Nitric oxide is involved in interleukin-1α-induced cytotoxicity in polarised human thyrocytes

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Abstract

Nitric oxide (NO) is a well-known mediator of autoimmune processes. In the thyroid gland, it is produced in response to interleukin 1 (IL-1) and may mediate cytokine action at an early stage of autoimmune thyroiditis. In this study, we have investigated whether NO is involved in cytokine-induced cytotoxic effects and epithelial barrier alterations in thyrocytes. Human thyroid epithelial cells were cultured as tight polarised monolayers on a permeable support and exposed or not to IL-1α (100 U/ml), alone or in combination with interferon-γ (IFN-γ; 100 U/ml) added to the basal compartment. NO production was not detected in control thyrocytes, but was significantly induced by the combination of IL-1α with IFN-γ, in a time-dependent fashion. Similarly, expression of the inducible isoform of nitric oxide synthase (NOSII), determined by immunoblot and immunofluorescence confocal microscopy, was not detected in control cells, but was markedly induced after 48-h exposure to both cytokines. This treatment significantly increased the release of cytosolic lactate dehydrogenase (LDH) in the apical and basolateral media and decreased transepithelial electrical resistance. Although IFN-γ was not sufficient to induce NO production, it could by itself decrease transepithelial resistance and synergised the IL-1α effect on LDH release. The NOS inhibitor, l-nitro-arginine-methyl ester, suppressed the cytokine-induced NO production and decreased the LDH release, but failed to prevent the loss of transepithelial resistance. These results indicated that human thyrocytes express NOSII and produce NO in response to IL-1α+IFN-γ and suggest that NO acts as a mediator of cytokine-induced cytotoxicity in the thyroid gland and may promote the exposure of autoantigens to the immune system. In contrast, NO does not appear to mediate the cytokine-induced disruption of the thyroid epithelial barrier.


Introduction

Hashimoto’s thyroiditis, a prototype of autoimmune disease, results from a complex multistage process involving many cell types, as well as a large array of cytokines (Weetman & McGregor 1994, Rasmussen et al. 2000). Among them, interleukin-1α (IL-1α), interferon-γ (IFN-γ), tumor necrosis factor-α and transforming growth factor-β have been reported to strongly modulate functions, morphology and growth of the thyroid gland (Weetman & McGregor 1994, Franzen et al. 1999, Gretzter et al. 2000, Rasmussen et al. 2000).

Some cytokine-induced effects in endocrine organs have been suggested to occur through nitric oxide (NO) production. This has been clearly shown in the endocrine pancreas where NO is involved as an important mediator of β-cell loss of function and reduced survival in response to cytokines (Southern et al. 1990, Bergmann et al. 1992, Corbett et al. 1992, 1993, Lindsay et al. 1995, Eizirik et al. 1996, Rabinovich & Suarez-Pinzon 1998). The thyroid tissue can also release NO in various conditions. Indeed, we have previously shown that the rat and human thyroid glands contain the enzymatic machinery for NO synthesis (Colin et al. 1995, 1997, 1998, Colin 1997). There is also evidence that increased production of NO at early stages of goitre formation could trigger initial steps leading to the expansion of the thyroid microvasculature (Colin et al. 1995, Gerard et al. 2000). In patients with Graves’ disease, the increased expression of the endothelial nitric oxide synthase III (NOSIII), particularly in thyrocytes, prompted the suggestion that the enlarged microcirculation observed in hyperfunctioning thyroid glands results from increased production of NO by epithelial cells (Colin et al. 1997). Likewise, we recently observed an increased
immunostaining for inducible nitric oxide synthase II (NOSII), predominantly localised in macrophages, in the early steps of iodine-induced thyroiditis in non obese diabetic mice, and suggested that NO overproduction could determine the evolution toward tissue destruction (Colin et al. 1998, Many et al. 2000).

