EXPERIMENTAL STUDY

Thyrocyte release of asymmetric dimethylarginine does not account for human thyrocyte inhibition of endothelial cell cyclic GMP

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Abstract

Background: The thyroid gland produces and responds to the signalling molecule nitric oxide (NO). The activity of NO synthase (NOS) may be regulated by endogenous NOS inhibitors such as asymmetric dimethylarginine (ADMA).

Objective: To investigate whether human thyrocytes are capable of regulating NOS activity via the production of ADMA.

Design: Human thyrocytes were incubated with human umbilical vein endothelial cells (HUVEC) in order to determine the effect on HUVEC NOS activity. HUVEC cGMP production over a 3-h period was measured as an indicator of NOS activity in the absence and presence of thyrocytes. To determine thyrocyte production of ADMA, samples of conditioned media were analysed by HPLC.

Results: The presence of primary human thyrocytes or immortalized human thyrocyte SGHTL-189 cells caused a significant inhibition of both basal (approximately 57% inhibition) and thrombin-stimulated (approximately 42% inhibition) HUVEC cGMP production. Both primary human thyrocytes and SGHTL-189 cells released ADMA (approximately 0.28 mg per 10^6 thyrocytes over a 3-day period). However, excess L-arginine, the natural substrate for NOS, was unable to overcome thyrocyte inhibition of HUVEC cGMP production.

Conclusion: These data indicate that human thyrocytes potently reduce endothelial cell cGMP concentrations, and that thyrocytes produce the endogenous NOS inhibitor, ADMA. However, the inhibition of endothelial cGMP is not mediated via thyrocyte production of a competitive NOS inhibitor.

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Introduction

Nitric oxide (NO), a ubiquitous free radical signalling molecule, is synthesized endogenously from l-arginine and molecular oxygen, in a reaction catalysed by the enzyme, NO synthase (NOS) (1). Three major NOS isoforms have been identified: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Many of the actions of NO are mediated via activation of the enzyme, soluble guanylyl cyclase (sGC), leading to increased production of the intracellular messenger, cyclic guanosine 3',5'-monophosphate (cGMP).

The thyroid gland is a highly vascularized organ, in which an extensive network of blood capillaries surrounds each thyroid follicle. Blood flow through the gland is tightly regulated, and plays an essential role in the maintenance of thyroid function. Thyroid hypertextrophy is commonly associated with prominent neovascularization and increased thyroid blood flow (2, 3). In such situations, endothelial cell proliferation occurs before the proliferation of follicular cells (4), suggesting that vascularization is necessary to sustain thyroid growth.

Nitric oxide is one of the factors that may play a part in the regulation of thyroid vascularity and blood flow. All three NOS isoforms are present within the rat thyroid gland (5), and primary human thyrocytes express the iNOS isoform after cytokine stimulation (6). Immunostaining for the eNOS isoform has been demonstrated in the thyroid follicular cells, in addition to the endothelial cells, of the human thyroid gland (7). Colin et al. (5) reported increased expression of both eNOS and nNOS in rats after goitre induction. Moreover, increased thyroid eNOS expression was reported in hyperthyroid patients, suggesting a possible role for NO in thyroid growth (7).

The activities of the three NOS isoforms can be competitively inhibited by l-arginine analogues such as N\(^\gamma\)-monomethyl-l-arginine (l-NMMA) and N\(^\delta\)N\(^\gamma\)-dimethyl-l-arginine (asymmetric dimethylarginine, ADMA).
ADMA, both of which are found in human plasma (8, 9). ADMA and its enantiomer, N^G-dimethyl-L-arginine (symmetric dimethylarginine, SDMA), are present in equimolar concentrations in human plasma (9), but SDMA does not inhibit NOS activity. Concentrations of both ADMA and SDMA increase in the plasma of patients with renal failure, and ADMA can reach concentrations sufficient to inhibit NOS activity (9). Increased plasma concentrations of ADMA have also been reported in preeclampsia (10), congestive heart failure (11), and hypercholesterolaemia (12), and may contribute to the hypertension and endothelial dysfunction seen in these diseases.

As NO may play an important part in thyroid vascularity and growth, we investigated whether thyrocytes are themselves capable of regulating NOS activity. We show by co-culture experiments with human thyrocytes and endothelial cells that thyrocytes profoundly inhibit endothelial cell production of cGMP. Moreover, we demonstrate that human thyrocytes produce the NOS inhibitor, ADMA. However, ADMA does not appear to be responsible for the inhibitory effect of thyrocytes on endothelial cell cGMP production.

