Potent inhibition of inducible nitric oxide synthase by geldanamycin, a tyrosine kinase inhibitor, in endothelial, smooth muscle cells, and in rat aorta

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Abstract We have examined whether specific protein tyrosine kinase (PTK) inhibitors (genistein, tyrphostin, or geldanamycin) prevent nitric oxide (NO•) production in rat smooth muscle cells (SMC), in murine brain endothelial cells (MBE), and in isolated rat aortas treated with endotoxin (LPS) and/or cytokines. Tyrphostin failed to inhibit either the release of nitrite in both endothelial and smooth muscle cells or vascular hyporeactivity in rat aorta, caused by immunostimulants. Genistein decreased nitrite production in MBE only at high concentration but had no effect on nitrite production in SMC and on the hypocontractility in aortic rings. In contrast, low concentrations of geldanamycin abolished the release of nitrite in MBE and in SMC treated with endotoxin and/or cytokines. Geldanamycin inhibited also the hypocontractility to phenylephrine in aortic rings treated with LPS or interleukin-1. This inhibitor failed to inhibit the release of nitrite and the vascular hyporeactivity once nitric oxide synthase (NOS) was induced by immunostimulants whereas methyl-L-arginine, an inhibitor of NOS, had significant effects. These data suggest that LPS- and cytokines-induced NO• production initiate a common signaling pathway involving a PTK that is inhibited by geldanamycin but not or slightly by tyrphostin or genistein at a point that precedes the induction of NOS.

Key words: Inducible nitric oxide synthase; Endothelial and smooth muscle cell; Artery; Tyrosine kinase inhibitor

1. Introduction

Nitric oxide (NO•) is involved as a signaling and effector molecule in several diverse biological systems [1]. Overproduction of NO• via the expression of an endotoxin- and cytokine-inducible nitric oxide synthase (iNOS) in smooth muscle cells and endothelial cells of the vascular wall leads to an inappropriate vasodilatation in endotoxin shock [2,3]. The intracellular signals that regulate the expression of iNOS have been only partially characterized in a small number of cell types. Increases in intracellular cyclic AMP levels enhanced interferon (IFN)-induced NOS expression in vascular smooth muscle cells [4] but partially reduced lipopolysaccharide (LPS)- and IFN-induced NO• production in murine macrophages [5]. Protein kinase C appears to be involved with iNOS expression in macrophages and hepatocytes [6,7], while in chondrocytes the activation of protein kinase C and protein kinase A is not sufficient to trigger iNOS expression [8]. Although both protein kinase C and protein kinase A have been implicated in mediating some effects of LPS in formation of inflammatory mediators, recent observations suggest that LPS activation operates independently from protein kinases C and A [8,9]. Tyrosine kinase inhibitors blocked LPS- or interleukin (IL-1)-induced NO• release in murine macrophages [10], pancreatic islet cells, and chondrocytes [8]. These inhibitors prevented both IL-1- and LPS-elicited cyclic GMP accumulation in vascular smooth muscle cells [11] and had protective effects in an in vivo model of septic shock [12]. Thus, protein tyrosine kinases seem to be essential in immunostimulant-induced NO• production, suggesting that tyrosine kinases may be potential targets to inhibit pathogenic effects of cytokines and LPS. Furthermore, it has been shown that the phosphorylated tyrosine can control gene expression by altering the activity of several transcription factors. We have investigated whether specific and chemically different protein tyrosine kinase inhibitors would prevent the production of NO• induced by NOS in vascular smooth muscle cells, in murine brain endothelial cells, and isolated vessels stimulated with cytokines or LPS.

2. Materials and methods

2.1. Cell culture

Vascular smooth muscle cells were kindly provided by Dr. Gross [13]. Cells in passage 10–15 were seeded into 24-well plates for the measurement of nitrite production. MBE cells were kindly provided by Dr. Nicolson and cultured in gelatin-coated 96-well dished with 100 μl culture medium until confluence. When cells reached confluence, the culture medium was aspirated and replaced with serum-free culture medium containing 0.1% (w/v) fatty acid-free bovine serum albumin to remove factors present in serum. The smooth muscle cells were exposed to IL-1α (30 ng/ml), LPS (50 ng/ml)+IFN (500 IU/ml), or vehicle for 24 h in the presence or absence of tyrosine kinase inhibitors (geldanamycin, genistein, or tyrphostin) added 1 h before the NOS inducers. MBE cells were exposed to TNF (10 ng/ml)+IFN (500 IU/ml), or vehicle in the presence or absence of tyrosine kinase inhibitors added 1 h before cytokines.