Isolated thyrocytes in culture, although producing very low levels of NO under basal conditions, release substantial amounts of NO after challenge by pro-inflammatory cytokines (Rasmussen et al. 1994, Kasai et al. 1995, Motohashi et al. 1996, Reimers et al. 1996). Production of NO, induced by IL-1β in rat FRTL cells (Reimers et al. 1996) or by a combination of IL-1α with IFN-γ in human monolayers, follows NOSII mRNA expression (Kasai et al. 1995). Although NO does not mediate the IL-1-induced inhibition of thyroid cell function (Rasmussen et al. 1994, Reimers et al. 1996), its possible role in cytokine-induced disruption of the thyroid epithelial barrier and cytotoxic effects has not yet been clearly demonstrated.

In the present study, we aimed to delineate the cytotoxic effects of pro-inflammatory cytokines on human thyroid cells. Based on the likelihood that NO participates in thyroid autoimmune processes, we tested the hypothesis of the involvement of NO as a mediator of cytokine actions.

Materials and Methods

**Cell cultures**

Human thyroid cells were isolated according to the method of Nilsson et al. (1996). Thyroid tissue was obtained from patients undergoing surgery for thyroid nodule or, for results reported in Fig. 2, from euthyroid patients with Graves’ disease. Isolated thyrocytes were suspended in modified Earle’s medium (MEM) or in Coon’s modified Ham–F12 medium containing 5% foetal bovine serum, penicillin (50 U/ml), streptomycin (50 µg/ml) and fungizone (2–5 µg/ml) (BRL-Gibco, Paisley, Strathclyde, UK). Coon’s modified medium was enriched with five factors (5H medium: insulin, bovine transferrin, hydrocortisone, glycyl-l-histidyl-l-lysine acetate and somatostatin; all reagents from Sigma Chemical Co, St Louis, MO, USA). Thyrocytes (± 5 µg DNA/filter) were plated in bicameral culture chambers (Transwell 3413, 0·33 cm²; Costar, Cambridge, MA, USA) on filters precoated with type I collagen (Roche Diagnostics, Brussels, Belgium) and cultured in a humidified atmosphere (5% CO₂) with or without thyrotophin (TSH). Tightness of the polarised monolayers was assayed by measuring the transepithelial electrical resistance (RTE) with a millicl-ERS ohm–meter (Millipore, Bedford, MA, USA). Culture medium was changed every 2 or 3 days. Cells with a significant RTE (>300 Ω cm²) were used for experiments between 5 and 10 days after isolation.

**Nitrite assay**

Nitrite accumulation in the apical and basal media was measured by a colorimetric assay after the Griess reaction using a commercially available kit (Promega, Madison, WI, USA).

**Lactate dehydrogenase (LDH) enzyme release assay**

Cytotoxicity of cytokines was estimated by the activity of cytoplasmic LDH released in the apical and basolateral culture media. LDH activity was measured as the amount of pyruvate consumed, by monitoring the decrease in absorbance at 340 nm due to NADH oxidation.

**Western blot**

Filter-cultured thyrocytes were suspended in 375 mM NaCl, 125 mM Tris–HCl, 1 mM EGTA, containing protease inhibitors (Sigma). Proteins (20 µg/lane) were separated by SDS-PAGE (7·5%) and transferred to nitrocellulose sheets (Bio-Rad, Watford, Herts, UK). Efficiency of transfer was routinely tested by Ponceau red staining and β-actin immunoreactivity. After blocking with 5% fat-free milk, membranes were incubated overnight at 4 °C with mouse monoclonal antibodies (1:5000 dilution; Transduction Laboratories, Lexington, KY, USA) against NOSII or NOSIII. Sodium phosphate-buffered saline (PBS) containing 0·05% Tween 20 (pH 7·4) was used for blocking, antibody dilution and for washings after each step of incubation. Membranes were then incubated with horseradish peroxidase-conjugated rabbit antimouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), and visualised by enhanced chemiluminescence (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).

