Endothelium-dependent responses in human isolated thyroid arteries from donors

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Abstract

The functional properties of the endothelium of human thyroid arteries remain unexplored. We investigated the intervention of nitric oxide (NO), prostacyclin (PGI₂) and endothelium-derived hyperpolarizing factor (EDHF) in the responses to acetylcholine and noradrenaline in isolated thyroid arteries obtained from multi-organ donors. Artery rings were suspended in organ baths for isometric recording of tension. The contribution of NO, PGI₂ and EDHF to endothelium-dependent relaxation was determined by the inhibitory effects of N^G-monomethyl-L-arginine (L-NMMA), indomethacin, and K⁺ channel inhibitors respectively. Acetylcholine induced concentration-dependent relaxation; this effect was not modified by indomethacin and was only partly reduced by L-NMMA, but was abolished in endothelium-denuded rings. The

relaxation resistant to indomethacin and L-NMMA was abolished by using either apamin combined with charybdotoxin, ouabain plus barium, or a high-K⁺ solution. Noradrenaline induced concentration-dependent contractions which were of greater magnitude in arteries denuded of endothelium or in the presence of L-NMMA.

In conclusion, the results indicate that in human thyroid arteries the endothelium significantly modulates responses to acetylcholine and noradrenaline through the release of NO and EDHF. EDHF plays a dominant role in acetylcholine-induced relaxation through activation of Ca²⁺-activated K⁺ channels, inwardly rectifying K⁺ channels and Na⁺-K⁺-ATPase.

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Introduction

The functional properties of the endothelium–nitric oxide (NO) system of human thyroid arteries remain largely unexplored. Nitric oxide synthase, the enzyme responsible for the formation of NO (Palmer *et al.* 1988) is present in the thyroid follicular cells and in endothelial cells of the human thyroid gland (Colin *et al.* 1997), and human thyrocytes produce the endogenous NO synthase inhibitor asymmetric-dimethyl-L-arginine (ADMA; Millatt *et al.* 2000). NO production, measured as plasma nitrite-plus-nitrate concentrations, is decreased in hyperthyroid patients when compared with controls (Hermenegildo *et al.* 2002). Hyperthyroid patients also revealed a significant increase in plasma levels of ADMA. Because ADMA increases the tone of peripheral vessels by inhibiting the basal release of NO from the endothelium (Vallance *et al.* 1989), it is conceivable that an increase in ADMA in hyperthyroidism might represent a compensatory mech-

anism to decrease NO production and, consequently, to counterbalance excessive peripheral vasodilatation (Hermenegildo *et al.* 2002). All these results are consistent with a role for NO in the vascular changes observed in thyroid dysfunction.

Receptor-dependent agonists, such as acetylcholine, induce endothelium-dependent smooth muscle relaxation by activating NO synthase and cyclooxygenase to produce NO and prostacyclin (PGI₂) respectively (Lüscher & Vanhoutte 1990, Suzuki & Cheng 1990, Cohen & Vanhoutte 1995, Shimokawa & Takeshita 1995, Vanhoutte 1996). In addition, after inhibition of NO synthase and cyclooxygenase, stimulation of the endothelium evokes smooth muscle relaxation that has been attributed to a factor called endothelium-derived hyperpolarizing factor (EDHF). Although the nature of EDHF is not known, experimental evidence suggests that the action of EDHF involves activation of endothelial K⁺ channels that are inhibited by the combination of apamin and

charybdotoxin (Petersson *et al.* 1997, Zygmunt *et al.* 1997, Edwards *et al.* 1998, Doughty *et al.* 1999). This implicates a role for small- and intermediate-conductance Ca^{2+} -activated K^+ channels (Zygmunt 1996) in endothelium-dependent relaxation. In addition, the release of EDHF leads to activation of smooth muscle inwardly rectifying K^+ channels and Na^+/K^+ -ATPase pumps (Edwards *et al.* 1998). The aim of the present study was to detect the respective intervention of endothelium-derived NO, PGI_2 and EDHF in the responses to acetylcholine and noradrenaline in human isolated thyroid arteries obtained from multi-organ donors.

Materials and Methods

Glandular branches of the superior thyroid artery were obtained from 14 multi-organ donors during procurement of organs for transplantation (ten men and four women; age range: 22–55 years; cause of death: nine head trauma, three intracranial hemorrhage, two myocardial infarction). The study was approved by the ethical committee of our institution. The vessels were immediately placed in a petri dish containing refrigerated (4°C) modified Krebs–Henseleit solution of the following composition (in mmol/l): NaCl, 115; KCl, 4.6; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.2; CaCl_2 , 2.5; NaHCO_3 , 25; glucose, 11.1; disodium EDTA, 0.01.

