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European Journal of Pharmacology 377 (1999) 193–197

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Increased nitroglycerin-induced relaxation by genistein in rat aortic rings

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Received 20 May 1999; accepted 1 June 1999

Abstract

The effect of genistein, a tyrosine kinase inhibitor, on nitroglycerin-induced relaxation was examined in rat aortic rings contracted by phenylephrine. In rat aortic rings, genistein (10^{-5} M and 3×10^{-5} M), a tyrosine kinase inhibitor, but not daidzein, an analogue of genistein, increased relaxation induced by nitroglycerin in a concentration-dependent manner. Iberiotoxin, an inhibitor of Ca^{2+}-activated K^+ channels, inhibited the relaxation induced by nitroglycerin, but it did not affect the effect of genistein. Glibenclamide, an inhibitor of ATP-sensitive K^+ channels, did not affect the relaxation induced by nitroglycerin. Theophylline, an inhibitor of cyclic AMP-dependent phosphodiesterase, increased the relaxation induced by nitroglycerin, and genistein (10^{-5} M) failed to affect the relaxation induced by nitroglycerin in the presence of theophylline. Genistein also inhibited the activity of cyclic AMP-dependent phosphodiesterase. In addition, 6-[4-(4′-pyridyl)amino phenyl]-4,5-dihydro-3(2*H*)-pyridazinone hydrochloride, an inhibitor of cyclic GMP-inhibitable cyclic AMP phosphodiesterase, inhibited the relaxation induced by nitroglycerin. These results suggest that, in the rat aortic rings, genistein inhibits cyclic AMP-dependent phosphodiesterase activities, resulting in the increase of the relaxation induced by nitroglycerin.

Keywords: Genistein; Nitroglycerin; Relaxation; Aorta, rat; Phosphodiesterase cAMP-dependent

1. Introduction

Nitroglycerin is known to cause relaxation of vascular smooth muscle by activation of guanylyl cyclase and subsequent increase of cyclic GMP formation (Ignarro and Kadowitz, 1985). It has also been reported that activation of large conductance Ca^{2+}-activated K^+ (K_{Ca}) channels may be involved in the relaxation due to nitroglycerin (Hamaguchi et al., 1992; Ishibashi et al., 1995; Pataricza et al., 1995). More recently, the involvement of ATP-sensitive K^+ (K_{ATP}) channels in the relaxant effect of nitroglycerin was also reported (Dumas et al., 1996).

The signal transduction pathway involving tyrosine kinase has recently been proposed in agonist-induced contraction of vascular smooth muscle (Tsuda et al., 1991; Saifeddine et al., 1992; Di Salvo et al., 1993; Sauro and Thomas, 1993; Abebe and Agrawal, 1995; Jinsi and Deth, 1995; Jinsi et al., 1996). In addition, tyrosine kinase was reported to be involved in the signaling pathway of lipopolysaccharide that triggers expression of nitric oxide (NO) synthase (Marczin et al., 1993; Nomura and Kitamura, 1993; Feinstein et al., 1994; Moritoki et al., 1995).

Recently, our preliminary experiment revealed that genistein, an inhibitor of tyrosine kinase (Akiyama et al., 1987), increased vasorelaxing effect of nitroglycerin. Since nitroglycerin is known to cause vasorelaxation by at least two mechanisms, increased cyclic GMP formation and activation of potassium channels, we have further investigated possible involvement of these two mechanisms in the effect of genistein on the relaxation induced by nitroglycerin.

