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# Effect of tyrosine kinase and tyrosine phosphatase inhibitors on aortic contraction and induction of nitric oxide synthase

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**Abstract**

We studied the effects of the tyrosine kinase inhibitors genistein and tyrphostin and the tyrosine phosphatase inhibitors sodium orthovanadate and phenylarsine oxide on endotoxin-mediated induction of nitric oxide (NO) synthase in rat aorta and its effects on vascular contractility. Genistein (i.p. 10 mg/kg) inhibited the ex vivo vascular hyporesponsiveness to noradrenaline and the aminoguanidine-sensitive nitrite accumulation induced by endotoxin (i.p. 5 mg/kg) in aortic rings. Low concentrations of genistein ($10^{-6}$ M) and tyrphostin ($3 \times 10^{-6}$ M) inhibited both endotoxin-induced hyporesponsiveness and nitrite and $NO_x$ accumulation in vitro in rat aorta without affecting control nitrite or $NO_x$ accumulation or contraction. Higher concentrations of genistein ($10^{-5}$ and $5.5 \times 10^{-5}$ M), sodium orthovanadate ($10^{-4}$ M) and phenylarsine oxide ($10^{-6}$ M) produced an irreversible depression of noradrenaline-induced contractions. In the presence of these drugs, endotoxin did not induce further depression of vascular contractility and did not increase nitrite or $NO_x$ production. In conclusion, there is a dissociation between the effects of these drugs on vascular smooth muscle contraction and NO synthase induction, the latter being more sensitive to inhibition by these drugs. Surprisingly, tyrosine phosphatase inhibitors produced similar effects to those of tyrosine kinase inhibitors, suggesting that there is a complex relationship between tyrosine kinases and phosphatases in the signalling pathway of agonist-induced vascular smooth muscle contraction and NO synthase induction. © 1997 Elsevier Science B.V.

*Keywords:* Lipopolysaccharide; Nitric oxide (NO) synthase, inducible; Aorta, rat; Genistein; Tyrphostin; Orthovanadate; Phenylarsine oxide

## 1. Introduction

Lipopolysaccharide is the endotoxin responsible for many of the symptoms of Gram-negative sepsis. It is a potent activator of the immune system and induces local or systemic inflammation and shock (Morrison and Ryan, 1979; Thiemermann and Vane, 1990; Petros et al., 1991). Septic shock is associated with a reduction in the pressor responses to vasoconstrictor agents, ultimately resulting in therapy-resistant hypotension (Groeneveld et al., 1988). Endotoxin activates soluble guanylate cyclase and increases the production of cGMP in both cultured (Marczin et al., 1993a,b) and native vascular smooth muscle cells (Fleming et al., 1990), inhibits agonist-induced contractions of isolated vascular preparations (Fleming et al., 1990; Szabo et al., 1993; Villamor et al., 1995) and promotes L-arginine-induced relaxation in rat thoracic aorta (Moritoki et al., 1995). These responses are prevented by protein synthesis inhibitors and L-arginine analogues, indicating that there is de novo synthesis of the inducible isoform of nitric oxide (NO) synthase and that NO has an autocrine action in vascular smooth muscle (see Thiemermann, 1994 for a review).

Recently, several reports have suggested an involvement of tyrosine kinase in the signal transduction pathway for the induction of NO synthase elicited by endotoxin or cytokines in several cell types including macrophages (Akarasereenont et al., 1994), endothelial cells (Radomski et al., 1990; Yang et al., 1994), chondrocytes (Geng et al., 1995), cultured rat aortic smooth muscle cells (Marczin et al., 1993b; Moritoki et al., 1995) and rat thoracic aorta (Moritoki et al., 1995). Tyrosine kinase activation also seems to be involved in the signal transduction pathway of

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other endotoxin-induced effects such as tumor necrosis factor-$\alpha$ release or cyclooxygenase-2 induction (Akarasereenont et al., 1994). Furthermore, tyrosine kinase activation is also involved in the signal transduction for the induction of NO synthase by the Gram-positive cell-wall component lipoteichoic acid (Kengatharan et al., 1996).

Tyrosine kinases are also involved as part of the signal transduction of agonist-induced vascular smooth muscle contraction (Hollenberg, 1994). Much of this evidence is based on the reported ability of tyrosine kinase inhibitors to reduce the vascular smooth muscle contraction induced by several agonists (Abebe and Agrawal, 1995; Filipeanu et al., 1995; Herrera et al., 1996). Therefore, on theoretical grounds, tyrosine kinase inhibitors may produce opposite effects on vascular smooth muscle in sepsis, i.e., relaxation by direct interference with smooth muscle contraction and reduction of endotoxin-induced vascular hyporesponsiveness by inhibition of NO synthase induction.

If the activation of a tyrosine kinase pathway has a role in a certain cellular event, tyrosine phosphatase may act as a counter-regulatory factor. Moreover, inhibitors of tyrosine phosphatases, by increasing tyrosine phosphorylation, may enhance tyrosine kinase-mediated events (Heffetz et al., 1990; Faure et al., 1992). Therefore, the activity of tyrosine phosphatases might potentiate the above mentioned tyrosine kinase-mediated events (induction of inducible NO synthase by endotoxin and vascular smooth muscle contraction).

