

**Title:**

Nitric oxide mediates abnormal responsiveness of thyroid arteries in methimazole-treated patients

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**Short title:**

Nitric oxide and human thyroid arteries

## **Abstract**

*Objective:* We studied the intervention of nitric oxide (NO), prostacyclin (PGI<sub>2</sub>) and endothelium-derived hyperpolarizing factor (EDHF) in mediating responses to acetylcholine in thyroid arteries from euthyroid (E) and methimazole-treated (MT) patients.

*Design and methods:* Branches of the superior thyroid artery were obtained from 19 E patients and 17 MT patients (euthyroid at the time of surgery) undergoing total thyroidectomy or hemithyroidectomy. Artery rings were suspended in organ baths for isometric recording of tension.

*Results and conclusions:* Acetylcholine caused endothelium-dependent relaxation of greater magnitude in arteries from MT patients (pD<sub>2</sub> 7.68±0.19 in E and 8.17±0.26 in MT, *P*<0.05). The relaxation was unaffected by indomethacin and partially reduced by the NO synthase inhibitor L-NMMA. This reduction was higher in arteries from MT patients (50±6 %) as compared to E patients (36±6 %) (*P*<0.05). Inhibition of K<sup>+</sup> channels using apamin combined with charybdotoxin or high K<sup>+</sup> solution abolished the relaxation resistant to L-NMMA and indomethacin. The maximal contractions to noradrenaline (in percentage of the response to 100 mM KCl) were lower in MT than in E patients (57±10 and 96±8, respectively, *P*<0.05). The hyporesponsiveness to noradrenaline in arteries from MT patients was corrected by L-NMMA. The results indicate: (1) thyroid arteries from MT patients show increased relaxation to acetylcholine and decreased contraction to noradrenaline due to overproduction of NO; (2) EDHF plays a prominent role in acetylcholine-induced relaxation through activation of Ca<sup>2+</sup>-activated K<sup>+</sup> channels; (3) the abnormal endothelium-dependent responses in arteries from MT patients are not corrected by medical treatment.

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## **Introduction**

Hyperthyroidism is associated with marked effects on the cardiovascular system including high cardiac output, increased heart rate, and a fall in peripheral vascular resistance (1). In addition, hyperthyroidism is characterized by alterations in vascular responsiveness. Experiments in aortic rings from hyperthyroid rats show reduced responses to contractile agents (2-4) and enhanced endothelium-dependent relaxations to acetylcholine (4) compared to euthyroid rats.

The mechanism by which thyroid dysfunction alters vascular responsiveness is still unknown. A recent study in rat isolated renal artery rings has shown that after short treatment (single injection) with triiodothyronine ( $T_3$ ), acetylcholine-induced relaxations mediated by endothelium-derived nitric oxide (NO) and by the endothelium-derived hyperpolarizing factor (EDHF) were significantly enhanced compared to controls. Following 8 week treatment with  $T_3$ , EDHF-mediated relaxation was impaired, whereas NO-mediated relaxation remained enhanced (5). These results show differences in vascular responses between acute and chronic hyperthyroidism and indicate that both NO and EDHF are responsible for the increased endothelium-dependent relaxation in hyperthyroidism.

In humans, during acetylcholine infusion, forearm blood flow, measured by plethysmography, increased more in hyperthyroid patients than in control subjects (6). In addition, untreated hyperthyroid patients showed a more pronounced fall in forearm blood flow after inhibition of basal NO synthesis by intra-arterial infusion of the NO-synthase inhibitor NG-monomethyl-L-arginine (L-NMMA). These results in human resistance vessels strongly suggest that NO might play a key role in the vascular changes observed in thyroid dysfunction. Moreover, after treatment with the antithyroid drug methimazole, thyroid hormone levels, basal forearm blood flow, and

vasodilatation in response to acetylcholine were restored to normal, thus supporting the conclusion that thyroid hormone elevation is specifically responsible for the vascular abnormalities observed in untreated hyperthyroidism (6).

