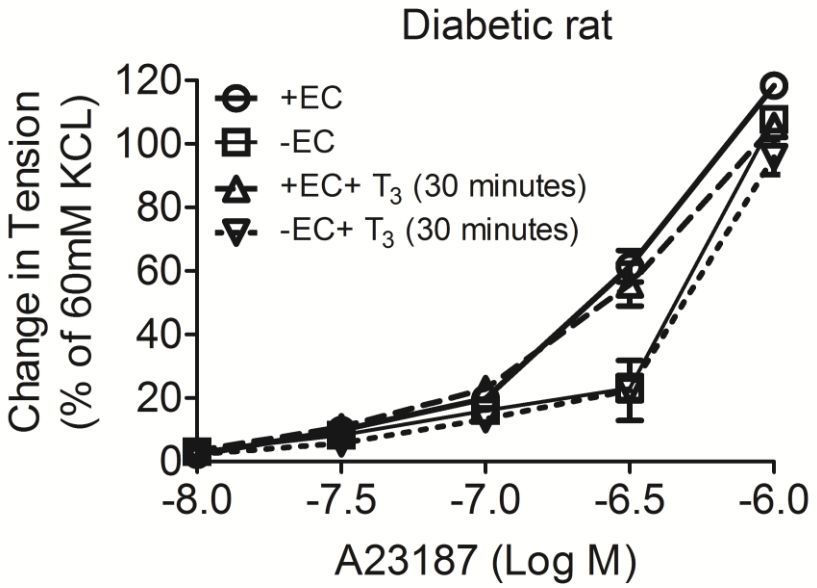

Diabetic rat
Change in Tension
(% of 60mM KCL)
120
100
80
60
40
20
0
+EC
-EC
+EC+ T3 (30 minutes)
-EC+ T3 (30 minutes)
-8.0
-7.5
-7.0
-6.5
-6.0
A23187 (Log M)

Fig. 5

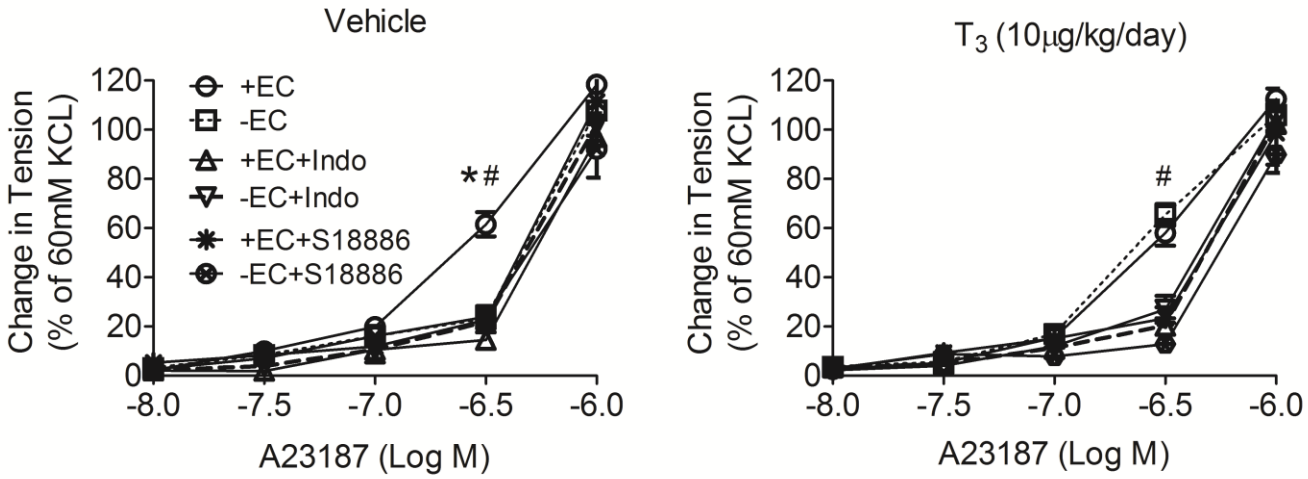
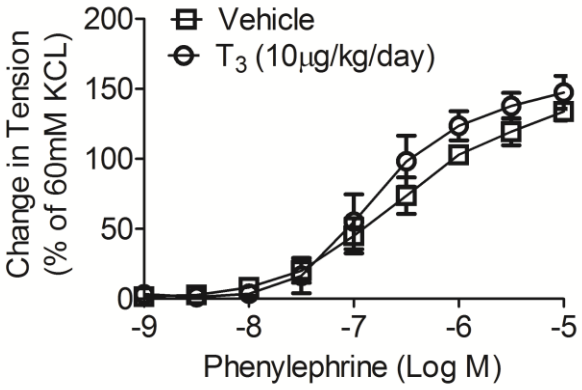