Materials and methods

Cell culture

Approval for the use of human thyroid tissue and human umbilical cords was obtained from the local Ethics Committee. Primary cultures of human thyrocytes were prepared by dispase type II digestion of surgical specimens from patients with multinodular goitre or Graves’ disease, as previously described (13). Primary thyrocytes were maintained in monolayer culture in Ham’s F10 medium containing 5% (v/v) newborn calf serum, 10 µg/ml insulin, 10 ng/ml somatostatin, 10 ng/ml glycine-histidine-lysine acetate, 3.6 ng/ml hydrocortisone, and 5 µg/ml transferrin (5H medium) (14). For co-culture studies, primary thyrocytes with the plasmid, pSV3neo, produced by electroporation-mediated transfection of human thyrocytes from a different partial thyroidectomy. The ment was performed with a fresh isolate of primary thyrocytes with the plasmid, pSV3neo, produced by electroporation-mediated transfection of primary human thyrocytes. SGHTL-189 cells were prepared from fresh umbilical cords by the method of Jaffe et al. (17), and cultured as previously described (18). Upon reaching confluence, HUVEC were passaged 1:4 once a week.

Human umbilical vein endothelial cells (HUVECs) were prepared from fresh umbilical cords by the method of Jaffe et al. (17), and cultured as previously described (18). Upon reaching confluence, HUVEC were passaged and seeded into 24-well tissue culture plates at a plating density of 5 x 10^4 cells/well. They were grown to confluence, then used for co-culture experiments at passage 1.

Chinese Hamster Ovary (CHO) cells (European Tissue Culture Collection, Porton Down, Wilts, UK) were maintained in monolayer culture in Medium 199 containing 5% (v/v) foetal calf serum, and were passaged 1:5 twice weekly.

Co-culture experiments

Co-culture procedure To potentiate the extracellular release of cGMP, all experiments were carried out in a hypotonic buffer consisting of 20 mmol/l HEPES, 25 mmol/l NaCl, 5 mmol/l KCl, 1.3 mmol/l CaCl_2, 0.44 mmol/l KH_2PO_4, 0.4 mmol/l MgSO_4, 0.34 mmol/l Na_2HPO_4, pH 7.4, with the addition of 0.1% (w/v) glucose, 0.1% (w/v) bovine serum albumin, and 0.3 mmol/l isobutylmethylxanthine (IBMX) (19).

Before co-culture experiments, confluent HUVEC were washed with phosphate-buffered saline (PBS), then pre-incubated for 15 min at 37°C in HEPES buffer containing 45 U/ml superoxide dismutase (SOD) and, where appropriate, 5 mmol/l l-arginine. SOD was included in all incubations to minimize the breakdown of NO by its reaction with superoxide free radicals (20).

Meanwhile, primary human thyrocytes or SGHTL-189 cells were removed from confluent 9 cm dishes, and resuspended in HEPES buffer, in which they were pre-incubated for 15 min. During pre-incubation, the thyrocytes were counted using a haemocytometer, and thyrocyte suspensions were diluted to the cell density appropriate for the experiment (4 x 10^5–2 x 10^6 cells/ml). After pre-incubation, additions of 45 U/ml SOD in the absence or presence of 5 mmol/l l-arginine, 5 U/ml thrombin, or both, were made to aliquots of the thyrocyte cell suspension, as appropriate. After removal of the HEPES buffer used for pre-incubation of the HUVEC, aliquots of thyrocytes (250 µl/well) were added to the wells containing HUVEC. In addition, HUVEC in other wells were incubated in the absence of thyrocytes, with HEPES buffer plus additions (SOD in the absence or presence of l-arginine, thrombin, or both) only.

The co-culture period was 3 h at 37°C, after which the incubation buffer was removed from each well and centrifuged at 1700 g for 15 min. Supernatants were then stored at −20°C until the determination of cGMP content. Similar experiments were performed using CHO cells in place of thyrocytes.

Cyclic GMP radioimmunoassay Measurement of extracellular cGMP in samples from co-culture experiments was performed by radioimmunoassay, using a rabbit polyclonal cGMP antiserum (a kind gift from Dr B C Williams, Western General Hospital, Edinburgh, UK). The sensitivity limit of the assay was approximately 20 fmol/assay tube, equivalent to 100 fmol/well. The inter- and intra-assay coefficients of variation were less than 10% over the linear range of the assay.
Cross-reactivity studies with the cGMP antiserum indicated 0.013% cross-reactivity with cyclic adenosine 3',5'-monophosphate (cAMP) (personal communication, Dr B C Williams). 