The endothelium-NO system of human thyroid arteries remains largely unexplored. A recent study from our laboratory has shown that in human thyroid arteries from multiorgan donors the endothelium modulates responses to acetylcholine and noradrenaline through the release of NO and EDHF (7). The EDHF component involved activation of  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels sensitive to the  $\text{K}^+$  channel blockers charybdotoxin and apamin. No data are available concerning endothelial function in thyroid arteries from euthyroid patients and patients with Graves' disease treated with methimazole. Such data would be relevant in the understanding of the regulation of intrathyroidal blood after medical treatment and the mechanisms underlying the vascular abnormalities in thyroid disease. Accordingly, the aim of the present study was to extend our previous observations by determining the responses to acetylcholine and noradrenaline of thyroid arteries from euthyroid patients and from hyperthyroid patients with Graves' disease after treatment with methimazole.

## **Material and methods**

### **Subjects**

Tissue samples were obtained from two groups of patients undergoing subtotal thyroidectomy or hemithyroidectomy. One group included 19 euthyroid (E) patients (free thyroxine  $1.24 \pm 0.04$  ng/dL, thyroid stimulating hormone  $2.8 \pm 0.4$  mIU/L) affected by multinodular goiters causing compression and obstructive symptoms (14 women, 5 men;  $54 \pm 3$  years). The other group included 17 patients affected by Graves' disease (15 women, 2 men;  $44 \pm 4$  years), who had been hyperthyroid for  $4 \pm 1.8$  months according to their medical history. Indications for surgery in this group included appearance of cyclic phases of exacerbation of the disease. The hyperthyroid patients had received methimazole for at least 12 months and were euthyroid at the time of surgery (free thyroxine  $1.14 \pm 0.04$  ng/dL, thyroid stimulating hormone  $2.6 \pm 0.6$  mIU/L). These patients are referred to as methimazole treated (MT) patients. The study was approved by the ethical committee of our institution and each patient gave informed written consent.

### **In vitro experiments**

Glandular branches of the superior thyroid artery were removed and cut into rings (3 mm in length, 1 to 1.5 mm outer diameter) and placed in refrigerated ( $4^{\circ}$  C) modified Krebs Henseleit solution of the following composition (in mmol/l): NaCl, 115; KCl, 4.6;  $MgCl_2 \cdot 6H_2O$ , 1.2;  $CaCl_2$ , 2.5;  $NaHCO_3$ , 25; glucose, 11.1 and disodium EDTA, 0.01. Each artery ring was prepared for isometric tension recording as previously described.<sup>(7)</sup> Changes in isometric force were recorded on a Macintosh computer (Apple Computer, Cupertino, Calif) by use of Chart version 3.4/s software and a MacLab/8e data acquisition system (ADInstruments, Mountain View, Calif). In some experiments, the endothelium was removed by inserting a stainless-steel wire into the

lumen. An optimal resting tension of 2 g was applied. The rings were allowed to attain a steady level of tension during a 2- to 3-hour accommodation period before testing.

Relaxations to acetylcholine (1nmol/l to 1 $\mu$ mol/l) were investigated in arteries precontracted with noradrenaline to 40-50 % of the contraction induced by 100 mmol/l KCl under the following conditions: 1: in the absence of inhibitors (control response); 2: in the presence of indomethacin (10  $\mu$ mol/l) to inhibit the production of prostacyclin (PGI<sub>2</sub>); 3: in the presence of indomethacin and N<sup>G</sup>-monomethyl-L-arginine (L-NMMA, 100  $\mu$ mol/l) to inhibit the production of PGI<sub>2</sub> and NO synthase, respectively; 4: in the presence of indomethacin, L-NMMA and KCl (20 mmol/l) to inhibit the production of PGI<sub>2</sub>, NO synthase and K<sup>+</sup> channel activity; 5: in the presence of methimazole (1 $\mu$ mol/l). To examine the nature of K<sup>+</sup> channel activation, relaxation to acetylcholine was obtained in the presence of indomethacin plus L-NMMA combined with one of the following inhibitors: iberiotoxin (0.1 $\mu$ mol/l), an inhibitor of large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels, charybdotoxin (0.1 $\mu$ mol/l), an inhibitor of both large- and intermediate-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels, apamin (1  $\mu$ mol/l), an inhibitor of small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels, and glibenclamide (10  $\mu$ mol/l), a selective blocker of ATP-sensitive K<sup>+</sup> channels. Control (in the absence of inhibitors) and experimental (after incubation for 20 minutes with inhibitors) responses were obtained from separate preparations.