Experiments started within 8 h after organs were removed. Arteries were cleaned of connective tissue and cut into rings (3 mm in length) under a dissecting microscope. In some experiments, the endothelium was removed mechanically by inserting a roughened stainless-steel wire into the lumen and gently rolling the vessel ring on wet filter paper. Each ring was suspended between two stainless-steel L-shaped pins in 4-ml organ baths containing modified Krebs solution. The solution was equilibrated with 95% oxygen and 5% carbon dioxide to give a pH of 7.3–7.4 and was maintained at 37°C with a circulating water jacket and a heat pump. One pin was fixed to the organ-bath wall and the other was connected to a strain gauge (model FT03; Grass Instrument Division of Astro-Med Inc, West Warwick, RI, USA). Changes in isometric force were recorded on a Macintosh computer (Apple Computer, Cupertino, CA, USA) by use of Chart version 3.4/s software and a MacLab/8e data acquisition system (ADInstruments, Mountain View, CA, USA). To establish the resting tension for maximal force development, we performed a series of preliminary experiments on thyroid artery rings which were exposed repeatedly to 100 mmol/l KCl. Basal tension was increased gradually until contractions were maximal. The optimal resting tension was 1 g. The rings were allowed to attain a steady level of tension during a 2- to 3-h accommodation period before testing. Functional integrity of the endothelium was confirmed routinely by the presence of relaxation induced

by acetylcholine (0.1 – $1\ \mu\text{mol/l}$) during contraction obtained with noradrenaline (1 – $3\ \mu\text{mol/l}$). Arteries in which the acetylcholine reversed the noradrenaline-induced tone by more than 70% were designated as endothelium intact and arteries in which acetylcholine caused less than 15% relaxation were designated as denuded (Martínez *et al.* 1994, Medina *et al.* 1997).

Relaxations to acetylcholine ($1\ \text{nmol/l}$ to $1\ \mu\text{mol/l}$) were investigated in arteries precontracted with noradrenaline to 40–60% 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 ($10\ \mu\text{mol/l}$) to inhibit the production of PGI_2 ; (3) in the presence of indomethacin and N^G -monomethyl-L-arginine (L-NMMA, $100\ \mu\text{mol/l}$) to inhibit the production of PGI_2 and NO synthase respectively; (4) in the presence of indomethacin, L-NMMA and KCl ($20\ \text{mmol/l}$) to inhibit the production of PGI_2 , NO synthase and K^+ channel activity respectively. To examine the nature of K^+ channel activation, concentration–response curves to acetylcholine were established in the presence of indomethacin plus L-NMMA combined with one of the following inhibitors: iberiotoxin ($0.1\ \mu\text{mol/l}$), an inhibitor of large-conductance Ca^{2+} -activated K^+ channels; charybdotoxin ($0.1\ \mu\text{mol/l}$), an inhibitor of both large- and intermediate-conductance Ca^{2+} -activated K^+ channels; apamin ($1\ \mu\text{mol/l}$), an inhibitor of small-conductance Ca^{2+} -activated K^+ channels, and glibenclamide ($10\ \mu\text{mol/l}$), a selective blocker of ATP-sensitive K^+ channels. Control (in the absence of inhibitors) and experimental (after incubation for 20 min with inhibitors) responses were obtained from separate preparations. In another set of experiments, we determined the effects of barium alone ($3\ \mu\text{mol/l}$, a blocker of inwardly rectifying K^+ channels) or combined with ouabain ($1\ \text{mmol/l}$, an inhibitor of Na^+/K^+ -ATPase).

Concentration–response curves for noradrenaline were determined in the absence and in the presence of L-NMMA ($100\ \mu\text{mol/l}$) in separate vascular preparations with or without endothelium.

Chemicals

All substances were purchased from Sigma.

Data analysis

All values are expressed as means $\pm$ S.E.M. Contractile effects were expressed as a percentage of the response to KCl ($100\ \text{mmol/l}$). Relaxation was expressed as a percentage of inhibition of noradrenaline-induced contraction. The concentrations of agonist producing half-maximum effect (EC_{50} values) were expressed as pD_2 ($-\log \text{EC}_{50}$). The pD_2 values were compared by an unpaired *t*-test

Figure 1 Concentration-response curves to acetylcholine in artery rings with ($n=7$) and without ($n=7$) endothelium and in the presence of L-NMMA (100 $\mu\text{mol/l}$, $n=6$) or indomethacin (10 $\mu\text{mol/l}$, $n=4$). Values are means $\pm$ S.E.M.

and two-way ANOVA. n values are presented as the number of donors. Statistical significance was accepted at $P < 0.05$.

Results

Acetylcholine caused concentration-dependent relaxations in arteries with an intact endothelium (Fig. 1). This relaxant effect was not modified by indomethacin and only partly reduced by L-NMMA ($P < 0.05$), but was abolished in endothelium-denuded rings (Fig. 1, Table 1).