Vehicle
Change in Tension (% of 60mM KCL)
120
100
80
60
40
20
0
+EC
-EC
+EC+Indo
-EC+Indo
+EC+S18886
-EC+S18886
*#
-8.0
-7.5
-7.0
-6.5
-6.0
A23187 (Log M)
T3 (10μg/kg/day)
Change in Tension (% of 60mM KCL)
#
A23187 (Log M)

Fig. 6


Vehicle
T3 (10μg/kg/day)
Change in Tension
(% of 60mM KCL)
200
150
100
50
0
-9
-8
-7
-6
-5
Phenylephrine (Log M)

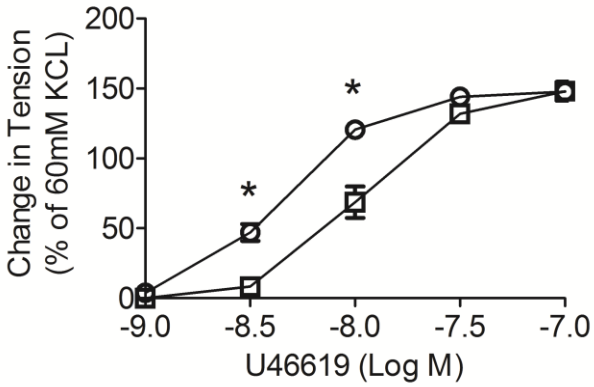

Change in Tension
(% of 60mM KCL)
200
150
100
50
0
*
*
-9.0
-8.5
-8.0
-7.5
-7.0
U46619 (Log M)

Fig. 7

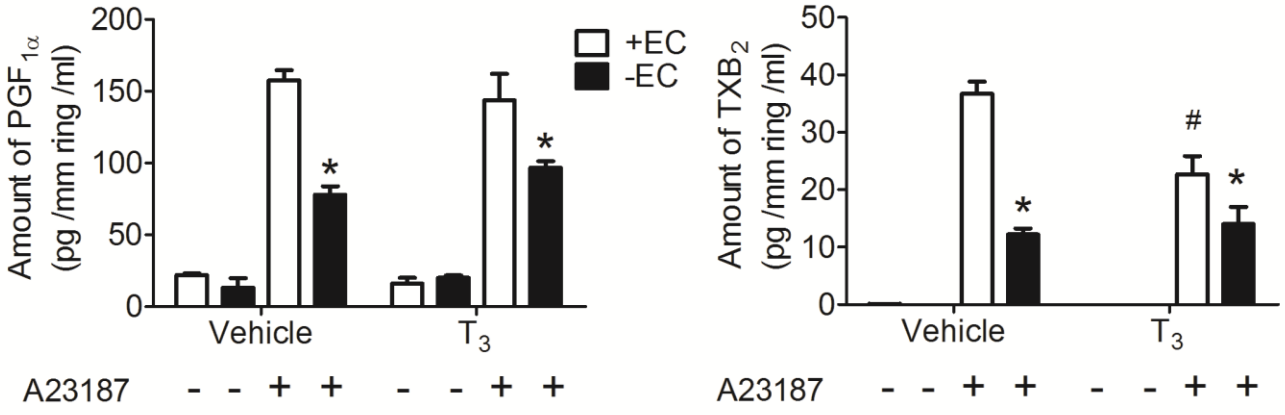

Amount of PGF1α (pg /mm ring /ml)
200
150
100
50
0
+EC
-EC
*
*
Vehicle
T3
A23187 - - + + - - + +
Amount of TXB2 (pg /mm ring /ml)
50
40
30
20
10
0
#
*
*
Vehicle
T3
A23187 - - + + - - + +