**Immunodetection of NOSII by confocal microscopy**

Thyrocytes cultured on Transwell-clear inserts (Costar 3470) were washed three times in PBS–Ca²⁺ (pH 7·4), fixed for 20 min in 4% paraformaldehyde in PBS, washed in PBS, preincubated for 1 h with blocking buffer, consisting of 1% bovine serum albumin, 1 mg/ml lysine, 0·01% saponin, 0·02% azide in PBS and permeabilised with 0·5% saponin for 30 min. The whole procedure was performed at room temperature. Cells were then incubated overnight at 4 °C with rabbit polyclonal anti-NOSII antibody (1:100 dilution in blocking buffer; Transduction Laboratories) and incubated for 1 h with Alexa 488-conjugated goat antirabbit IgG (1:1000 dilution; Molecular Probes, Leiden, The Netherlands). Filters were mounted on glass with Moviol (Calbiochem, La Jolla, CA, USA) and examined with a confocal microscope (Bio–Rad MRC 1024).


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Other reagents

Recombinant human IFN-γ was purchased from Promega, recombinant human TSH from Genzyme (San Carlos, CA, USA), recombinant human IL-1α from R&D Systems (Abingdon, Oxon, UK), bovine TSH, NADH and L-nitro-arginine-methyl ester (L-NAME) from Sigma.

Data analysis

Data are means ± S.E.M. of quadruplicates. All experimental procedures were repeated at least twice. Statistical analyses were performed using Student’s t-test and P<0.05 was considered statistically significant.

Results

Evidence for a cytokine-stimulated NOS-dependent pathway in polarised human thyrocytes

To examine whether NO acts as a mediator of some cytokine effects, polarised human thyrocytes were incubated for 48 h with 100 U/ml IL-1α, alone or in combination with 100 U/ml IFN-γ, a concentration known to induce maximal nitrite production (Kasai et al. 1995). Co-incubation with L-NAME, a competitive non-specific inhibitor of all NOS isoforms, was used to test for the suppression of the cytokine-induced effects (Fig. 1). Production of nitrite, the stable end-product of NO generation, was observed neither in basal conditions nor in IFN-γ-conditioned media. In contrast, nitrite release was induced after IL-1α treatment, and synergised by IFN-γ. In the presence of L-NAME, nitrite accumulation was greatly reduced, indicating that IL-1α, alone or in combination with IFN-γ, induces the release of NO from human thyroid cells through an l-arginine/NOS-dependent pathway.

To investigate whether the cytokine-induced NO release was related to the thyroid gland, cells were incubated for 2 days with IL-1α+IFN-γ, without or with 0·1 mU/ml TSH, or 2 mM methyl-mercapto-imidazole (MMI) (Fig. 2). Nitrite accumulation was not modified, indicating that the cytokine-induced NOS pathway is independent of TSH or of thyroperoxidase activity. In this experiment, 7·5 mM L-NAME induced per se a release of nitrite. We therefore used a lower concentration (2·5 mM) in further experiments.

We previously found that the three NOS isoforms are expressed in the rat thyroid gland. Among them, the inducible isoform NOSII exhibited the lowest level of basal expression (Colin et al. 1995). To examine the possibility that NOSII might be involved in the cytokine-induced release of NO, we examined by immunofluorescence and confocal microscopy the effects of IL-1α+IFN-γ on NOSII expression (Fig. 3). No immunostaining was seen in control polarised thyrocytes. On the contrary, in cells incubated for 48 h with the two cytokines, an intense and diffuse cytosolic signal was found, demonstrating the induction of NOSII expression. These results were confirmed by Western blotting (Fig. 4). A band of 130 kDa corresponding to NOSII was clearly detected in lysates of thyrocytes incubated in the presence of 0·1 mU/ml TSH with IL-1α (Fig. 4, lane 3), especially when combined with IFN-γ (Fig. 4, lane 4). In contrast, there was no visible signal in lysates of control cells, or cells incubated with IFN-γ alone (Fig. 4, lane 2). Comparable, if not stronger, results were obtained in the presence of L-NAME. Higher concentrations of TSH (10 mU/ml) during the last 2 days did not modify the cytokine-induced signal (Fig. 4, lanes 9–10). These results indicate that IL-1α induces NOSII expression in human thyroid cells and that IFN-γ exerts a synergistic effect, independently of TSH-activated intracellular pathways. They also suggest a short-loop negative feedback regulation between NO and the expression of NOSII. Parallel blots were probed with an anti-NOSIII antibody to verify the specificity of the induction. Results were negative in all conditions (not shown).