**Determination of dimethylarginine production**

The concentration of ADMA and SDMA in conditioned media from primary thyrocytes and SGHTL-189 cells was determined by high performance liquid chromatography (HPLC) (18, 21).

**Collection of conditioned medium** Sub-confluent monolayer cultures of primary human thyrocytes and SGHTL-189 cells in 9 cm dishes were washed with PBS, and the medium was replaced with 10 ml fresh 5H medium per plate. The cells were then incubated at 37°C for up to 7 days, during which time the medium remained unchanged. At the appropriate time-points, conditioned medium was collected from the plates, centrifuged at 1700 g for 10 min, and the supernatant stored at −20°C. Samples (10 ml) of unconditioned 5H medium were also collected. After removal of the conditioned medium, thyrocytes were washed with PBS, detached from the plate using trypsin/EDTA, and counted using a haemocytometer. Samples of 3-h conditioned HEPES buffer were collected in the same manner from confluent 9 cm dishes of thyrocytes.

**Partial purification of methylarginines** Amino acids were extracted from conditioned media samples by passage through BondElut SCX columns (Anachem, Luton, Beds, UK) and elution with ammonia/methanol, as previously described (9). Before extraction, l-homoarginine (0.5 μg) was added to each sample to determine the extraction efficiency of the SCX columns.

**HPLC analysis** Separation of amino acids was performed as previously described (18, 21). Samples of standard solutions of l-homoarginine, ADMA, and SDMA were included amongst each set of samples.

**Analysis of HPLC results** The area under each HPLC peak was calculated by computerized integration after calibration with the authentic standards. From the peak area of the l-homoarginine added to the experimental samples, the extraction efficiency of each SCX column was determined and used to calculate the ADMA or SDMA content of each sample. Results were expressed either as μg ADMA or SDMA per ml conditioned medium, or were corrected for cell number and expressed as μg ADMA or SDMA produced per 10⁶ thyrocytes.

**Statistical analysis** All co-culture studies were performed in triplicate, and all experimental samples were assayed in duplicate. Data are reported as mean ± S.E.M. Statistical significance was assessed using the Student’s unpaired two-tailed t-test, or the Mann–Whitney test for data that were not normally distributed. Differences were considered significant where P < 0.05.

**Results**

**Effect of thyrocyte–endothelial cell co-culture on HUVEC cGMP production**

Co-culture experiments were performed in which increasing numbers of primary thyrocytes were incubated with confluent HUVECs for 3 h. The amount of cGMP produced by thyrocytes alone was very low (24.8 ± 3.1 fmol/well, n = 6 experiments); in contrast, HUVEC extracellular cGMP production was 399 ± 65 fmol/well (n = 3 experiments). In the presence of thyrocytes, HUVEC cGMP production was markedly reduced (Fig. 1); the percentage inhibition increased with the number of thyrocytes, and reached statistical significance at a primary thyrocyte density of 5 × 10⁴ cells/well. From haemocytometer counts of wells containing confluent HUVECs alone, this cell density was calculated to represent a ratio of two HUVECs per thyrocyte. The degree of thyrocyte inhibition of endothelial cell cGMP production reached a plateau of approximately 60% at a thyrocyte density of 5 × 10⁵ cells/well; this thyrocyte density was used for all subsequent studies.

Further experiments were performed in which confluent HUVECs were incubated with primary human...
thyrocytes or SGHTL-189 cells for 3 h, in either the absence or the presence of thrombin (5 U/ml), as indicated. HUVECs were incubated alone (open bars) or in the presence of 5×10⁵ primary human thyrocytes (hatched bars) or SGHTL-189 cells (solid bars). Extracellular cGMP production was measured by radioimmunoassay. Results are expressed relative to basal HUVEC cGMP production, and are means±S.E.M. from at least three experiments. *P<0.001 compared with HUVECs only; †P<0.005 compared with HUVECs+thrombin (t-test).

Similar experiments were performed in which HUVECs were incubated for 3 h in the absence or the presence of eNOS activator, thrombin (5 U/ml). HUVEC production of cGMP increased by an average 3.5-fold in the presence of thrombin (Fig. 2). In contrast, the low basal level of thyrocyte cGMP production was unchanged by thrombin stimulation (data not shown). In the wells of HUVECs incubated with thyrocytes, both basal and thrombin-stimulated HUVEC cGMP production were significantly reduced (Fig. 2). Primary thyrocytes and SGHTL-189 cells inhibited HUVEC cGMP production to a similar degree (inhibition of basal cGMP: 61% and 54%, respectively; inhibition of thrombin-stimulated cGMP: 43% and 40%, respectively).