Fig. 8

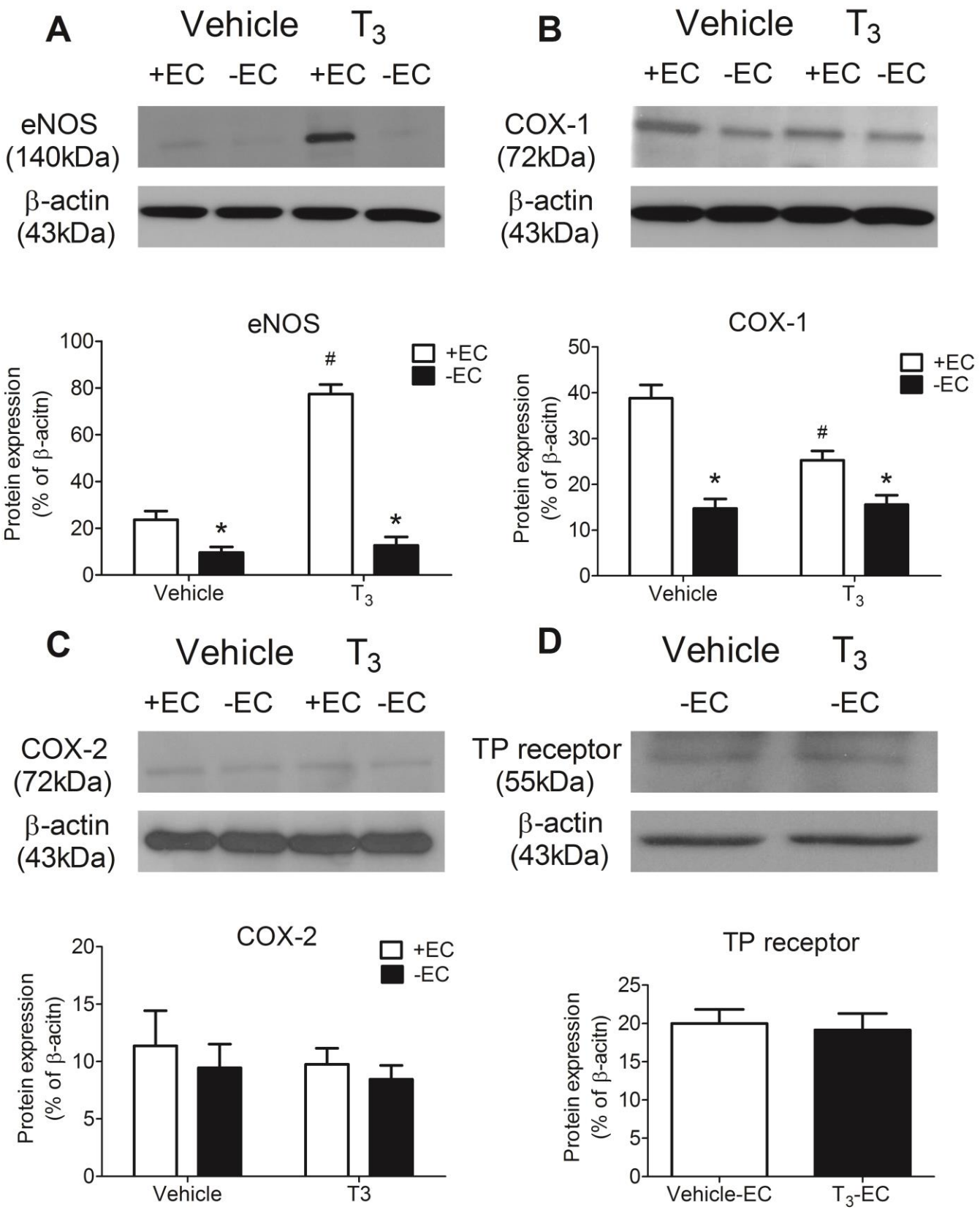