In the present paper we studied the involvement of the tyrosine kinase pathway in the induction of NO synthase by endotoxin and in vascular smooth muscle contractility. The aims of the present study were to assess (a) whether tyrosine kinase inhibition prevents NO synthase induction without affecting vascular smooth muscle contraction and (b) the interactions of tyrosine phosphatase inhibitors (sodium orthovanadate and phenylarsine oxide) with NO synthase induction and vascular contractility.

## 2. Methods

### 2.1. Tissue preparation

The descending thoracic aortae from male Wistar rats (250–300 g) were used in this study for both ex vivo and in vitro studies. Rats were killed by a blow on the head and the aortae were quickly dissected and placed in a modified physiological saline solution (PSS) of the following composition (mM): NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2, $KH_2PO_4$ 1.2 and glucose 11. The aorta was cleaned of fat and connective tissue and cut into 3 mm rings. In some preparations the endothelium was removed mechanically by rubbing the intimal surface of the vessels with a metal rod. The endothelium removal procedure was verified by the lack of relaxant effects of $10^{-6}$ M acetylcholine in arteries pre-contracted by noradrenaline.

### 2.2. Tension measurement

Two L-shaped stainless-steel wires were inserted into the arterial lumen and the rings were placed in Allhin organ chambers filled with PSS at 37°C and gassed with 95% $O_2$ and 5% $CO_2$. One wire was attached to the chamber and the other to an isometric force–displacement transducer (Letigraph 2000, Letica) as previously described (Duarte et al., 1994). The rings were stretched to a resting tension of 1 g and allowed to equilibrate for 60–90 min. During this period tissues were re-stretched and washed every 30 min with warm PSS. After equilibration, concentration–response curves for noradrenaline ($10^{-9}$–$10^{-5}$ M) were made by increasing the concentration in the organ chamber in cumulative increments after a steady-state response had been reached with each increment. In some experiments aortic rings were treated with $10^{-4}$ M L-NAME 20 min before the addition of noradrenaline.

### 2.3. Nitrite accumulation

Nitrite or $NO_x$ (nitrite + nitrate) accumulation, as indicators of NO synthase activity, was measured by the Griess reaction. $NO_x$ was determined after the reduction of nitrate to nitrite by incubation with acid washed (0.1 M HCl, three times just prior to use) cadmium powder in an ammonium chloride/borax buffer (final concentration 0.01 and 0.0043 M, respectively) for 15 min (Wishnock et al., 1996). The concentrations of nitrite in the incubation media were determined by adding 500 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine in 5% orthophosphoric acid) to 500 μl of sample and measuring the absorbance at 560 nm. Nitrite concentrations were calculated by comparison with a standard curve of absorbance at 560 nm of different concentrations of sodium nitrite.

### 2.4. Ex vivo studies

Rats were injected i.p. with vehicle (dimethylsulfoxide in saline) plus saline, vehicle plus endotoxin (5 mg/kg), genistein (10 mg/kg) plus saline or genistein (10 mg/kg) plus endotoxin (5 mg/kg) and were killed after 6 h by a blow on the head. The aortae were removed. Nitrite and tension measurements were performed with different sets of rats. Tension measurements were performed with endothelium-intact rings just after dissection. For nitrite measurements aortae were cut into 4 rings, two of which were denuded of endothelium by gently rubbing the internal surface of the vessel with a metal rod. The rings were incubated in 2 ml PSS containing gentamicin (50 μg $ml^{-1}$, to avoid bacterial contamination), L-arginine ($10^{-5}$ M, the precursor of NO) and cycloheximide ($10^{-5}$ M, a protein synthesis inhibitor to prevent further NO synthase induction) in the presence or absence of $10^{-4}$ M aminoguanidine (a selective inhibitor of the inducible NO

synthase, Griffiths et al., 1993) at 37° C and gassed with 95% $O_2$ and 5% $CO_2$ for 24 h. Nitrite production in the incubation media in the absence of aminoguanidine is expressed as a percentage of that in the presence of aminoguanidine. Thus, values above 100% (aminoguanidine-sensitive nitrite accumulation) were considered to reflect NO production as a result of inducible NO synthase activity.

### 2.5. In vitro studies

The aortic rings were denuded of endothelium and incubated in 2 ml PSS containing gentamicin (50 μg/ml) and L-arginine ($10^{-5}$ M) in the presence of vehicle, endotoxin (1 μg/ml), genistein ($10^{-6}$, $10^{-5}$ or $5.5 \times 10^{-5}$ M), endotoxin (1 μg/ml) plus genistein ($10^{-6}$, $10^{-5}$ or $5.5 \times 10^{-5}$ M), tyrphostin ($3 \times 10^{-6}$ M), endotoxin (1 μg/ml) plus tyrphostin ($3 \times 10^{-6}$ M), sodium orthovanadate ($10^{-4}$ M), endotoxin (1 μg/ml) plus sodium orthovanadate ($10^{-4}$ M), phenylarsine oxide ($10^{-6}$ M) or endotoxin (1 μg/ml) plus phenylarsine oxide ($10^{-6}$ M) for 20 h. The solution was maintained at 37°C and gassed with 95% $O_2$ and 5% $CO_2$. The nitrite concentration in the incubation media was measured as described above and the rings were used to measure vascular reactivity. $NO_\times$ values were measured in a separate set of rings.