2.2. Nitrite assay

Nitrite production, an indicator of NO• synthesis, was determined by colorimetric assay. Aliquots of conditioned medium from confluent cells (50 μl) were mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 2% phosphoric acid). Nitrite concentrations were determined by comparison with OD_{540} of standard solutions of sodium nitrite prepared in cell culture medium.

2.3. Organ chamber studies

Aortic rings were prepared from male Wistar rats (250–300 g) as described previously [14]. The rings were suspended in 10-ml organ

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Abbreviations: NO•, nitric oxide; PTK, protein tyrosine kinase; SMC, smooth muscle cells; MBE, murine brain endothelial cells; NOS, nitric oxide synthase; LPS, endotoxin, lipopolysaccharides; IFN, interferon γ; TNF, tumor necrosis factor α; IL-1, interleukin-1

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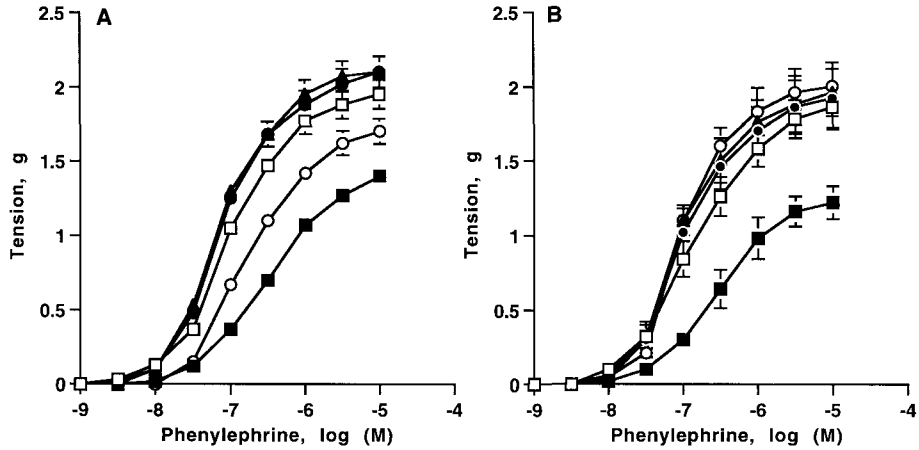

Fig. 1. Concentration-contraction curves evoked by phenylephrine in control (□) and immunostimulant-treated (■) rat aortic rings with endothelium [IL-1 (40 ng/ml) (A) or LPS (100 ng/ml) (B)]. Effects of geldanamycin 0.1 μM (○), 0.5 μM (●), and 1 μM (▲) on contractions evoked by this α-agonist in immunostimulant-treated rings (added 1 h before the induction). Results are presented as mean ± S.E.M. of five or six separate experiments and are shown in absolute values.

chambers under an optimal tension of 2.5–3 g. The tension was recorded with an isometric force transducer connected to an input board in an IBM 386/30 MHz personal computer. Rings were maintained at 37°C in Krebs-Ringer solution containing (composition in mM) NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; CaEDTA, 0.016; and glucose, 11.1 (control solution). The presence of the endothelium was verified by the addition of acetylcholine (10^{-6} M) in arteries contracted with phenylephrine (10^{-6} M). The preparation were then rinsed three times with warm control solution, rested 30 min before a concentration-contraction curve to phenylephrine was obtained. To induce NOS activity, before suspension in the organ chambers aortic rings were incubated in 1 ml of DMEM/F12 containing IL-1α (40 ng/ml), LPS (100 ng/ml), or vehicle for 5 h in the presence or absence of protein tyrosine kinase inhibitors added 1 h before the induction.

2.4. Statistics

Results are expressed as means ± S.E.M. Statistical evaluation of the data was performed by Student's *t*-test for paired or unpaired observations. *P* values less than 0.05 were considered significant.