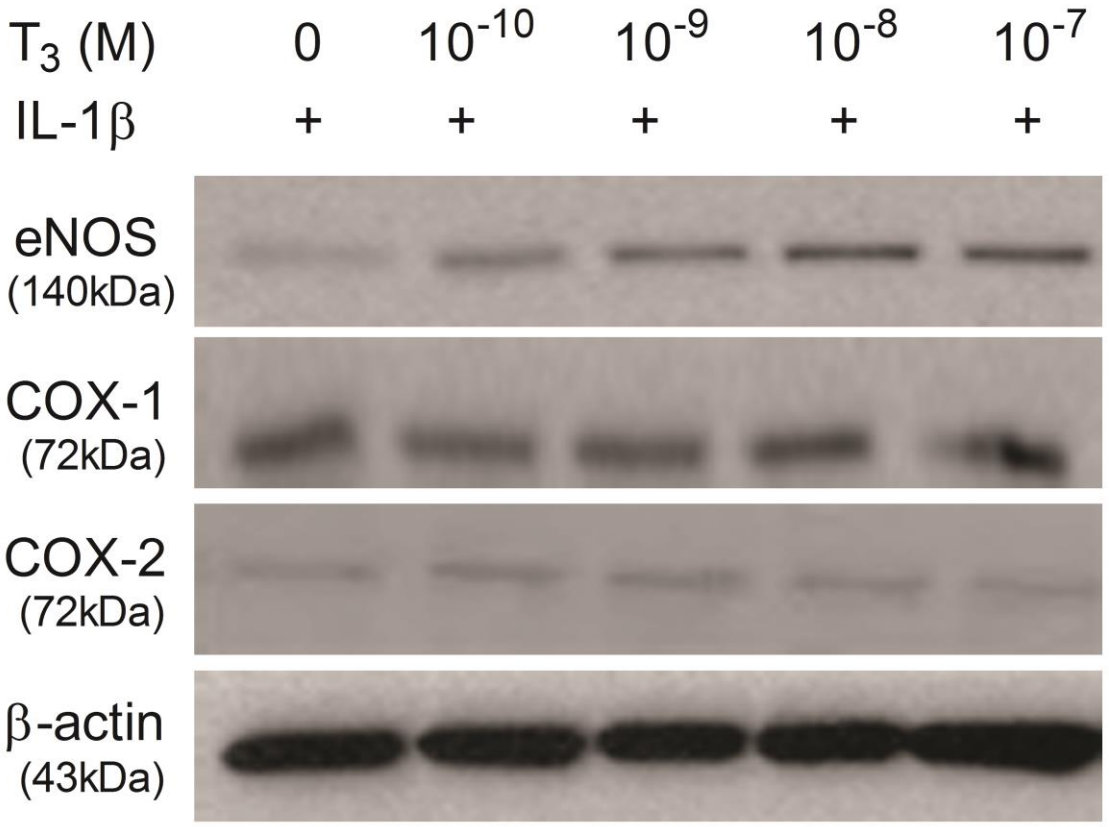