The remaining endothelium-dependent maximal relaxation (E_{max}), resistant to indomethacin and NO synthase inhibition, was further reduced by pretreatment with 20 mmol/l KCl or the combination of charybdotoxin and apamin (Fig. 2; before treatment: pD_2 7.33 ± 0.20 , E_{max} $72 \pm 5\%$; after KCl: pD_2 7.30 ± 0.13 , $P > 0.05$, E_{max} $23 \pm 3\%$, $P < 0.05$; after charybdotoxin plus apamin: pD_2 7.45 ± 0.20 , $P > 0.05$, E_{max} $27 \pm 4\%$, $P < 0.05$). Incubation with charybdotoxin or apamin alone, or the

Table 1 pD_2 and maximal responses values for acetylcholine and noradrenaline

	pD_2	E_{max} (%)
Treatment		
Acetylcholine		
Control	7.76 ± 0.20	88 ± 3 (7)
L-NMMA	$7.33 \pm 0.11^*$	$72 \pm 5^*$ (6)
Noradrenaline		
Control	5.98 ± 0.29	102 ± 11 (7)
L-NMMA	$6.52 \pm 0.31^*$	$120 \pm 6^*$ (6)

Values are means $\pm$ S.E.M.

* $P < 0.05$ versus control.

Number of donors in parenthesis.

combination of apamin and iberiotoxin, did not change the acetylcholine-induced relaxation significantly ($P > 0.05$; Fig. 2). Glibenclamide, a blocker of ATP-sensitive K^+ channels, had no significant effect on acetylcholine-induced relaxation (data not shown).

The relaxation to acetylcholine in arteries exposed to L-NMMA plus indomethacin was slightly decreased in the presence of barium (Fig. 3; before barium: pD_2 7.33 ± 0.20 , E_{max} $72 \pm 5\%$; after barium, pD_2 7.15 ± 0.30 , $P > 0.05$, E_{max} $43 \pm 6\%$, $P < 0.05$). In the presence

Figure 2 Concentration-response curves to acetylcholine in the absence (control, $n=6$) and in the presence of KCl (20 mmol/l, $n=5$), charybdotoxin (0.1 $\mu\text{mol/l}$, $n=6$), apamin (1 $\mu\text{mol/l}$, $n=6$), apamin plus charybdotoxin ($n=6$) and apamin plus iberiotoxin (0.1 $\mu\text{mol/l}$, $n=6$). L-NMMA and indomethacin were present throughout. Values are means $\pm$ S.E.M.

Figure 3 Relaxation to acetylcholine in the absence (control, $n=7$) and in the presence of barium ($3 \mu\text{mol/l}$, $n=5$) and barium plus ouabain (1 mmol/l , $n=5$). Indomethacin and L-NMMA were present throughout. Values are means $\pm$ S.E.M.

of barium plus ouabain the relaxation to acetylcholine was further reduced (E_{max} $12 \pm 6\%$, $P < 0.05$).

There was no significant difference in the contractile response to 100 mM KCl between intact and endothelium-denuded artery rings (2162 ± 270 vs $2475 \pm 225 \text{ mg}$, $n=6$ for each group, $P > 0.05$). Noradrenaline produced concentration-dependent contractions which were of greater magnitude in arteries without endothelium (Fig. 4, Table 1). Treatment with the NO synthase inhibitor L-NMMA induced a parallel leftward shift (about 3 times) ($P < 0.05$) of the response to noradrenaline in arteries with endothelium and did not modify significantly ($P > 0.05$) the concentration-response curve to noradrenaline in arteries without endothelium (not shown). The potentiation induced by L-NMMA in arteries with endothelium was completely reversed by L-arginine (1 mmol/l , Fig. 4).

Discussion

The present study in human thyroid arteries demonstrates that the endothelium modulates the responses to acetyl-

Figure 4 Concentration-response curves to noradrenaline in artery rings with ($n=7$) and without ($n=6$) endothelium and in the presence of L-NMMA ($100 \mu\text{mol/l}$, $n=6$) and L-NMMA plus L-arginine (1 mmol/l , $n=5$). Values are means $\pm$ S.E.M.

choline and noradrenaline. The relaxation to acetylcholine appears to be endothelium dependent because mechanical removal of the endothelium inhibited the response. The findings of the present study indicate that the order of pD_2 values for noradrenaline and acetylcholine in thyroid arteries corresponds to those determined in other human arteries (Martinez *et al.* 1994, Medina *et al.* 1997, Segarra *et al.* 2002).