3. Results

3.1. Release of nitrite

Treatment of confluent cultures of smooth muscle cells from rat aortas with IL-1 (30 ng/ml) or LPS (50 ng/ml)+IFN (500 IU/ml) for 24 h caused the accumulation of nitrite in the incubation medium (Tables 1–3). Treatment of MBE cells with TNF (10 ng/ml)+IFN (500 IU/ml) also caused the release of nitrite (Tables 1–3). Exposure for 24 h to geldanamycin (0.01, 0.1, or 0.3 μM), genistein (1, 10, or 50 μM), or tyrphostin (1, 10, or 50 μM) minimally affected the basal levels of nitrite anions in conditioned medium of unstimulated endothelial or smooth muscle cells (data not shown). Geldanamycin, added 1 h before the NOS inducer, elicited a concentration-dependent and significant inhibition of IL-1- and LPS+IFN-induced nitrite release of smooth muscle cells (Table 1). In a similar manner, low concentrations of geldanamycin (0.01, 0.03, or 0.1 μM) significantly inhibited NO• production in culture medium of MBE cells treated with TNF+IFN (Table 1). When geldanamycin (0.1 μM) was added 16 h after the inducers, the accumulation of nitrite was not significantly different in the presence or absence of this tyrosine kinase inhibitor (data not shown). The presence of genistein (1 μM or 10 μM) slightly increased the accumulation of nitrite in the incubation medium of vascular smooth muscle cells stimulated by IL-1 or LPS+IFN, while 50 μM of this inhibitor had no effect (Table 2). Only the high concentration (50 μM) of genistein significantly reduced the release of nitrites induced by MBE cells treated with cytokines (Table 2). Similarly, the release of nitrite stimulated by IL-1 was greater in the incubation medium of vascular smooth muscle cells in the presence than in the absence of tyrphostin (10 μM) (Table 3). Tyrphostin (1 or 50 μM) failed to inhibit the effects of IL-1 (Table 3). The pre-exposure of vascular smooth muscle cells to tyrphostin (1, 10, or 50 μM) was also associated with an increase of the accumulation of nitrite induced by LPS+IFN (Table 3). Pretreatment of MBE cells with tyrphostin (1, 10, or 50 μM) did not change the release of nitrites of these cells stimulated with cytokines (Table 3). If methyl-L-arginine was included in the tissue culture medium, nitrites remained at

Table 1
Effects of increasing concentrations of geldanamycin (μM) on release of nitrite (μM) from MBE (1.5×10^4) and rat SMC (1.0×10^5) exposed to cytokines and/or endotoxin for 24 h

MBE		SMC		SMC	
TNF+IFN	47.7 ± 5.8	IL1	19.3 ± 3	LPS+IFN	16.9 ± 3
+0.01	40.5 ± 10.7	+0.01	21.1 ± 5.4	+0.01	19.2 ± 3
+0.03	26.0 ± 6.9	+0.1	2.3 ± 0.1	+0.1	1.9 ± 0.4
+0.1	1.0 ± 0.6	+0.3	1.8 ± 0.3	+0.3	1.8 ± 0.4

Results are shown as mean ± S.E.M. of 3–5 separate experiments performed in triplicate.