Concentration-response curves for noradrenaline were determined in the absence and in the presence of L-NMMA (100  $\mu$ mol/l).

### **Chemicals**

All substances were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of the drugs were freshly prepared every day.

## **Data analysis**

All values are expressed as means  $\pm$  SEM. Contraction was expressed as a percentage of the response to KCl (100 mmol/l). Relaxation was expressed as a percentage of inhibition of noradrenaline-induced contraction. EC<sub>50</sub> values (concentration of agonist producing half-maximum effect) were expressed as pD<sub>2</sub> (-log EC<sub>50</sub>). The pD<sub>2</sub> values were compared by an unpaired *t* test and two-way analysis of variance (ANOVA). *n* values are presented as the number of patients. Statistical significance was accepted at *P*<0.05.

## Results

Acetylcholine caused concentration-dependent relaxations which were of greater magnitude in arteries from MT patients (Fig. 1, Table I). There was no significant difference in the response to the endothelium-independent vasodilator sodium nitroprusside (Table I). In endothelium-denuded rings the relaxant response to acetylcholine was abolished. L-NMMA reduced acetylcholine-induced relaxation in endothelium-intact rings; the inhibition was greater in arteries from MT patients (50% in MT vs 36% in E,  $P<0.05$ ). Indomethacin did not change the relaxation to acetylcholine.

The remaining endothelium-dependent relaxation after exposure to L-NMMA and indomethacin was further reduced by 20 mmol/l KCl or the combination of charybdotoxin and apamin whereas apamin with iberiotoxin had no significant effects (Fig. 2). Inhibition of relaxation by charybdotoxin and apamin was greater in arteries from MT patients compared to E patients (90% in MT versus 65% in E patients,  $P<0.05$ ). Incubation with either apamin, charybdotoxin, iberiotoxin or the blocker of ATP-sensitive  $K^+$  channels glibenclamide had no effect on acetylcholine-induced relaxation (not shown).

There was a marked hyporesponsiveness of thyroid arteries from MT patients to noradrenaline (Fig. 3, Table I). L-NMMA increased maximal contractile responses in arteries from the two groups; however, the increment was more pronounced in arteries from MT patients (135% in MT vs 35% in E,  $P<0.05$ ). There was no significant difference in the contractile response to 100 mmol/l KCl between arteries from E and MT patients ( $1950\pm 221$  mg vs  $2092\pm 215$  mg;  $n=12$  for each group;  $P>0.05$ ).

Methimazole had no effect *per se* in arteries from E and MT patients. This was assessed by the absence of effects of methimazole (1  $\mu$ mol/l) on acetylcholine-induced

relaxation (Table I) and by the lack of relaxing effect (less than 10%) of methimazole (10 nmol/l to 5  $\mu$ mol/l) on arteries contracted with noradrenaline.

## Discussion

The present results in human thyroid arteries reveal that the relaxation to acetylcholine was significantly greater in arteries from MT patients. In addition, the reduction by L-NMMA of the acetylcholine-induced relaxation was higher in arteries from MT patients, thus suggesting that a greater release of NO was involved. The remaining relaxation, insensitive to L-NMMA and indomethacin, may result from the release by acetylcholine of EDHF (8). The involvement of NO and EDHF in acetylcholine-induced relaxation has been recently observed in the isolated renal artery from hyperthyroid rats (5).