### 2.6. Drugs

The following drugs were used: (±)-noradrenaline bitartrate, acetylcholine chloride, lipopolysaccharide from *Escherichia coli* (serotype 055:B5), L-arginine, gentamicin sulphate, sulphanilamide, naphthylethylenediamine, cycloheximide, sodium nitrite, L-NAME ($N^G$-nitro-L-arginine-methyl ester), aminoguanidine, tyrphostin 25, sodium orthovanadate and phenylarsine oxide (Sigma, Madrid). Genistein, tyrphostin and phenylarsine were dissolved in dimethylsulfoxide to prepare a 0.1 M stock solution. All other drugs were dissolved in distilled deionized water. Ascorbic acid ($10^{-4}$ M) was added to the stock solution of noradrenaline to prevent oxidation. Further dilutions were made in Krebs solution for in vitro studies or in saline for in vivo studies.

### 2.7. Statistical analysis

Results are expressed as means ± S.E.M. of measurements in $n$ arteries. Cumulative concentration–response curves for noradrenaline in each ring were fitted to the logistic equation $y = E_{max}/(1 + \exp(-K(x + pD_2))$, where the $E_{max}$ represents the maximal tension induced by noradrenaline, $K$ the slope of the curve and $pD_2$ the negative logarithm of the concentration of NA producing 50% of $E_{max}$. Statistically significant differences were calculated by means of an unpaired Student's $t$-test. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Ex vivo studies

#### 3.1.1. Effects of genistein on endotoxin-induced hyporesponsiveness to noradrenaline

As shown in Fig. 1A, endothelium-intact aortic rings from rats treated with vehicle plus endotoxin (5 mg/kg) and killed 6 h afterwards showed a significant reduction in their maximal response to noradrenaline ($E_{max} = 1158 \pm 26$ mg vs. $804 \pm 4$ mg, in vehicle plus saline and vehicle plus endotoxin-treated rats, respectively, $n = 6$, $P < 0.01$). A similar depression of noradrenaline-induced contractions was observed in aortae from rats treated with genistein (10 mg/kg) plus saline ($E_{max} = 801 \pm 10$ mg, $P < 0.05$ vs. vehicle plus saline, $n = 6$). However, the reactivity to

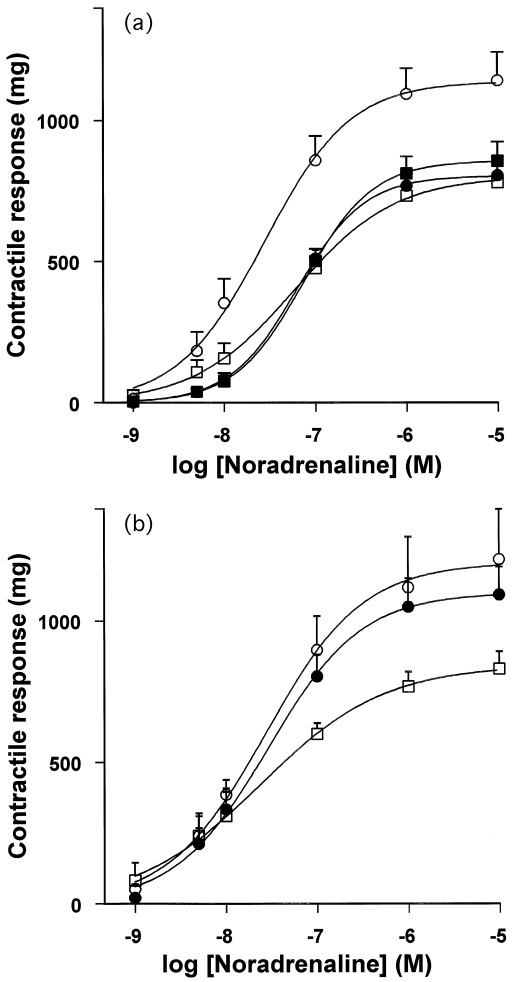


Fig. 1. Effects of genistein on the ex vivo vascular hyporesponsiveness induced by endotoxin. Concentration–response curves for noradrenaline were made for endothelium-intact aortae from rats treated for 6 h with vehicle plus saline (○), vehicle plus endotoxin (5 mg/kg, i.p., ●), genistein plus saline (□) or genistein plus endotoxin (■). The experiments were carried out in the absence (A) or in the presence (B) of $10^{-4}$ M L-NAME in the organ bath. Data are expressed as means ± S.E.M. of 6 observations.

noradrenaline of rings from genistein plus endotoxin-treated rats was similar to that of rings from rats treated with genistein plus vehicle. Therefore, in genistein-treated rats, endotoxin was unable to induce further hyporesponsiveness.