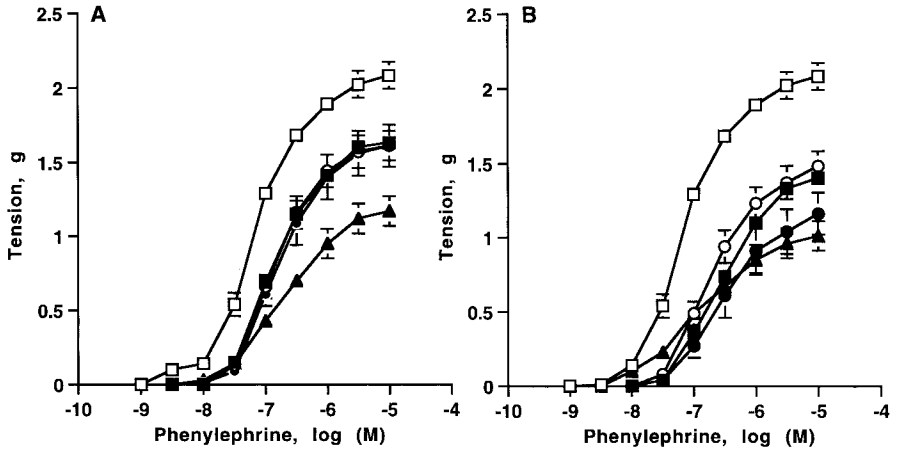

Fig. 2. Concentration-contraction curves evoked by phenylephrine in control (□) and immunostimulant-treated (■) rat aortic rings with endothelium [IL-1 (40 ng/ml) (A) or LPS (100 ng/ml) (B)]. Effects of genistein 1 μM (○), 10 μM (●), and 100 μM (▲) on contractions evoked by this α-agonist in immunostimulant-treated rings (added 1 h before the induction). Results are presented as mean ± S.E.M. of five or six different experiments and are shown in absolute values.

basal levels after LPS+IFN or IL-1 stimulation, in the presence or absence of tyrosine kinase inhibitors in smooth muscle cells (data not shown).

3.2. Vascular reactivity

The incubation of aortic rings with endothelium for 5 h in culture medium containing 40 ng/ml of IL-1 or 100 ng/ml of LPS shifted the concentration-contraction curves evoked by phenylephrine significantly to the right and decreased the maximal responses (E_{max}: control, 1.95 ± 0.1 g; IL-1, 1.14 ± 0.18 g; LPS, 1.22 ± 0.11 g) (Fig. 1). Treatment of aortic rings with geldanamycin (0.5 μM) or tyrphostin (100 μM) for 6 h had no significant effects on contractions evoked by phenylephrine (pEC_{50}: 7.18 ± 0.07 and 7.34 ± 0.06 or 7.1 ± 0.04 without and with geldanamycin or tyrphostin, respectively) (E_{max}: 2.08 ± 0.13 g and 2.1 ± 0.04 g or 1.9 ± 0.04 g without and with geldanamycin or tyrphostin, respectively) while genistein (100 μM) significantly decreased maximal responses to the α_1-agonist (E_{max}: 1.4 ± 0.13 g in the presence of genistein) without changing the pEC_{50} (7.05 ± 0.05). Geldanamycin (0.1 μM) added 1 h before IL-1 partially reversed the hyporeactivity to phenylephrine and significantly increased the maximal responses from 1.14 ± 0.18 g to 1.7 ± 0.12 g (Fig. 1A). Higher concentrations of this inhibitor (0.5 μM and 1 μM) completely restored contractions evoked by this α_1-agonist (Fig. 1A). Treatment with a low concentration of geldanamycin (0.1 μM) induced a complete inhibition of the hypocontractility to phenylephrine in rings incubated with LPS (Fig. 1B). No additional inhibitory effects were obtained with higher doses of geldanamycin (0.5 μM or 1 μM) (Fig. 1B). In contrast, genistein or tyrphostin (1 μM) did not affect the vascular hypocontractility induced by LPS or IL-1 (Figs. 2 and 3) and 100 μM of these inhibitors significantly potentiated the vascular hyporeactivity induced by cytokines or LPS (Figs. 2 and 3). When geldanamycin (0.5 μM) was added 4 h after LPS or IL-1 into the culture medium, the contractions evoked by phenylephrine were not significantly different in the presence or the absence of this tyrosine kinase inhibitor (pEC_{50}: IL-1, 6.81 ± 0.08 and 6.73 ± 0.07; LPS, 6.8 ± 0.08 and 6.79 ± 0.07 without and with geldanamycin, respectively).

4. Discussion

These findings suggest that geldanamycin may interfere with the signaling process which induces NOS, by altering the activity of a protein tyrosine kinase at a point that precedes the induction of NOS and that protein tyrosine kinase activation may be involved in the induction of NOS expression by cytokines and endotoxin in endothelial or smooth muscle cells and isolated arteries. However, genistein and tyrphostin, two chemically distinct tyrosine kinase inhibitors, do not appear to be inhibitors of NOS since they failed to inhibit the NO• production in endothelial or smooth muscle cells and in isolated vessels induced with immunostimulants.