Our results in thyroid arteries from patients indicate that the EDHF component involves activation of  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels sensitive to charybdotoxin and apamin, a finding similar to that observed in thyroid arteries from donors (7). The nature and mechanisms of action of EDHF remain unknown. Several candidates for EDHF have been proposed including endothelium-derived  $\text{K}^+$  ions (9), epoxyeicosatrienoic acid (10), hydrogen peroxide (11) and C-type natriuretic peptide (12). In human left internal mammary artery, EDHF is 11,12-epoxyeicosatrienoic acid produced by one or more isoforms of cytochrome P450 (CYP450), and accounts for 40% of endothelium-dependent relaxation (13). Experiments in liver microsomes suggest a metabolism-dependent inhibition of P450 enzymes by methimazole (14,15). In the present study we did not determine whether cytochrome P450 enzymes play a role in endothelium -dependent relaxation of human thyroid arteries and therefore have no direct evidence that long treatment with methimazole inhibited these enzymes. However, if methimazole was an EDHF inhibitor in our experiments, the acetylcholine relaxation insensitive to L-NMMA and indomethacin should have been reduced in thyroid arteries from MT patients.

An important feature of the present results is that although the hyperthyroid patients were euthyroid at the time of surgery, the responsiveness of thyroid arteries was clearly abnormal. This implies that abnormal endothelium-dependent responses of arteries from MT patients can be observed when plasma levels of thyroid hormones have been corrected by treatment. The observation, however, does not rule out a possible relationship between thyroid hormone levels and endothelium dysfunction in other vascular beds. In relation to this, it has been shown that the abnormal responsiveness of human forearm resistance vessels of hyperthyroid patients is corrected when euthyroidism is restored by medical treatment (6). In support of our results in thyroid arteries, an increased blood flow of the superior thyroid artery has been demonstrated in patients with Graves disease, maintained in a euthyroid status with methimazole, prior to thyroid surgery (16). The increase in blood flow in the superior thyroid artery is positively correlated with microvessel density (17) and with vascular endothelial growth factor expression in hyperplastic follicular cells (18). These previous reports, together with the present results, indicate that vascularity in the thyroid gland does not parallel the methimazole-induced normalization of thyroid hormone levels, and that responsiveness of thyroid vessels does not follow the pattern of forearm resistance vessels. The abnormal response of thyroid vessels could be the result of sustained vascular expansion and endothelial cell proliferation caused by exposure to excess thyroid hormones during the period of time preceding medical therapy. These changes would lead to overproduction of NO and EDHF. In support of this proposal it has been shown in patients undergoing thyroid surgery that NO synthase is expressed in thyroid vascular endothelial cells and thyroid follicular cells and its levels are higher in hyperthyroid patients (euthyroid at the time of surgery) compared with euthyroid patients (19). Recent experiments in the rat have shown that

after 8 weeks treatment with  $T_3$ , endothelial NO synthase expression was markedly enhanced in the aorta and was associated with an increase in NO-mediated relaxation in the renal artery (5). These effects are probably due to the increased fluid shear stress at the endothelial surface (20). In contrast to the results observed in the rat renal artery (5) EDHF-mediated relaxation was enhanced in MT patients. This may also be the consequence of a chronic elevation in shear stress, as this stimulus is known to enhance the expression of potassium channels on endothelial cells (21).

We observe a decreased contractile response to noradrenaline in arteries from MT patients. Inhibition of NO synthase augmented the contractile responses. This effect is attributed to the inhibition by L-NMMA of the depressant influence of endothelial NO released by noradrenaline through stimulation of  $\alpha_2$  adrenoceptors on endothelial cells (22) or through indirect mechanisms involving a signal conducted from smooth muscle to adjacent endothelial cells (23). The magnitude of the effect of L-NMMA on contraction elicited by noradrenaline was greater in arteries from MT patients thus indicating increased endothelial NO production. It is possible that EDHF is also formed after noradrenaline-induced contraction (24). The study of this possibility has not been attempted in the present report.