Endotoxin-induced hyporesponsiveness was inhibited when $10^{-4}$ M L-NAME was present in the organ bath 20 min before the administration of noradrenaline (Fig. 1B) ($E_{max} = 1206 \pm 24$ mg vs. $1099 \pm 23$ mg, in rings from vehicle plus saline and vehicle plus endotoxin-treated rats, respectively, $n = 6$, $P > 0.05$), indicating that NO was responsible for the endotoxin-induced hyporesponsiveness. However, genistein-induced hyporesponsiveness could not be attributed to an increased production of NO since the hyporesponsiveness was not inhibited by the presence of L-NAME in the organ bath ($E_{max} = 843 \pm 14$ mg in genistein plus vehicle, $P < 0.05$ vs. vehicle plus vehicle, $n = 6$).

### 3.1.2. Effects of genistein on endotoxin-induced nitrite accumulation

Fig. 2 shows the aminoguanidine-sensitive nitrite accumulation in the incubation media of endothelium-intact and endothelium-denuded aortae from rats treated for 6 h with genistein and endotoxin. After 24 h of incubation in the presence of $10^{-4}$ M aminoguanidine, endothelium-intact and endothelium-denuded rings from rats treated with vehicle plus saline produced $1.53 \pm 0.15$ and $1.71 \pm 0.19$ nmol of nitrite per mg of wet tissue, respectively ($P > 0.05$ intact vs. denuded), which represents the nitrite production not attributable to inducible NO synthase. Nitrite accumulation in endothelium-intact or endothelium-denuded rings from vehicle plus saline or genistein plus saline-treated rats was not significantly different in the presence or absence of aminoguanidine. In contrast, endotoxin increased aminoguanidine-sensitive nitrite accumulation (values in the absence of aminoguanidine were about 150% of those in its presence, $P < 0.05$). This increase was similar in endothelium-intact and endothelium-denuded rings, indicating that the endothelium did not significantly contribute to aminoguanidine-sensitive nitrite production. The endotoxin-induced increase in aminoguanidine-sensitive nitrite accumulation was fully abolished when rats were treated with genistein and this effect was independent of the presence or absence of endothelium.

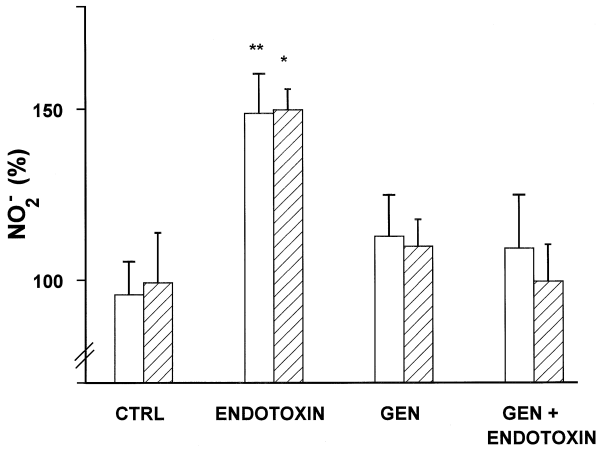


Fig. 2. Effects of genistein on the ex vivo aminoguanidine-sensitive nitrite accumulation induced by endotoxin in the incubation media of endothelium-denuded (open bars) and endothelium-intact (hatched bars) aortae. Aortic rings from rats treated for 6 h with vehicle plus saline (CTRL), vehicle plus endotoxin (5 mg/kg, i.p., ENDOTOXIN), genistein (10 mg/kg, i.p.) plus saline (GEN) and genistein plus endotoxin (GEN + ENDOTOXIN) were incubated for 24 h in Krebs solution containing $10^{-5}$ M L-arginine and $10^{-5}$ M cycloheximide in the presence or absence of $10^{-4}$ M aminoguanidine. Results (means ± S.E.M. of 6 experiments) reflect nitrite accumulation detected in the absence of aminoguanidine, expressed as a percentage of that in the presence of aminoguanidine. * and ** $P < 0.05$ and $P < 0.01$ ENDOTOXIN vs. CTRL.

## 3.2. In vitro studies

### 3.2.1. Effects of genistein, tyrphostin, sodium orthovanadate and phenylarsine oxide on endotoxin-induced hyporesponsiveness to noradrenaline