2. Materials and methods

2.1. Mechanical response

Male Wistar rats weighing 150–170 g were killed by cervical dislocation under ether anesthesia. The aortas were isolated, and excess fats and connective tissue were

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00412-4

removed. Vessels were cut into rings of about 3 mm in length. Preparations were mounted in organ baths containing 25 ml of a modified Krebs solution of the following composition (mM); NaCl, 120.3; KCl, 4.8; $CaCl_2$, 1.2; $MgSO_4$, 1.3; KH_2PO_4, 1.2; $NaHCO_3$, 24.2; and glucose, 5.8, at pH 7.4. The tissue bath solution was maintained at 37°C and bubbled with a 95% O_2 and 5% CO_2 mixture. Stainless steel hooks were put through the aortic ring, one attaching the muscle to a stainless steel rod and the other to a transducer adjusted to give an initial stretched tension of 2 g. Changes in isometric tension were recorded through force-displacement transducers (Grass FT-03) connected to a six-channel Grass polygraph.

The aortic rings were contracted by phenylephrine (3×10^{-6} M) before the addition of vasorelaxing agents. Some tissues were pretreated by inhibitors for 20 min before addition of phenylephrine. The presence of endothelium was confirmed by the presence of acetylcholine (10^{-6} M)-induced relaxation (> 80%) in the aorta precontracted by phenylephrine (3×10^{-6} M).

2.2. Cyclic AMP-dependent phosphodiesterase activity

Cyclic AMP-dependent phosphodiesterase activity was determined by a procedure of Poch (1971). Rat aorta was homogenized at 4°C in 5 vol. of 1.15% KCl. The homogenate was centrifuged at 100,000 × *g* for 40 min. The supernatant was dialyzed overnight against 10 mM Tris–HCl buffer (pH 7.5) at 4°C and stored at −70°C. The protein was determined by Lowry et al. (1951). The dialyzed supernatant was used as enzyme preparation. The reaction was started by addition of enzyme preparation (16 μg protein/ml) in the incubation mixture containing 40 mM Tris–HCl (pH 7.4), 5 mM Mg Cl_2, 1 mM EGTA, 0.15 μM cyclic [^{3}H]AMP in a total volume of 0.5 ml (30°C for 10 min). Some incubation mixtures contained genistein (10^{-5} M). The reaction was terminated by boiling for 5 min. 0.05 ml of snake venom (1 mg/ml) was added and the mixture was incubated at 30°C for 10 min to convert 5′[^{3}H]AMP formed by phosphodiesterase to [^{3}H]adenosine. Suspension (1 ml) of ion exchange resin (AG 1-X2, Bio-Rad) was added and after 10 min, the mixture was centrifuged at 1000 × *g* for 5 min. 0.25 ml of the supernatant was placed in scintillation vials containing 10 ml of Aquasol 2 (New England Nuclear) and the radioactivity was measured.

2.3. Chemicals

The following drugs were used: nitroglycerin (Warner-Lambert, Morris Plain, NJ, USA), glibenclamide (Upjohn, Kalamazoo, MI, USA), genistein (LC Laboratories, Woburn, MA, USA), daidzein (LC Laboratories), iberiotoxin (Research Biochemicals International, Natick, MA, USA), phenylephrine, acetylcholine, theophylline, and snake venom (*Crotalus atrox*) (Sigma, St. Louis, MO, USA), 6-[4-(4′-pyridyl)amino phenyl]-4,5-dihydro-3(2*H*)-pyridazinone hydrochloride (MCI-154) (Mitsubishi Pharmaceutical, Tokyo, Japan).

2.4. Statistical analysis

Maximal contractions induced by phenylephrine just before the addition of relaxing agents were taken as 100%. The data are presented as the mean ± S.E.M. The data are analyzed by Student's two-tailed *t*-test and analysis of variance, followed by Dunnett's test.

3. Results

In rat aortic rings precontracted by phenylephrine (3×10^{-6} M), nitroglycerin (10^{-9}–3×0^{-5} M) caused relaxation in a biphasic manner (Fig. 1). Pretreatment of the aortic rings by genistein at 10^{-5} M and 3×10^{-5} M, but not daidzein (10^{-5} M), significantly increased the relaxation induced by nitroglycerin at 10^{-6}–10^{-5} M (Fig. 1). Pretreatment of the aortic rings by iberiotoxin (2×10^{-8} M), but not glibenclamide (10^{-5} M), significantly inhibited the relaxation induced by nitroglycerin (3×10^{-8}–10^{-4} M) (Fig. 2). In the presence of iberiotoxin (2×10^{-8} M), genistein (10^{-5} M) still significantly increased the relaxation induced by nitroglycerin (3×10^{-6}–10^{-4} M; Fig. 2).