The design of our in vitro experiments does not allow to study long-term effects of methimazole. Under conditions of acute exposure, methimazole failed to induce changes in contracted and noncontracted arteries. The range of concentrations of methimazole used ( $10^{-8}$  –  $3 \times 10^{-6}$  mol/l) include the mean intrathyroid methimazole concentration (300 ng/g of thyroid tissue) determined, prior to subtotal thyroid resection, in patients with Graves' disease (euthyroid at the time of surgery) treated with methimazole for 11.7 months (25).

In conclusion, the results indicate: (1) thyroid arteries from MT patients (euthyroid at time of surgery) show increased relaxation to acetylcholine and decreased contraction to noradrenaline due to overproduction of NO; (2) EDHF plays a prominent role in acetylcholine-induced relaxation through activation of Ca<sup>2+</sup>-activated K<sup>+</sup> channels; (3) the abnormal endothelium-dependent responses in arteries from MT patients are not corrected by medical treatment.

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**Table I.-** pD<sub>2</sub> and maximal responses values for acetylcholine, sodium nitroprusside and noradrenaline in arteries from euthyroid (E) and methimazole treated (MT) patients

Treatment	E		MT	
	pD <sub>2</sub>	E <sub>max</sub> (%)	pD <sub>2</sub>	E <sub>max</sub> (%)
Acetylcholine				
Control	7.68±0.19	80±6 (9)	8.17±0.26†	96±2† (10)
L-NMMA	7.91±0.35	51±9* (7)	7.47±0.19*	48±8* (7)
Methimazole	7.58±0.20	82±8 (4)	8.21±0.35†	96±3† (4)
Sodium nitroprusside	8.16±0.46	100 (6)	8.28±0.34	100 (6)
Noradrenaline				
Control	5.83±0.18	96±8 (7)	5.45±0.11†	57±10† (7)
L-NMMA	6.01±0.10	130±13* (7)	5.53±0.16†	134±15* (7)

pD<sub>2</sub> , negative logarithm of molar concentration required to produce half-maximal response

\*  $P < 0.05$  versus control.

†  $P < 0.05$  versus euthyroid patients.

Number of patients in parenthesis.

## LEGENDS

Figure 1. Effects of endothelium removal ( $n=9$ ), indomethacin ( $n=7$ ), and L-NMMA ( $n=7$ ) on relaxation to acetylcholine in thyroid arteries from euthyroid (E) and methimazole treated (MT) patients.

Figure 2. Relaxation to acetylcholine in the absence (control,  $n=8$ ) and in the presence of KCl 20 mM ( $n=5$ ), apamin plus charybdotoxin ( $n=6$ ) and apamin plus iberiotoxin ( $n=6$ ) in thyroid arteries from euthyroid (E) and methimazole treated (MT) patients. L-NMMA and indomethacin were present throughout.

Figure 3. Contractile responses to noradrenaline in the absence (control,  $n=7$ ) and in the presence of L-NMMA ( $n=7$ ) in thyroid arteries from euthyroid (E) and methimazole treated (MT) patients.