The exposure of endothelial or smooth muscle cells to endotoxin or cytokines for several hours was associated with the accumulation of nitrite in the incubation medium. These results indicate that mediators of immune and inflammatory responses stimulate the induction of NOS in vascular smooth muscle cells as well as in most other types of mammalian cells [15,16]. The observation that the release of nitrite caused by different cytokines or endotoxin in endothelial and smooth

Table 2
Effects of increasing concentrations of genistein (μM) on release of nitrite (μM) from MBE (1.5×10^4) and rat SMC (1.0×10^5) exposed to cytokines and/or endotoxin for 24 h

MBE		SMC		SMC	
TNF+IFN	47.7 ± 5.8	IL1	14.4 ± 1.3	LPS+IFN	9.8 ± 1.4
+1	53.0 ± 8.0	+1	16.1 ± 2.1	+1	17.6 ± 3.3
+10	46.8 ± 6.9	+10	20.8 ± 3.2	+10	21.1 ± 4.6
+50	28.3 ± 6.7	+50	13.4 ± 1.6	+50	10.5 ± 1.6

Results are shown as mean ± S.E.M. of 3–5 separate experiments performed in triplicate.

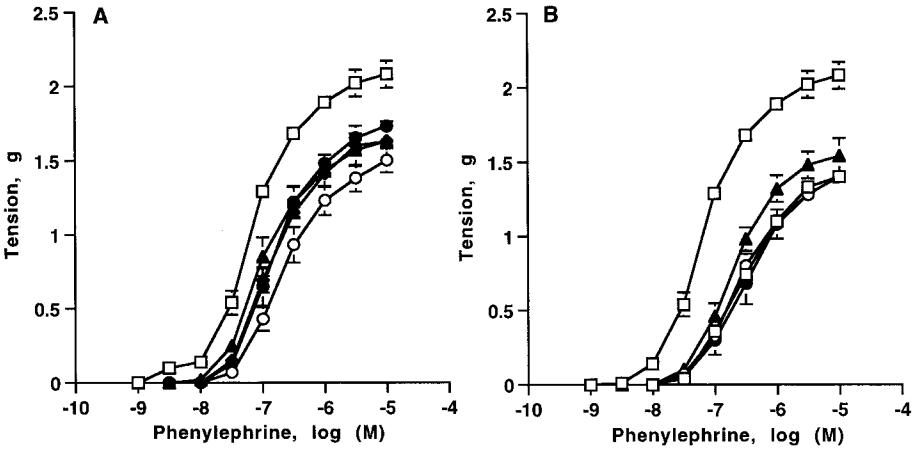

Fig. 3. Concentration-contraction curves evoked by phenylephrine in control (□) and immunostimulant-treated () rat aortic rings [IL-1 (40 ng/ml) (A) or LPS (100 ng/ml) (B)]. Effects of tyrphostin 1 μM (○), 10 μM (●), and 100 μM (▲) on contractions evoked by this α-agonist in immunostimulant-treated rings (added 1 h before the induction). Results are presented as mean ± S.E.M. of five or six separate experiments and are shown in absolute values.

muscle cells is reduced in a dose-dependent manner by the pretreatment with geldanamycin, a potent and specific tyrosine kinase inhibitor [9,11], suggests that the induction and/or activity of NOS may be dependent on a tyrosine kinase. The stimulation of rat aortas for several hours with LPS or IL-1 reduced their responsiveness to vasoconstrictors, as previously reported [14]. The fact that this vascular hypocontractility was completely inhibited by the presence of a tyrosine kinase inhibitor, geldanamycin, indicated the involvement of tyrosine kinases in the NO• synthesis induced by NOS. In addition, the regulatory mechanism involving protein tyrosine kinases is not restricted to a specific immunostimulant but functions during both IL-1 and LPS signaling. However, the ability of geldanamycin to inhibit IL-1- and LPS-induced NO• production differs in its potency, reflecting a differential contribution of tyrosine kinases to the effects of LPS and IL-1. Furthermore, this agent does not appear to be an inhibitor of NOS since it failed to inhibit nitrite accumulation in vascular smooth muscle cells and the hypocontractility to vasoconstrictors once NOS was induced by LPS or IL-1; in contrast, methyl-L-arginine, an inhibitor of NOS [14], had significant effects.