In aortic rings precontracted by phenylephrine (3×10^{-6} M), theophylline (3×10^{-4} M) significantly increased the relaxation induced by nitroglycerin (10^{-9}–10^{-7} M) (Fig. 3). In the aortic rings pretreated by theophylline (3×10^{-4}

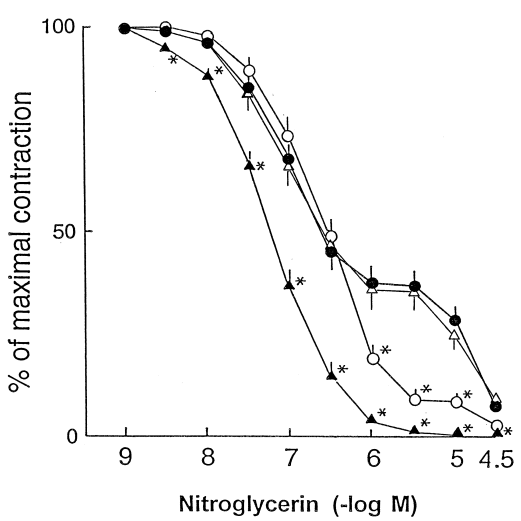

Fig. 1. The effect of genistein on the relaxation induced by nitroglycerin. Rat aortic rings precontracted by phenylephrine were relaxed by nitroglycerin (●) ($n = 4$). Some tissues were pretreated by genistein at 10^{-5} M (○), 3×10^{-5} M (▲) or daidzein at 10^{-5} M (△) ($n = 4$). * Significantly different from the control (●) ($P < 0.05$).

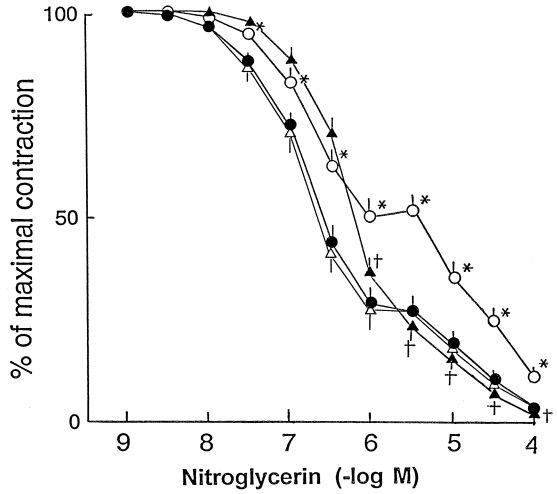

Fig. 2. The effects of iberiotoxin and genistein on the relaxation induced by nitroglycerin. Rat aortic rings precontracted by phenylephrine were relaxed by nitroglycerin (●) ($n = 4$). Some tissues were pretreated by iberiotoxin (2×10^{-8} M) (○) ($n = 8$) or genistein (10^{-5} M) plus iberiotoxin (2×10^{-8} M) (▲) or glibenclamide (10^{-5} M) (△) ($n = 4$). * Significantly different from the control (●) ($P < 0.05$). + Significantly different from the group with iberiotoxin alone (○) ($P < 0.05$).