Aortic rings were incubated with vehicle or endotoxin (1 μg/ml) in the presence or absence of genistein ($10^{-6}$, $10^{-5}$ or $5.5 \times 10^{-5}$ M), tyrphostin ($3 \times 10^{-6}$ M) or sodium orthovanadate ($10^{-4}$ M) for 20 h and then transferred to the organ bath. Thereafter, the cumulative concentration–response curve for noradrenaline was made in the absence of these drugs. Aortic rings incubated for 20 h with endotoxin showed a decreased maximal contractile response to noradrenaline ($E_{max} = 990 \pm 47$ mg, $n = 16$ and $746 \pm 60$ mg, $n = 15$, vehicle and endotoxin-treated arteries, respectively, $P < 0.05$, Fig. 3A). Incubation with $10^{-6}$ M genistein for 20 h produced no change in the maximal response to noradrenaline in control arteries but suppressed the hyporesponsiveness induced by endotoxin ($E_{max} = 967 \pm 72$ mg, $n = 11$ and $1059 \pm 55$ mg, $n = 7$, in untreated and endotoxin-treated arteries, respectively, $P > 0.05$, Fig. 3B). Incubation with genistein at higher concentrations ($10^{-5}$ and $5.5 \times 10^{-5}$ M) concentration dependently reduced the contractile responses to noradrenaline (Fig. 3C and D). Endotoxin, when co-incubated with these high concentrations of genistein, did not induce any further change in the contractile response to noradrenaline (Fig. 3C and D). Incubation with $3 \times 10^{-6}$ M tyrphostin produced a significant reduction in the contractile response induced by low concentrations of noradrenaline but had no effect on its maximal response ($E_{max} = 899 \pm 50$ mg, $n = 7$). In the presence of tyrphostin, endotoxin had no effect on the concentration–response curve for noradrenaline, i.e., tyrphostin inhibited endotoxin-induced hyporesponsiveness to noradrenaline (Fig. 3E).

Incubation with the tyrosine phosphatase inhibitor sodium orthovanadate ($10^{-4}$ M) decreased the maximal contractile response to noradrenaline as compared with that of control arteries ($506 \pm 46$, $n = 9$, $P < 0.01$, Fig.