Treatment with L-NMMA, an inhibitor of NO synthase (Rees *et al.* 1989, 1990) reduced the relaxation to acetylcholine in arteries with endothelium thus indicating the intervention of NO in this effect. However, the NO component, sensitive to L-NMMA, accounted for only 20% of the maximum relaxation to acetylcholine. Moreover, treatment with the cyclooxygenase inhibitor indomethacin did not change the endothelium-dependent relaxation, indicating that PGI_2 does not modulate the relaxation response under these conditions. Therefore, the remaining relaxation, insensitive to L-NMMA and indomethacin, appears to be the major component of endothelium-dependent relaxation. This NO/ PGI_2 -independent relaxation may result from the release by acetylcholine of EDHF (Feletou & Vanhoutte 1988, Taylor & Weston 1988). The large EDHF-induced

relaxation in response to acetylcholine observed in the thyroid artery is comparable to that shown in the radial artery (Segarra *et al.* 2000) and in subcutaneous resistance arteries (Coats *et al.* 2001).

There is no clear agreement on the identity or the mechanisms by which EDHF relaxes vascular smooth muscle. In humans, the existence of EDHF has been demonstrated *in vitro* and *in vivo* (Urakami-Harasawa *et al.* 1997, Miura *et al.* 1999, Coats *et al.* 2001, Katz & Krum 2001). Several candidates for EDHF have been proposed including endothelium-derived K^+ ions (Edwards *et al.* 1998), epoxyeicosatrienoic acid (Campbell *et al.* 1996, Fisslthaler *et al.* 1999) hydrogen peroxide (Matoba *et al.* 2000, 2002, Yada *et al.* 2003), anandamide (Randall *et al.* 1996) and C-type natriuretic peptide (Chauhan *et al.* 2003). Experimental evidence in small arteries suggests that receptor-dependent agonists, such as acetylcholine, may induce the release of EDHF by opening endothelial K^+ channels that are sensitive to charybdotoxin plus apamin but are unaffected by iberiotoxin alone or iberiotoxin combined with apamin (Edwards *et al.* 1998, Doughty *et al.* 1999, Coats *et al.* 2001, Scotland *et al.* 2001). These results in small arteries indicate that large-conductance, Ca^{2+} -activated K^+ channels are not involved in the acetylcholine-induced release of EDHF. Our results in relatively large human thyroid arteries indicate that the EDHF component involves activation of Ca^{2+} -dependent K^+ channels, sensitive to charybdotoxin and apamin, a finding similar to that observed in human subcutaneous arteries (Coats *et al.* 2001).

In certain vascular preparations, EDHF stimulates smooth muscle inwardly rectifying K^+ channels and $Na^+-K^+-ATPase$ (Edwards *et al.* 1998). This induces hyperpolarization and relaxation. In our experiments, the combination of barium plus ouabain abolished the relaxation resistant to L-NMMA and indomethacin. These results indicate that EDHF released by acetylcholine is associated with activation of inwardly rectifying K^+ channels and $Na^+-K^+-ATPase$.

We observe that treatment with L-NMMA, an inhibitor of NO synthase, augmented the contractile responses to noradrenaline. Inhibitors of NO synthase have been previously reported to increase noradrenaline-induced constriction in several experimental preparations (Angus *et al.* 1986, Jones *et al.* 1993, Martínez *et al.* 1994, Segarra *et al.* 1999). This effect is attributed to the inhibition by L-NMMA of the depressant influence of endothelial NO released by noradrenaline through stimulation of α_2 adrenoceptors on endothelial cells (Angus *et al.* 1986) or through indirect mechanisms involving a signal conducted from smooth muscle to adjacent endothelial cells (Dora *et al.* 1997). It has been hypothesized that the rise in smooth muscle cell Ca^{2+} generates a diffusion gradient that drives Ca^{2+} through myoendothelial cell junctions and into the endothelial cells, thereby causing the synthesis of NO (Dora *et al.* 1997). It is also possible that the EDHF is

also formed after noradrenaline-induced constriction. The study of this possibility has not been attempted in the present report. However, experiments in rat small mesenteric arteries have shown that the release of EDHF can be evoked indirectly by stimulation of α_1 adrenergic receptors on the smooth muscle cells (Dora *et al.* 2000).

The present results may have implications for understanding how the increased release of endothelium-derived NO and EDHF might affect tissue perfusion in the thyroid gland. Thyroid vascular expansion and endothelial cell proliferation in goitrous rats are significantly inhibited after treatment with the NO synthase inhibitor L-NMMA (Colin *et al.* 1997), thus suggesting a role for NO in the abnormal vascular changes in thyroid hyperplasia. It remains to be determined whether EDHF, due to its powerful relaxation effects, may represent a novel mechanism for regulating vascularity and maintaining a high tissue perfusion in the thyroid gland.

In conclusion, we have demonstrated that in human thyroid arteries the endothelium significantly modulates responses to acetylcholine and noradrenaline through the release of NO and EDHF. EDHF-mediated relaxation involves activation of charybdotoxin- and apamin-sensitive K^+ channels.

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