Fig. 1

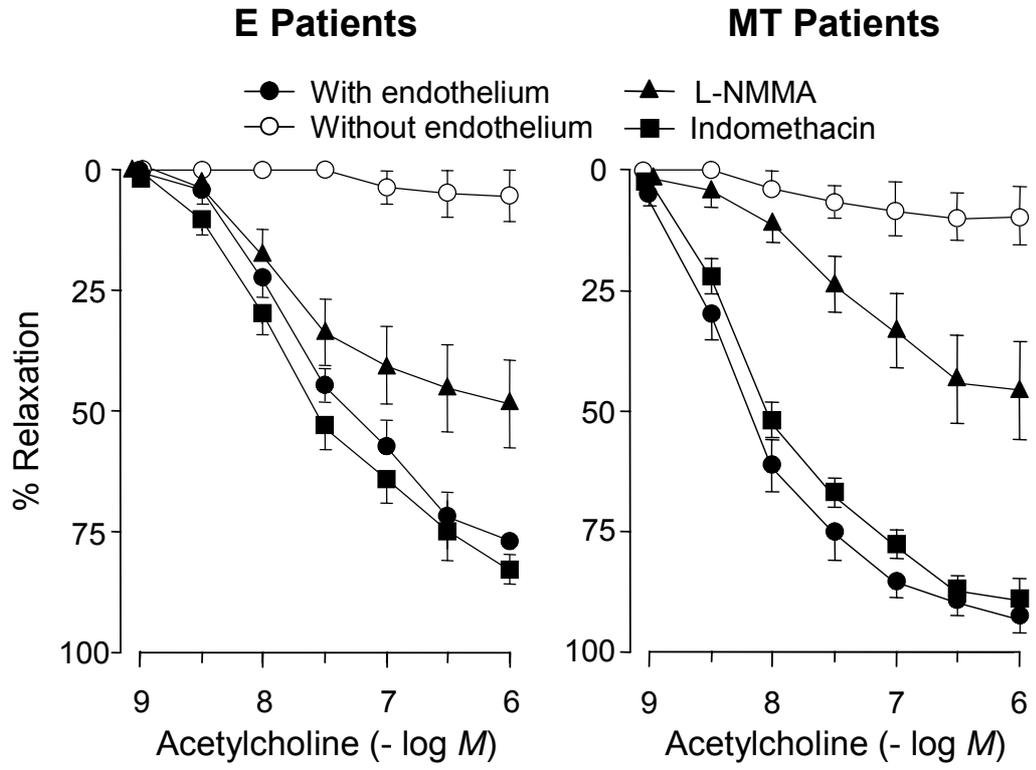


Fig. 2

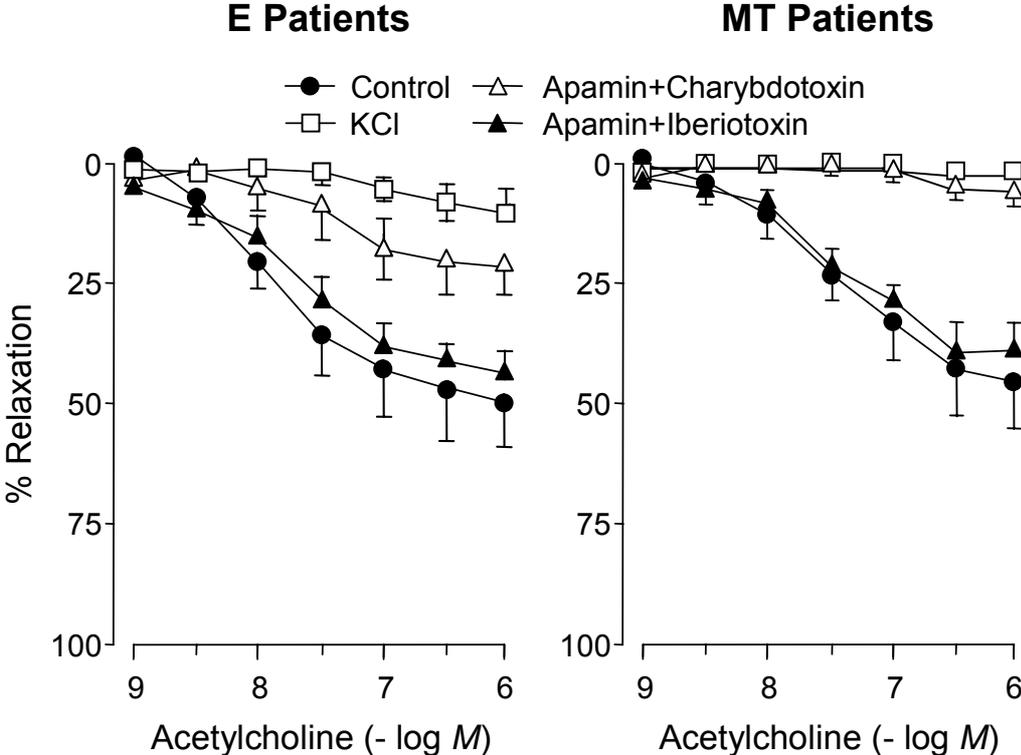


Fig. 3