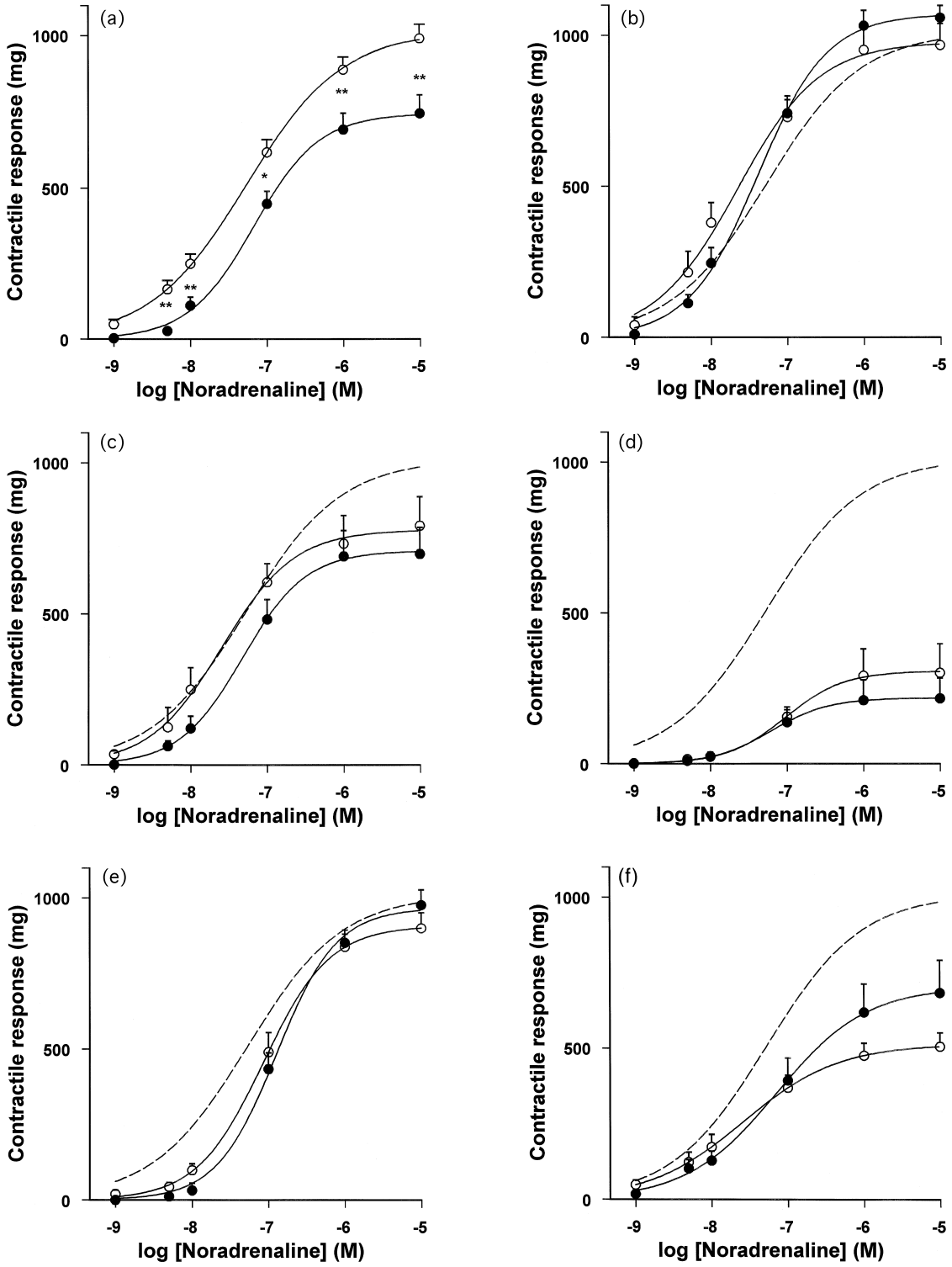


Fig. 3. Effects of tyrosine kinase and tyrosine phosphatase inhibitors on the in vitro vascular hyporesponsiveness induced by endotoxin. Concentration–response curves for noradrenaline were made with endothelium-denuded aortae incubated for 20 h with vehicle (○) or endotoxin (1 μg/ml, ●) in the absence (A) or in the presence of (B) $10^{-6}$ M, (C) $10^{-5}$ M and (D) $5.5 \times 10^{-5}$ M genistein, (E) $3 \times 10^{-6}$ M tyrphostin and (F) $10^{-4}$ M orthovanadate. Data are expressed as means ± S.E.M. of 8–23 experiments. $^{**}P < 0.01$ represent significant differences with respect to endotoxin-treated arteries. The dashed lines in (B–F) indicate the curve obtained with vehicle in (A) for comparative purposes.

3F). In arteries incubated with endotoxin plus sodium orthovanadate, the contractile response was not significantly different (although it tended to be higher) from that obtained in arteries incubated with sodium orthovanadate alone, i.e., in the presence of sodium orthovanadate endotoxin did not modify the reactivity to noradrenaline. In

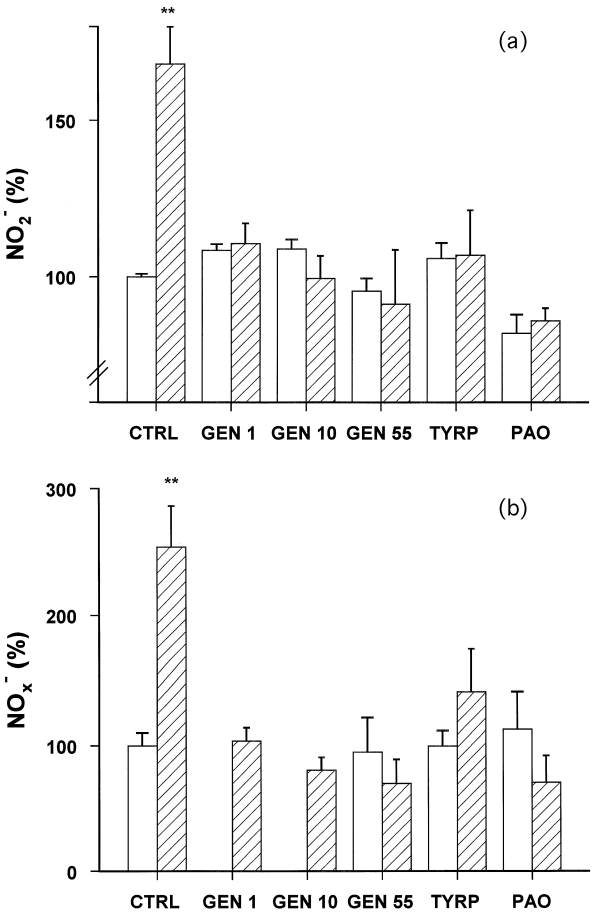


Fig. 4. Effects of tyrosine kinase and tyrosine phosphatase inhibitors on the in vitro nitrite (A) and $NO_x$ (B) accumulation induced by endotoxin. Nitrites and $NO_x$ were measured in the incubation media of endothelium-denuded aortae incubated for 20 h in the absence (open bars) or presence of endotoxin (1 μg/ml, hatched bars) in the absence (CTRL) or in the presence of genistein $10^{-6}$ M (GEN 1), $10^{-5}$ M (GEN 10) or $5.5\times10^{-5}$ M (GEN 55), $3\times10^{-6}$ M tyrphostin (TYRP) or $10^{-6}$ M phenylarsine oxide (PAO). The effects of $10^{-6}$ and $10^{-5}$ M genistein on $NO_x$ accumulation were not tested in the absence of endotoxin. Data (means ± S.E.M. of 6–23 experiments) are expressed as a percentage of values for aortic rings incubated in the presence of vehicle under control conditions. $^{**}P < 0.01$ vehicle vs. endotoxin.

arteries incubated in the presence of phenylarsine oxide ($10^{-6}$ M), alone or in combination with endotoxin, no contractile responses to noradrenaline could be elicited.