Thus, geldanamycin likely prevents the production of NO• in immunostimulant-activated endothelial cells, smooth muscle cells, and isolated arteries by inhibiting the expression of inducible nitric oxide. These results are in line with previous findings showing that the phosphorylated state of a tyrosine can control gene expression by altering the activity of several transcription factors [17]. The fact that previous studies [11] and our results showed that geldanamycin added after inducers exposure did not inhibit the NO• production suggests that tyrosine kinase inhibitors had no direct effect on iNOS activity after its induction and involvement of tyrosine activity is an early event. In contrast to geldanamycin, genistein and tyrphostin, two chemically distinct tyrosine kinase inhibitors [8,9,11,18], do not or slightly alter the signaling process leading to the induction of NOS since they do not inhibit LPS- or IL-1-induced the release of nitrite in endothelial and smooth muscle cells, or the vascular hypocontractility in isolated arteries. Doses of these two agents, which in previous studies inhibited cyclic GMP production in vascular smooth muscle cells [11] and nitrite production in macrophages [10] stimulated with IL-1 or LPS potentiated the accumulation of nitrite in vascular smooth muscle cells and the hyporeactivity to phenylephrine in aortic rings stimulated with cytokines or endotoxin in this study. These results in conjunction with those obtained with geldanamycin indicate that a specific tyrosine kinase susceptible to geldanamycin inhibition may be involved in LPS- and IL-1-mediated signal transduction. Among the possible targets for the tyrosine kinases are NF-kB/IkB complexes [21,22]. NF-kB is a critical transcription factor of several genes that are involved in immune and inflammatory responses [23,24]. In addition, previous observations demonstrating that the promoter of mouse iNOS contains at least two NF-kB sites [25] and that an inhibitor of NF-kB activation, pyrrolidine dithiocarbonate, abolished IL-1-induced iNOS mRNA expression and NO• production in mesangial cells [26] are in line with our data and confirm that a tyrosine kinase may selectively participate in the signaling pathway required for endotoxin and cytokines to induce the expression of iNOS to generate NO• production. Why the effects of genistein and tyrphostin on the modulation of NOS

Table 3
Effects of increasing concentrations of tyrphostin (μM) on release of nitrite (μM) from MBE (1.5×10^4) and rat SMC (1.0×10^5) exposed to cytokines and/or endotoxin for 24 h

MBE		SMC		SMC	
TNF+IFN	47.7 ± 5.8	IL1	14.4 ± 1.3	LPS+IFN	9.8 ± 1.4
+1	47.5 ± 6.3	+1	14.3 ± 1.7	+1	17.8 ± 3.2
+10	47.9 ± 6.8	+10	17.0 ± 3.1	+10	16.9 ± 3.3
+50	46.1 ± 7.4	+50	12.8 ± 2.4	+50	12.8 ± 3.5

Results are shown as mean ± S.E.M. of 3–5 separate experiments performed in triplicate.

in this study differ from those seen in other reports is not clear, but differences in the types of cells or methods of stimulation used might be responsible. However, previous reports support the finding that geldanamycin and its closely related analogue, herbimycin A, differ in their mechanism of action from genistein and tyrphostin [9,18]. Furthermore, there are a few data showing that the effects of genistein and tyrphostin might also result from some nonspecific action [19,20]. Genistein may depress contraction in the swine carotid artery by altering intracellular calcium concentration [20] and increase production of IL-1 in macrophages [10], this could explain, in part, the potentiation of the effects of IL-1 and LPS in vascular smooth muscle cells and in isolated arteries obtained in this study.

These results demonstrate that protein tyrosine kinases are essential in the regulation of iNOS activity in vascular smooth muscle cells in murine brain endothelial cells, and in isolated vessels and that tyrosine kinase inhibitors may be useful in the treatment of diseases such as septic shock. However, except for geldanamycin, many commonly used protein tyrosine kinase inhibitors failed to inhibit the induction of NOS.

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