A
Vehicle
T3
+EC
-EC
+EC
-EC
eNOS
(140kDa)
β-actin
(43kDa)
B
Vehicle
T3
+EC
-EC
+EC
-EC
COX-1
(72kDa)
β-actin
(43kDa)
eNOS
Protein expression
(% of β-acitn)
100
80
60
40
20
0
+EC
-EC
#
*
*
Vehicle
T3
COX-1
Protein expression
(% of β-acitn)
50
40
30
20
10
0
+EC
-EC
#
*
*
Vehicle
T3
C
Vehicle
T3
+EC
-EC
+EC
-EC
COX-2
(72kDa)
β-actin
(43kDa)
D
Vehicle
T3
-EC
-EC
TP receptor
(55kDa)
β-actin
(43kDa)
COX-2
Protein expression
(% of β-acitn)
20
15
10
5
0
+EC
-EC
Vehicle
T3
TP receptor
Protein expression
(% of β-acitn)
25
20
15
10
5
0
Vehicle-EC
T3-EC

Fig. 9


T3 (M)
0
10-10
10-9
10-8
10-7
IL-1β
+
+
+
+
+
eNOS
(140kDa)
COX-1
(72kDa)
COX-2
(72kDa)
β-actin
(43kDa)

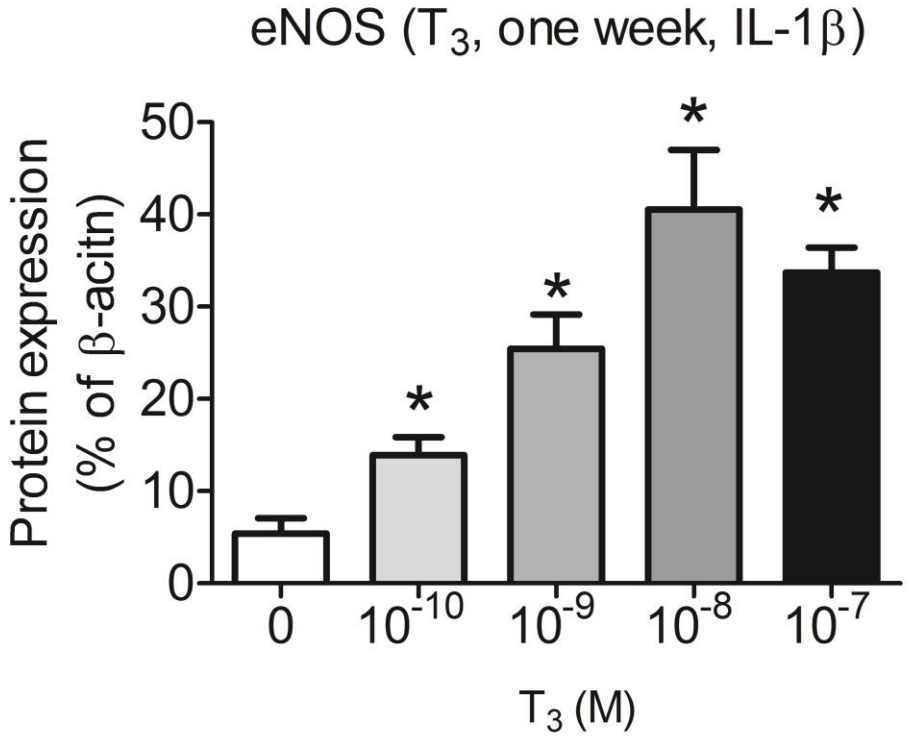

eNOS (T3, one week, IL-1β)
Protein expression
(% of β-acitn)
50
40
30
20
10
0
*
*
*
*
0
10-10
10-9
10-8
10-7
T3 (M)