*3.2.2. Effects of genistein, tyrphostin, sodium orthovanadate and phenylarsine oxide on endotoxin-induced nitrite and $NO_x$ accumulation*

After 20 h, nitrite and $NO_x$ accumulation in the incubation media of control arteries averaged $1.62 \pm 0.31$ and $3.16 \pm 0.32$ nmol $mg^{-1}$ wet tissue ($n = 23$ and $n = 10$), respectively. Fig. 4A and B show that incubation with endotoxin significantly increased nitrite ($167.6 \pm 12.9$% of control, $n = 15$, $P < 0.01$) and $NO_x$ accumulation ($252.8 \pm 33.4$% of control, $n = 9$, $P < 0.01$). The presence of genistein ($10^{-6}$, $10^{-5}$ and $5.5 \times 10^{-5}$ M) or tyrphostin ($3 \times 10^{-6}$ M) in the incubation media did not modify nitrite or $NO_x$ accumulation in control arteries but inhibited the increase induced by endotoxin. The effects of sodium orthovanadate on nitrite production could not be measured since it interfered with the Griess reaction (it reacted with the Griess reagent to yield a colored solution increasing the absorbance at 560 nm). In the absence of endotoxin, phenylarsine oxide ($10^{-6}$ M) induced a weak reduction in nitrite ($82 \pm 9$% of control, $P < 0.05$) but not in $NO_x$ accumulation. Moreover, phenylarsine oxide inhibited the endotoxin-induced increase in nitrites and $NO_x$.

## 4. Discussion

In the present paper we studied the involvement of the tyrosine kinase pathway in the induction of NO synthase by endotoxin in rat aortae. The main results of this study can be summarized as follows. First, endotoxin induced, both in vivo and in vitro, a decrease in the contractile response to noradrenaline of rat aortic rings, an effect which could be inhibited by the NO synthase inhibitor L-NAME. These effects were accompanied by an increase in nitrite accumulation, the metabolite of NO, in the incubation media. Low concentrations of the tyrosine kinase inhibitors genistein and tyrphostin inhibited both endotoxin-induced hyporesponsiveness and nitrite accumulation in rat aorta without affecting control nitrite accumulation or contraction. Higher concentrations of genistein resulted in an irreversible depression of noradrenaline-induced contractions. When given in vivo, genistein by itself attenuated noradrenaline-induced contraction and prevented further endotoxin-induced hyporesponsiveness and the endotoxin-induced increase in nitrite accumulation. Second, in the absence of endotoxin, incubation with the tyrosine phosphatase inhibitors sodium orthovanadate and phenylarsine oxide depressed vascular contractility. In the presence of these drugs, endotoxin did not induce further depression of vascular contractility and did not increase nitrite production. Therefore and surprisingly, tyrosine phosphatase inhibitors produced effects similar to those of tyrosine kinase inhibitors.

The inhibitory effects of endotoxin on the contractile responses to vasoconstrictors have been attributed to the induction of NO synthase (McKenna, 1990; Fleming et al., 1990; Julou-Schaeffer et al., 1990; Thiemermann, 1994; Moritoki et al., 1995; Villamor et al., 1995). Accordingly, in our experiments, aortae from rats treated with endotoxin or aortic rings treated in vitro with endotoxin demonstrated a decreased contractile response to noradrenaline, an effect which was inhibited by L-NAME, and these effects were associated with endothelium-independent increases in nitrite and $NO_x$ levels. Recently, several reports have demonstrated that in vitro inhibition of tyrosine kinase inhibits the induction by endotoxin of several enzymes

such as cyclooxygenase-2 in endothelial cells and macrophages (Akarasereenont et al., 1994, 1995) and inducible NO synthase in macrophages (Akarasereenont et al., 1994), endothelial cells (Radomski et al., 1990), cultured rat aortic smooth muscle cells (Marczin et al., 1993a; Moritoki et al., 1995) and rat thoracic aorta (Moritoki et al., 1995). In the present experiments, low concentrations of the tyrosine kinase inhibitors genistein ($10^{-6}$ M) and tyrphostin ($3 \times 10^{-6}$ M) inhibited the hyporesponsiveness to noradrenaline and the nitrite and $NO_x$ accumulation induced by endotoxin in vitro, which is consistent with previous evidence suggesting that tyrosine phosphorylation is part of the signal transduction mechanism of NO synthase induction by endotoxin. Inhibition of tyrosine kinase by genistein (10 mg/kg, i.p.) has also been shown to reduce the expression of the inducible NO synthase protein in lung homogenates and the delayed hypotensive response in rats with endotoxin-induced shock (Ruetten and Thiemmerman, 1997). In this study, we showed that genistein (10 mg/kg, i.p.) is able to inhibit the induction of NO synthase by endotoxin in vascular smooth muscle in vivo, which may account for the inhibition of endotoxin-induced hypotension.