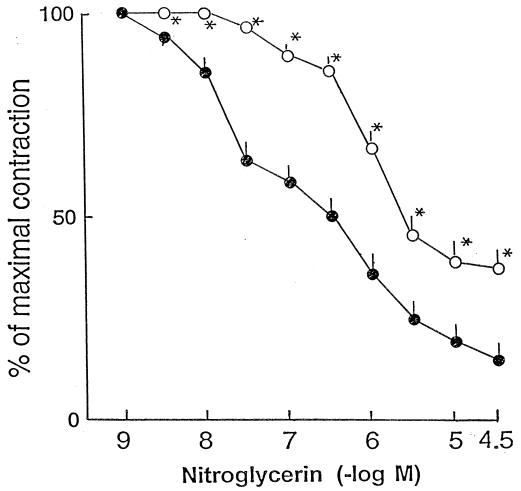

Fig. 4. The effect of MCI-154 on the relaxation induced by nitroglycerin. Rat aortic rings precontracted by phenylephrine were relaxed by nitroglycerin (●) ($n = 4$). Some tissues were pretreated by MCI-154 (10^{-6} M) (○) ($n = 4$). * Significantly different from the control (●) ($P < 0.05$).

M), genistein (10^{-5} M) failed to affect the relaxation induced by nitroglycerin (10^{-8}–3×10^{-7} M) (Fig. 3). In the aortic rings, pretreatment by MCI-154 (10^{-6} M) inhibited the nitroglycerin-induced relaxations (Fig. 4). The activity of cyclic AMP-dependent phosphodiesterase was examined. The incubation of the enzyme preparation with genistein (10^{-5} M) significantly inhibited the activity of cyclic AMP-dependent phosphodiesterase as compared to the control (control: 0.592 ± 0.053 nmol/mg protein/min, genistein: 0.319 ± 0.037 nmol/mg protein/min, $n = 6$).

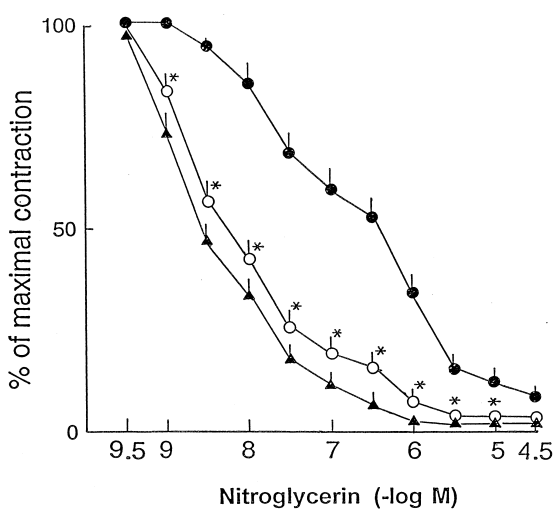

Fig. 3. The effects of genistein and theophylline on the relaxation induced by nitroglycerin. Rat aortic rings precontracted by phenylephrine were relaxed by nitroglycerin (●) ($n = 4$). Some tissues were pretreated by theophylline (3×10^{-4} M) (○) or theophylline (3×10^{-4} M) plus genistein (10^{-5} M) (▲) ($n = 4$). * Significantly different from the control (●) ($P < 0.05$).

4. Discussion

In the present study, genistein, an inhibitor of tyrosine kinase (Akiyama et al., 1987), was shown to increase the relaxation induced by nitroglycerin in rat aortic rings. Daidzein, an analogue of genistein which is inactive against tyrosine kinase at the concentration used in the present study (Akiyama et al., 1987), did not affect the relaxation induced by nitroglycerin. These results suggest that tyrosine kinase may be partly involved in the relaxation induced by nitroglycerin. The biphasic relaxant curve of nitroglycerin observed in the present study has been reported previously in bovine mesenteric artery (Ahlner et al., 1988), rat aorta (Malta, 1989), and bovine tracheal smooth muscle (Hamaguchi et al., 1992). In the rat aorta, the first relaxant phase, but not the second phase, was inhibited by oxyhaemoglobin (Malta, 1989). It was suggested that there are two distinct mechanisms of relaxation for nitroglycerin, involving cyclic GMP as the second messenger (Malta, 1989).