Fig. 10

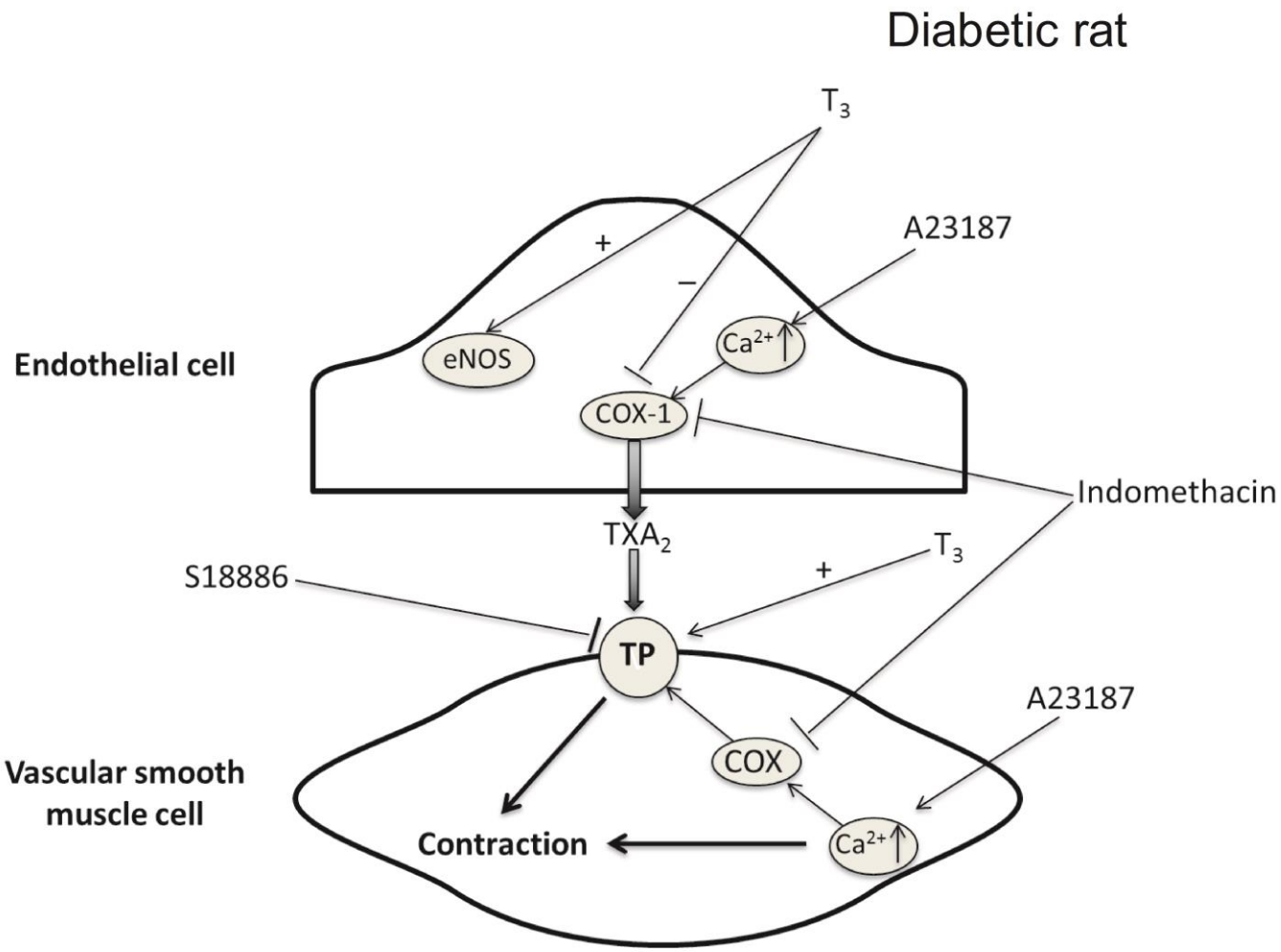

Diabetic rat
T3
A23187
+
–
Endothelial cell
eNOS
Ca2+
COX-1
Indomethacin
TXA2
S18886
T3
+
TP
A23187
COX
Vascular smooth muscle cell
Contraction
Ca2+

Table 1

Effects of various pharmacological inhibitors on the relaxation evoked byT_3 in rat mesenteric arteries[a].