Cytokine-induced alteration in the epithelial barrier integrity is not mediated by NO

Evidence accumulated over recent years indicates that IL-1α affects the epithelial barrier integrity by altering immunostaining...
junctional complexes (Nilsson et al. 1998, Gretzer et al. 2000). To test whether this effect is mediated by NO, we measured variations of RTE in polarised thyrocytes incubated with cytokines in the presence or absence of L-NAME. Compared with controls, we observed a marked reduction of RTE in cells treated with cytokines. Kinetic analysis of the IL-1β+IFN-γ-induced effects showed that a similar delay of 15 h was required to cause both nitrite accumulation and RTE reduction, suggesting that both events could be linked to each other (Fig. 5). We therefore tried to dissociate those two effects by incubating cells with IL-1β, IFN-γ or both with or without L-NAME (Fig. 6). Although greater in IL-1β or in IL-1β+IFN-γ-treated cells, the drop in RTE was also observed in cells incubated with IFN-γ alone and the loss of transepithelial resistance remained unaffected by the addition of L-NAME. These results indicate that cytokine-induced alterations of the epithelial barrier are not mediated by NO.

Cytokine-induced cytotoxic effects are mediated by NO

It is well accepted that cytokines can promote an inflammatory reaction in endocrine tissues by releasing autoantigens from destroyed cells. In the pancreas for instance, recent evidence has pointed out oxygen- or nitrogen-based free radicals as potent mediators of cytokine-induced β-cell destruction (Rabinovitch & Suarez-Pinzon 1998). To test whether cytokines can be directly toxic for thyroid epithelial cells, we measured the release of LDH, a cytosolic enzyme, from thyroid cells cultured with or without IL-1α+IFN-γ (Fig. 7). LDH activity was much greater in media from IL-1α+IFN-γ-incubated cells than from control thyrocytes. Furthermore, we observed in a separate experiment, that IFN-γ alone was unable to induce LDH release, but aggravated the IL-1α-induced effects (data not shown). LDH release was partially mediated by NO, since it was significantly reduced by L-NAME (Fig. 7). These results indicate that, besides altering junctional complexes, pro-inflammatory cytokines are directly toxic for thyroid epithelial cells. This effect is mediated, at least in part, through the activation of an l-arginine/NOS-dependent pathway.

Discussion

We have shown in the present study that IL-1α, alone or combined with IFN-γ, induces the expression of NOSII in human thyrocytes and that NO produced in such conditions, by contributing to the cytokine-induced cell death, might be involved in the initiation of an autoimmune reaction. IFN-γ by itself was unable to induce NO production, but acted synergistically with IL-1α.

Several papers have already reported that the l-arginine/NOSII pathway is activated by cytokines within thyroid epithelial cells (Rasmussen et al. 1994, Kasi et al. 1995, Reimers et al. 1996). We have shown here that, although the NOSII enzyme is not expressed in normal thyrocytes, NO production is detected after their exposure to IL-1α+IFN-γ for 15 h. This delay likely reflects de novo NOSII enzyme synthesis, as in other
experimental models (Hughes et al. 1990, Ellman et al. 1993). As shown by immunoblot, L-NAME inhibition of NO production induced an up-regulation in NOSII concentration, indicating the existence of a negative feedback loop between NO and NOSII expression, as already reported for NOSII activity (Assreuy et al. 1993) and expression (Sheffler et al. 1995) in macrophages and might explain how NO limits by itself the extent and the duration of cytokine-induced NOSII expression.