Nitroglycerin is known to cause relaxation of smooth muscle through increased cyclic GMP formation by activation of guanylyl cyclase (Ignarro and Kadowitz, 1985). K_{Ca} channels can be activated by cyclic GMP through activation of cyclic GMP-dependent protein kinase (Khan et al., 1993; Robertson et al., 1993; Archer et al., 1994). NO was also reported to directly activate K_{Ca} channels in rabbit aortic smooth muscle cells (Bolotina et al., 1994). In addition, activation of K_{Ca} channels may be involved in the relaxation induced by nitroglycerin (Ishibashi et al., 1995; Pataricza et al., 1995). The present study also demonstrated that the inhibition of K_{Ca} channels by iberi-

otoxin, an inhibitor of K_{Ca} channels (Galvez et al., 1990), inhibited the relaxation induced by nitroglycerin. However, the effect of genistein on the relaxation induced by nitroglycerin was still apparent even in the presence of iberiotoxin. These results, therefore, indicate that the activation of K_{Ca} channels may not be involved in the effect of genistein to increase the nitroglycerin-induced relaxation. In addition, since glibenclamide, an inhibitor of K_{ATP} channels, did not affect the relaxation induced by nitroglycerin, a possible involvement of K_{ATP} channels in the effect of genistein on the relaxation induced by nitroglycerin may be eliminated.

The present study also indicated that inhibition of cyclic AMP-dependent phosphodiesterase by theophylline, an inhibitor of cyclic AMP-dependent phosphodiesterase, increased the relaxation induced by nitroglycerin. In this experimental condition, genistein failed to affect nitroglycerin-induced relaxation already increased by theophylline. Therefore, it is conceivable that the mechanism by which genistein increases the nitroglycerin-induced relaxation may be similar to that of theophylline. It has been suggested that, in rat pinealocytes, cyclic AMP metabolism may be tonically controlled by tyrosine kinase acting on cyclic AMP-dependent phosphodiesterase (Ho et al., 1995). In the present study, genistein also inhibited the activity of cyclic AMP-dependent phosphodiesterase. These results, therefore, suggest that the effect of genistein on the relaxation induced by nitroglycerin is most likely due to the inhibition of cyclic AMP-dependent phosphodiesterase.

The mechanism in which the increased cyclic AMP levels caused by genistein affects the relaxation induced by nitroglycerin was further investigated in the present study. Cyclic GMP inhibits cyclic GMP-inhibitable cyclic AMP phosphodiesterase (Thompson, 1991), resulting in the potentiation of isoproterenol-induced relaxation (Maurice et al., 1991). It was reported that nicorandil, through an increased level of cyclic GMP, increased cyclic AMP levels in the rat aorta in the absence or presence of isoproterenol (Satake et al., 1995). It was further reported that MCI-154, an inhibitor of cyclic GMP-inhibitable cyclic AMP phosphodiesterase (Kitada et al., 1987), potentiated the isoproterenol-induced relaxation and inhibited the potentiating effect of nicorandil on the isoproterenol-induced relaxation (Satake et al., 1995). In the present study, MCI-154 inhibited the relaxation induced by nitroglycerin. This suggests that the nitroglycerin-induced relaxation is partly due to the inhibition of cyclic GMP-inhibitable cyclic AMP phosphodiesterase, resulting in the increased level of cyclic AMP. Given all the results above, it is suggested that the nitroglycerin-induced relaxation is increased by genistein mostly due to the increased level of cyclic AMP caused by the inhibition of cyclic AMP-dependent phosphodiesterase by genistein. The level of cyclic AMP is further increased following the inhibition of cyclic GMP-inhibitable cyclic AMP phosphodiesterase by the nitroglycerin-induced increase in cyclic GMP levels.

5. Conclusion

In the rat aortic rings, genistein, an inhibitor of tyrosine kinase, increased the relaxation induced by nitroglycerin. This may be due to the inhibition of cyclic AMP-dependent phosphodiesterase activities.

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