Similar experiments were performed in which HUVECs were incubated with CHO cells for 3 h in the absence or presence of thrombin (5 U/ml). The presence of CHO cells did not significantly affect either basal or thrombin-stimulated HUVEC cGMP production (Fig. 3).

HPLC analysis was performed on samples of 3-day conditioned culture medium from primary human thyrocytes and SGHTL-189 cells (Table 1). Unconditioned 5H medium contained small amounts of ADMA and SDMA, presumably originating from the serum in the medium. No significant increase in SDMA concentrations occurred after a 3-day incubation of the 5H medium with thyrocytes of either type. However, the amount of ADMA present in the 3-day conditioned medium from thyrocytes was significantly greater than that in unconditioned medium (average increases 8.2-fold and 6.4-fold, n=3, P<0.05, for primary thyrocytes and SGHTL-189 cells, respectively). In time-course experiments, the amount of ADMA produced by SGHTL-189 cells increased over a 7-day culture period, reaching significance compared with unconditioned medium at day 3 (Fig. 4). After 7 days in culture, the amount of ADMA produced was approximately 1 µg per 10⁶ cells.

Table 1

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<tr>
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<th>ADMA content (µg/ml)</th>
<th>ADMA content (µg/10⁶ cells)</th>
<th>SDMA content (µg/ml)</th>
<th>SDMA content (µg/10⁶ cells)</th>
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<tr>
<td>Unconditioned 5H medium</td>
<td>0.021 ± 0.006</td>
<td>–</td>
<td>0.017 ± 0.003</td>
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<tr>
<td>3-Day conditioned medium</td>
<td></td>
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<tr>
<td>Primary thyrocytes</td>
<td>0.172 ± 0.065*</td>
<td>0.266 ± 0.0004</td>
<td>0.032 ± 0.008</td>
<td>0.034 ± 0.006</td>
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<td>SGHTL 189 cells</td>
<td>0.134 ± 0.02*</td>
<td>0.283 ± 0.101</td>
<td>0.020 ± 0.002</td>
<td>0.027 ± 0.005</td>
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*P<0.05 compared with unconditioned 5H medium (Mann–Whitney test).
Further HPLC analysis was performed to determine whether ADMA was released by thyrocytes into the HEPES buffer during the 3-h co-culture period. Unconditioned HEPES buffer contained no detectable dimethylarginines (n=2). HEPES buffer that had been incubated with SGHTL-189 cells for 3 h also contained no detectable dimethylarginines (n=2). A 3-h incubation of HEPES buffer with primary thyrocytes resulted in the production of very small amounts of ADMA (0.003±0.002 μg/ml; 0.003±0.002 μg/10⁶ cells; n=3).

**Co-culture of HUVECs and thyrocytes in the presence of L-arginine**

To determine whether thyrocyte inhibition of endothelial cell cGMP production could be overcome by excess L-arginine, SGHTL-189 cells were incubated for 3 h with unstimulated and thrombin-stimulated HUVECs, both in the absence and in the presence of L-arginine (5 mmol/l). The presence of L-arginine had no significant effect on the degree of thyrocyte inhibition of HUVEC cGMP production in either the absence or the presence of thrombin (Fig. 5).

**Discussion**

The abundant blood supply of the thyroid gland is essential for the maintenance of thyroid function. In animal models of thyroid hypertrophy, the mitotic activity of the thyroid endothelial cells increases before thyrocyte proliferation (4). Moreover, during involvment of experimentally induced goitre, the thyroid vasculature regresses much earlier than the thyroid epithelium (22). These observations suggest the existence of a close relationship between thyroid follicular and endothelial cells, whereby each cell type may influence the proliferation, and perhaps the function, of the other cell type. In support of such a relationship are reports of endothelial cell growth factors released by thyroid cells (23, 24), and evidence suggesting that endothelial cells can stimulate the proliferation of thyroid epithelial cells (25).

The present report provides further evidence for the existence of a functional relationship between thyrocytes and endothelial cells. We found that human thyrocytes, but not CHO cells, potently inhibit endothelial cell production of cGMP, the downstream effector for many of the actions of endothelial-derived NO. Thyrocytes inhibited approximately 50% of endothelial cell cGMP production over a 3-h period, affecting both basal and thrombin-stimulated cGMP. As endothelial cell cGMP production was markedly increased in the presence of thrombin, a well-known activator of eNOS (26), it is highly likely that the cGMP measured reflects stimulation of sGC by NO, rather than stimulation of the particulate guanylyl cyclase by other mediators.