Noteworthy, neither TSH nor the oxidative status of the thyrocytes modulated NO production in polarised human thyrocytes, suggesting that, in contrast to NOSI and NOSIII expression (Colin et al. 1995, Gerard et al. 2000), NOSII expression is unrelated to the differentiated status of the gland. In addition, cytokine-induced NOSII appeared by confocal microscopy to be scattered throughout the cytoplasm, a pattern different from the apical membrane localisation of NOSIII in thyroid tissue from hyperthyroid patients (Colin et al. 1997). It is therefore likely that, depending upon the pathophysiological condition of the gland and in response to the activation of one or another isoform of NOS, NO is released in various amounts and maybe in particular cellular compartments where it may play distinctive and specific roles.

Another finding reported in the present paper refers to the observation that IL-1α-induced (alone or in combination with IFN-γ) release of LDH by human thyrocytes was prevented by L-NAME. This provides evidence for a role of NO as a mediator in cytokine cytotoxicity. This has already been reported in the ovulatory process (Ellman et al. 1993), after oxidative stress injuries in cardiac myocytes (Ing et al. 1999) or in other autoimmune endocrinopathies, such as type-1 diabetes (Mandrup-Poulsen et al. 1993, Rabinovitch & Suarez-Pinzon 1998, Sjöholm 1998, Suarez-Pinzon et al. 2001). We suggest that pro-inflammatory cytokines may promote thyroid autoimmunity by inducing NOSII expression and the production of NO, followed by target cell lysis and release of autoantigens. This hypothesis is further supported by our recent in vivo observations showing that macrophages and
thyrocytes express NOSII in the early steps of an experimental model of Hashimoto-like thyroiditis and that preventing the evolution of the disease with IL-9, a Th2 cytokine, also impedes the expression of NOSII (Colin et al. 1998, Many et al. 2000). Data reported here do not allow us to state the mechanism of NO-induced cellular damages. In pancreatic islets, cytokines exert cytotoxic effects with cellular features of necrosis and apoptosis, although NO seems mostly to be involved in cell necrosis (Grey et al. 1999, Liu et al. 2000, Saldeen 2000, Zumsteg et al. 2000). Additional experiments are required to clarify this point in our model. Nevertheless, there was no massive tissue destruction or great morphological alterations in cells remaining on filters, suggesting that polarised thyroid epithelial cells are somewhat resistant to the cytotoxic effects of cytokines, as compared with other cellular systems such as pancreatic cells (Rabinovitch & Suarez-Pinzon 1998). This could explain why, depending on the concentration and the duration of exposure to cytokines, some authors have reported little impact of IL-1

Figure 4 Western blot analysis of IL-1α+IFN-γ-induced NOSII expression. Thyrocytes were cultured in 5H Coon’s modified Ham F-12 medium for 5 days, supplemented with 0·1 mL/ml TSH for 2 days, and then for 2 additional days with 100 mL/ml IL-1α (lane 3), 100 mL/ml IFN-γ (lane 2) or their combination (lane 4), in the absence (lanes 1–4) or presence (lanes 5–8) of 2·5 mM L-NAME. Some inserts were treated with both cytokines in the presence of 10 mL/ml TSH (lanes 9–10). Cells were lysed and 20 µg proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a NOSII antibody. Purified NOSII was used as positive control (lane 11).