Treatment	pEC_{50} of T_3[b]	Concentration[c]	Target[d]	References[e]
Vehicle	4.96±0.19	-	-	-
Rp-8-Br-cAMPs	4.89±0.22	10^{-4}M	protein kinase A	Keung et al., 2005
KT5823	4.86±0.11	10^{-6}M	protein kinase G	Keung et al., 2005
L-NAME	4.93±0.13	3 X 10^{-4}M	eNOS, iNOS	Shi et al., 2007a
1400W	4.93±0.09	10^{-5}M	iNOS	Zhang et al., 2012
ODQ	4.98±0.05	10^{-5} M	sGC	Li et al., 2013
Charybdotoxin	5.14±0.45	10^{-7}M	IK_{Ca}, BK_{Ca}	Edwards et al., 1998
Iberiotoxin	4.89±0.23	10^{-7}M	BK_{Ca}	Li et al., 2013
$BaCl_2$	4.95±0.12	10^{-6}M	K_{IR}	Li et al., 2013
Glibenclamide	4.95±0.20	10^{-5}M	K_{ATP}	Li et al., 2013
ouabain	5.05±0.08	5 × 10^{-7}M	Na^+-K^+-ATPase	Li et al., 2013
nifedipine	4.85±0.13	10^{-7}M	L-type Ca^{2+}-channel	Cognard et al., 1990
calhex 231	5.10±0.32	3 X 10^{-6} M	CaSR	Weston et al., 2008
thapsigargin	4.79±0.18	10^{-6} M	SERCA	Chan et al., 2011
pertussis toxin	5.02±0.06	400ng/ml	$G_{\alpha i}$ proteins	Wong et al., 2010
D-Trp-SP[f]	4.95±0.08	10^{-5}M	$G_{\alpha q}$ proteins	Mukai et al., 1992

[a] Preparations without endothelium were contracted with U46619 (3×10^{-8}M).

[b] Values are means ± S.E.M.; n=6 to 10.

[c] Incubation during 30 min, except for pertussis toxin (two h), prior to the contraction with U46619.

[d] Abbreviations: BK_{Ca}, large conductance Ca^{2+}-activated potassium channel; CaSR, calcium sensing receptor; eNOS, endothelial nitric oxide synthase; IK_{Ca}, intermediate conductance Ca^{2+}-activated potassium channel; iNOS, inducible nitric oxide synthase; K_{ATP}, ATP-sensitive K^+ channel; K_{IR}, inwardly-rectifying K^+ channel; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPases; sGC, soluble guanylyl cyclase.

[e] Justifying the used concentration of the inhibitors.

[f] D-Trp-SP: [D-$Trp^{7,9,10}$]-Substance P.

Table 2

Body weight, blood glucose and serum T_3 level in vehicle and T_3 treated diabetic rats[a].

	Vehicle		T_3 treated[c]	
	Before	STZ[b]	Before	STZ[b]
Body weight (g)	571.9±5.9	423.4±9.0[d]	552.9±7.7	414.9±10.5[d]
Blood glucose (mmol/L)	5.8±0.2	25.0±0.7[e]	5.7±0.2	23.6±0.8[e]
Serum T_3 (ng/dL)	-	226.8±20.1	-	411.3±27.4[f]

[a] Data shown as means ± S.E.M.; n=10 to 20.

[b] Streptozotocin (STZ; 30 mg/kg; intraperitoneally; once per day for three consecutive days) was given followed by vehicle or T_3 treatment.

[c] Daily intraperitoneal injection of T_3 (10μg/kg/day) for four weeks.

[d] Statistically significant difference in body weight between before and four weeks after streptozotocin treatment ($P<0.05$).

[e] Statistically significant difference in blood glucose level between before and four weeks after streptozotocin treatment ($P< 0.05$).

[f] Statistically significant difference in serum T_3 level between vehicle and T_3 treated diabetic rats ($P< 0.05$).

Table 3

pEC_{50} to phenylephrine or U46619 in femoral arteries from vehicle and T_3 treated diabetic rats[a].

	Phenylephrine		U46619	
	Vehicle[b]	T_3 treated[b]	Vehicle[b]	T_3 treated[b]
pEC_{50}	6.55±0.30	6.76±0.21	7.94±0.09	8.53±0.06[c]

[a] Data shown as means ± S.E.M.; n=5 to 10.

[b] Vehicle or T_3 treatment (10μg/kg/day; intraperitoneally) was given on diabetic rats for four weeks.

[c] Statistically significant difference in pEC_{50} to U46619 between vehicle and T_3 treated diabetic rats ($P< 0.05$).