Increasing evidence suggests that the thyroid gland both produces and responds to NO. The rat thyroid gland expresses all three NOS isoforms (5), and eNOS expression has been demonstrated in human thyrocytes (7). We have previously shown that NO donors stimulate the production of cGMP in both primary human thyrocytes and SGHTL-189 cells, indicating that the NO-cGMP signalling pathway may play a role in thyroid function (16). Indeed, NO was shown to act
via cGMP to inhibit iodide uptake in primary cultures of calf thyrocytes (27). cGMP-independent effects of NO on iodine organification (6) and thyrocyte growth (28) have also been reported, and we have recently demonstrated that NO donors enhance thyroid peroxidase activity in primary human thyrocytes (29). Thus NO appears to act via multiple pathways to influence several aspects of thyroid function and growth.

A potential mechanism whereby thyroid NO production may be controlled is the synthesis of an endogenous NOS inhibitor such as ADMA. In the present study, both primary human thyrocytes and immortalized human thyrocyte SGHTL-189 cells were found to produce ADMA. The amount of ADMA produced by SGHTL-189 cells over a 7-day period was approximately 10.4 μg/10^7 thyrocytes, equivalent to 0.05 μmol/10^7 thyrocytes. The minimum concentration of ADMA reported to inhibit NOS activity in vitro is 2 μM (9, 18). It is not possible to predict the actual molar concentration of ADMA produced by thyrocytes within the thyroid gland. However, local or intracellular concentrations of ADMA, produced by thyrocytes in vivo may reach levels sufficiently high to affect the NOS activity of thyrocytes and thyroid endothelial cells. In this way, thyrocyte-derived ADMA, perhaps in conjunction with endothelial-cell-derived ADMA (18), may play a role in the regulation of thyroid function.

In spite of the demonstration of the production of ADMA by thyrocytes, ADMA does not appear to mediate the thyrocyte inhibition of endothelial cell cGMP production observed in the present study. The natural substrate for NOS, L-arginine (5 mmol/l), was unable to overcome the inhibitory effect, despite the fact that this concentration of L-arginine is more than 1000-fold greater than the published K_m for L-arginine of eNOS, 2.9 μmol/l (30). In addition, the amount of ADMA produced by thyrocytes over the 3-h period of the co-culture experiments was extremely small, and unlikely to be sufficient to cause the significant degree of inhibition of cGMP production observed. As a competitive NOS inhibitor does not appear to be involved, it seems most likely that thyrocytes produce a factor or factors that directly affect(s) the amount of cGMP produced by HUVECs in response to unchanged levels of NO production. Such a factor could act by inhibiting the activity of sGC, or by increasing the activity of cGMP phosphodiesterase (PDE), which breaks cGMP down to GMP. Although IBMX was included in all experiments, to eliminate PDE activity, it is possible that cGMP was degraded by thyrocyte production or activation of one of the IBMX-insensitive cyclic nucleotide PDEs (31). The gene expression of sGC has been shown to be downregulated in vascular smooth muscle cells by cAMP (32), the intracellular messenger produced by thyrocytes in response to thyroid-stimulating hormone (TSH). However, basal levels of cAMP production by both primary human thyrocytes and SGHTL-189 cells are very low (16). In addition, it seems unlikely that the inhibition of endothelial cell cGMP production observed in the present study was due to a change in gene expression, as the effect occurred rapidly over a 3-h period.

Human thyrocyte inhibition of endothelial cell cGMP production may have important implications for the control of thyroid vascularization. For example, studies have suggested a role for TSH-induced vascular endothelial growth factor (VEGF) in the abnormal proliferation of thyroid endothelial cells that occurs in goitre formation (24, 33). Importantly, the mitogenic effect of VEGF on cultured endothelial cells has been shown to be mediated via NO–cGMP activation of cGMP-dependent protein kinase (34). Therefore, by inhibiting endothelial cell production of cGMP in response to NO, thyrocytes may reduce VEGF-induced proliferation, and thus provide a feedback loop to limit thyroid vascularization.

In summary, we have shown that human thyrocytes have a potent inhibitory effect on endothelial cell production of cGMP. Although we demonstrated that human thyrocytes produce the endogenous NOS inhibitor, ADMA, the effect on cGMP production does not appear to be mediated by a competitive NOS inhibitor. However, the study provides evidence of a functional relationship between thyrocytes and endothelial cells, and this may have a role in regulating thyroid growth.

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References