Figure 5 Kinetics of IL-1α+IFN-γ effects on RTE and nitrite production. Thyrocytes were grown in MEM medium and 0·1 mL/ml TSH was added at day 4. IL-1α+IFN-γ (100 mL/ml each) were added or not at day 9. (A) RTE and (B) nitrite accumulation in apical and basal medium were measured at 6, 15 and 24 h after cytokine addition. Values are means ± s.e.m. (n=4). *P<0·01 effect of cytokines.
on thyroid cell viability (Rasmussen et al. 1993, Mandrup-Poulsen et al. 1996, Nilsson et al. 1998, Bretz et al. 1999). The sensitivity of cells to the deleterious effects of cytokines may in fact depend upon the target organ but also on the species, and even on the strain of experimental animals (Eizirik et al. 1993, 1994, 1996, Mandrup-Poulsen et al. 1996). However, a few cellular alterations are sufficient to release enough autoantigens so as to induce autoimmunity. This could result from limited cellular shedding, as reported after cytokine exposure of proximal tubular cells of human kidney, a process mediated by NO (Glynne & Evans 1999). Tyrosine nitration could provide another explanation for NO cytotoxicity. Indeed, nitrite is a substrate for mammalian peroxidases and formation of peroxinitrite by oxidation can contribute to tyrosine nitration and cytotoxicity (van der Vliet et al. 1997).

Previous data reported by Nilsson et al. (1998) and Gretzer et al. (2000) clearly demonstrated that IL-1α alters the integrity of tight and polarised monolayers of thyroid cells by disrupting junctional complexes. Their results indicated severe changes in the junction-associated cytoskeleton, especially in the tight junction protein ZO-1 (Nilsson et al. 1998). Because IL-1α was not yet known to induce NO release from thyrocytes, they suggested that NO could act as mediator in this IL-1-induced effect, acting downstream of the IL-1 receptor activation. Their results were reproduced in the present study. For instance, we observed a dramatic drop in RTE values in cells incubated with cytokines. However, the variations in RTE suggested that the alterations of the junctional complexes induced by IL-1α and/or IFN-γ are not mediated by NO. First, the drop in RTE values observed after cytokine incubation was not prevented by the NOS inhibitor, L-NAME. Furthermore, there was no correlation between IFN-γ-induced decrease in RTE values and NO production. Although nitrite accumulation and RTE decrease appear after a comparable delay after the cytokine challenge, they should be considered as independent events resulting from the activation of different intracellular pathways.

In conclusion, our results indicate that, in the presence of IL-1α+IFN-γ, human thyrocytes express NOSII and secrete large amounts of NO. The reduction in LDH enzyme release after NOS inhibition suggests that cytokine-induced cytotoxic effects are at least partially mediated by NO. In contrast, the absence of RTE protection by L-NAME treatment suggests that cytokine-induced alteration of the thyroid barrier integrity is not

Figure 6 IL-1α+IFN-γ decreased the RTE via an NO-independent pathway. Thyrocytes cultured in Coon’s modified Ham F-12 medium were treated with 0·1 mU/ml TSH from day 5 (same experiment as in Fig. 1). Cells were then treated with IL-1α, alone or combined with IFN-γ (100 mU/ml each), or with IFN-γ for 2 days, in the presence or absence of 2·5 mM L-NAME. RTE before incubation with cytokines was 468 ± 10 Ω·cm². Values are means ± S.E.M. (n=4). *P<0·005 effect of cytokines.

Figure 7 IL-1α+IFN-γ increased the LDH release via an NO-dependent pathway. Thyrocytes cultured in MEM medium were treated with 0·1 or 10 mU/ml TSH from day 7. After 6 additional days, they were treated for 65 h with IL-1α+IFN-γ (100 mU/ml each) in the presence or absence of 2·5 mM L-NAME. LDH activity was assayed in apical and basal media and results were pooled since there was no polarity in the LDH release. Values are means ± S.E.M. (n=4). *P<0·01 effect of cytokines; +P<0·01 effect of L-NAME.
directly related to NO production. The pleiotropic effects of cytokines on thyroid cells therefore result from the activation of a variety of second messengers that determine their specificity of action.

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