Incubation with higher concentrations of genistein ($10^{-5}$ and $5.5 \times 10^{-5}$ M) reduced in a concentration-dependent manner the contractile responses to noradrenaline even when genistein was not present when the concentration–response curves were made. This effect, which was also observed in aortae from rats treated with genistein, seems to be unrelated to NO production since it was not inhibited by L-NAME and was not associated with increased levels of nitrites in the incubation media. Several reports have shown that tyrosine kinase inhibitors, such as genistein, inhibited the contractions evoked in rat aortic rings by several agents, i.e., phenylephrine, noradrenaline, phorbol 12-myristate 13-acetate and high-KCl (Abebe and Agrawal, 1995; Filipeanu et al., 1995; Herrera et al., 1996). In the present study, the potency of long-term incubation with genistein to inhibit noradrenaline-induced contractions (calculated $IC_{50} = 2.9 \times 10^{-5}$ M) was similar to that previously reported for relaxation of phenylephrine- and noradrenaline-induced contractions in rat aorta ($IC_{50} = 6.9 \times 10^{-5}$ M, Filipeanu et al., 1995; $4.2 \times 10^{-5}$ M, Herrera et al., 1996). However, the relationship between the vasodilator response of genistein and its inhibitory effects on tyrosine kinase is not clear. In fact, several other mechanisms for its vasodilator activities have been proposed: inhibition of $Ca^{2+}$ entry or $Ca^{2+}$ release induced by agonists (Sargeant and Sage, 1993; Filipeanu et al., 1995), a direct effect upon the contractile proteins (Ozaki et al., 1993) and inhibition of other kinases, such as protein kinase C (Hidaka and Kobayashi, 1992; Herrera et al., 1996). Furthermore, since, after long-term incubation with genistein, the reduced contractile responses were observed in the absence of the drug, it is also possible that genistein may induce irreversible changes in the arteries or, due to its high lipophilicity, may accumulate in the preparation. Therefore, the present results demonstrate that there is a dissociation between the effects of genistein and tyrphostin on vascular smooth muscle contraction and NO synthase induction, this latter effect being much more sensitive to inhibition by these drugs. Furthermore, over a certain range of concentrations, these drugs can inhibit the vascular hyporesponsiveness induced by endotoxin without producing vasodilator effects.

The role of tyrosine kinase-induced phosphorylation in the induction of NO synthase in rat aortic smooth muscle was also studied by assessing the effects of the tyrosine phosphatase inhibitors sodium orthovanadate and phenylarsine oxide. Inhibition of tyrosine phosphatase would be expected to enhance tyrosine kinase-mediated responses (Laniyonu et al., 1994). In fact, acute exposure to sodium orthovanadate contracts vascular smooth muscle (Filipeanu et al., 1995) and increases noradrenaline-induced contractions (Abebe and Agrawal, 1995), i.e., actions opposite to those of tyrosine kinase inhibitors. Surprisingly, long-term incubation with tyrosine phosphatase inhibitors produced similar effects to those of tyrosine kinase inhibitors both on vascular contractility and NO synthase induction. Incubation with sodium orthovanadate decreased the contractile response to noradrenaline and, under these conditions, endotoxin was unable to induce any further decrease, whereas phenylarsine oxide fully abolished noradrenaline-induced responses. Sodium orthovanadate and phenylarsine oxide have been recently reported to induce endothelium-dependent relaxations (Nakaike et al., 1996; Fleming et al., 1996). However, this mechanism cannot account for the reduced contractions in response to noradrenaline in our experiments, since the experiments were performed with endothelium-denuded arteries. Furthermore, phenylarsine oxide did not increase nitrite and $NO_x$ levels but inhibited the endotoxin-induced increase. Unfortunately, nitrite levels could not be measured with orthovanadate since it interfered with the Griess reaction. Therefore, the relationship between tyrosine kinases and tyrosine phosphatases regarding vascular smooth muscle contraction and NO synthase induction is apparently more complex than expected. A possible explanation for these contradictory results is the following. Phosphorylation of a tyrosine residue in the C-terminus fragment of tyrosine kinases of the Src family decreases their activity, the dephosphorylation of this residue by tyrosine phosphatases being a prerequisite for enzyme activation (Yanagi et al., 1996). Vanadate and phenylarsine, by inhibiting the tyrosine phosphatase(s), might increase phosphorylation at this inhibitory site and thus may decrease tyrosine kinase activity. Therefore, tyrosine phosphatase inhibitors may behave as indirect tyrosine kinase inhibitors. Accordingly, Nakaike et al. (1996) have reported that vanadate causes a dramatic decrease in the amount of the active form of the Src family tyrosine kinases in pig coronary arteries. Nevertheless, although the drugs used to inhibit tyrosine kinases and

phosphatases were selected from different chemical groups and act through different mechanisms, a possible lack of specificity of the inhibitors used cannot be ruled out.

In conclusion, the tyrosine kinase inhibitors genistein and tyrphostin inhibited endotoxin-mediated induction of NO synthase and the subsequent vascular hyporesponsiveness in rat aorta. In addition, they also depressed noradrenaline-induced contractile responses. However, there was a dissociation between the effects of these drugs on vascular smooth muscle contraction and NO synthase induction, the latter being much more sensitive to inhibition by these drugs. Tyrosine phosphatase inhibitors (sodium orthovanadate and phenylarsine oxide) produced similar effects to those of tyrosine kinase inhibitors, which suggests that there is a complex relationship between tyrosine kinases and phosphatases in the signalling pathway of agonist-induced vascular smooth muscle contraction and NO synthase induction.

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