

Tyrosine kinases participate in α_{1A} -adrenoceptor-mediated vasoconstriction in perfused rat hindlimb¹

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KEY WORDS alpha-1 adrenergic receptors; protein-tyrosine kinase; hindlimb; erbstatin; quercetin; sodium fluoride; vanadates; tetradecanoyl acetate

AIM: To determine whether or not tyrosine kinase is involved in the signal transduction of α_{1A} -adrenoceptors. **METHODS:** Effects of various pharmacological probes on norepinephrine (NE)-induced vasopressor responses were determined in the perfused rat hindlimb. **RESULTS:** The putative tyrosine kinase inhibitors, genistein, and tyrphostin, significantly inhibited the vasopressor responses induced by NE but not that induced by KCl. The protein-tyrosine-phosphatase inhibitor, sodium orthovanadate, selectively potentiated the vasopressor responses induced by NE. Neither genistein nor tyrphostin had effect on the contraction elicited by phorbol 12-myristate 13-acetate. In contrast, both genistein and tyrphostin attenuated the vasopressor responses evoked by NaF. **CONCLUSION:** The genistein- and tyrphostin-sensitive tyrosine kinases participate in α_{1A} -adrenoceptor-mediated vasoconstriction in perfused rat hindlimb.

It has traditionally been regarded that there are different signal transduction pathways between the G protein-coupled receptors and tyrosine kinase-coupled receptors, but it has been recently reported that there was cross-talk between them^[1]. Two groups of tyrosine kinase inhibitors have been described: compounds interacting with the ATP binding site, such as genistein (a quercetin derivatives) and those which interact with the substrate binding site, such as the tyrphostins (synthetic analogues of

erbstatin). They are useful pharmacological probes in investigating the role of tyrosine kinases in diverse signal transduction.

The α_1 -adrenoceptor has been classified by pharmacological evidences into three subtypes termed α_{1A} -, α_{1B} -, and α_{1D} -adrenoceptor^[2]. Our previous studies have demonstrated that α_{1A} -adrenoceptors are the major functional receptors mediating vasopressor response induced by exogenous norepinephrine (NE) in rat perfused hindlimb vasculature^[3]. It is well accepted that the responses mediated by α_1 -adrenoceptors are elicited through the activation of phospholipase C (PLC) and its related signal transduction. It has been reported^[4,5] that the NE-induced contraction was attenuated by genistein or tyrphostin in rat aorta, in which α_{1D} -adrenoceptors dominate NE-induced contractile response^[6]. This prompted us to investigate the possible involvement of tyrosine kinases in the intracellular signaling pathways of α_{1A} -adrenoceptor-mediated contraction in rat hindlimb vascular smooth muscle.

MATERIALS AND METHODS

NE, yohimbine, propranolol, desmethylinipramine, normetanephrine, indometacin, genistein, quercetin, tyrphostin A₄₇, sodium fluoride, phorbol 12-myristate 13-acetate (PMA), potassium chloride, sodium orthovanadate (Na_3VO_4) were purchased from Sigma.

Preparation of perfused rat hindlimb^[7]

Wistar rats ($n = 75$, 180 - 200 g), Certificate No 013056, were supplied by the Experimental Animal Center of Beijing Medical University. Rats were anesthetized with pentobarbital sodium ($60 \text{ mg} \cdot \text{kg}^{-1}$, ip). Flow was restricted to two hindlimbs by cannulation with PE-50 polyethylene tubes to two bilateral common iliac arteries. Perfusion was performed in a temperature-controlled cabinet, and the perfusion medium was temperature-equilibrated through a heat exchanger at 28 °C. The perfusion Krebs solution

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contained NaCl 118, KCl 4.7, CaCl₂ 1.27, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 8.3 mmol·L⁻¹, and 2 % hetastarch, pH 7.4. Saturated with 95 % O₂ + 5 % CO₂. The perfusion pressure was monitored through a junction located as close as practicable to the iliac canal, using a pressure transducer connected to a polygraph. Desmethylinipramine 1 μmol·L⁻¹, normetanephrine 1 μmol·L⁻¹ (to block neuronal and extraneuronal uptake of NE, respectively), indometacin 10 μmol·L⁻¹ (to inhibit prostanoid production), propranolol 10 μmol·L⁻¹ (to block β-adrenoceptors), and yohimbine 0.1 μmol·L⁻¹ (to block α₂-adrenoceptors) were included in the perfusion solution.

Effects of tyrosine kinase- and phosphatase-inhibitor on vasoconstrictor responses
After equilibration, the preparations were contracted with KCl (80 mmol·L⁻¹) to condition the tissues for 5 min and then washed. After further equilibrated, contractile responses to NE, KCl, NaF, and PMA were obtained in separate preparations in a cumulative fashion. Osmolarity changes that could be induced by the addition of high concentration of KCl and NaF did not appear to have effects on the hindlimb vascular beds since similar concentrations of NaCl had no detectable action. When KCl was used as a contractile agent, the tissues were preincubated with phentolamine 1 μmol·L⁻¹ for 20 min to block the effect of neurally released NE. The involvement of tyrosine kinases in the contractile response of the various agents was assessed by repeating the concentration-response curves (CRC) after pretreatment of the tissues with genistein (3, 10, and 30 μmol·L⁻¹), quercetin (3, 10, 30, and 100 μmol·L⁻¹), tyrphostin A₄₇ (1, 3, 10, and 30 μmol·L⁻¹), Na₃VO₄ (30 and 100 μmol·L⁻¹) for 30 min. Responses to the contractile agents in the absence and presence of genistein, quercetin, tyrphostin, or Na₃VO₄ were expressed as developed perfusion was determined from a calibrated recorder tracing, and concentration-response relationships were determined from these readings, so that a graphical representation could be made. EC₅₀ and EC₂₀ (concentration producing half- and 20 percent of maximal contraction, respectively) values were

calculated by nonlinear regression analysis of the CRC using all the data points. No alterations in responsiveness with time were observed in these experiments.

Statistics Results were expressed as $\bar{x} \pm s$. Comparisons were made using ANOVA and *t*-test. The pD₂ and pD₅ values represented the -lg EC₅₀ and -lg EC₂₀.

RESULTS

Effects of genistein and tyrphostin A₄₇ on the NE-induced vasopressor responses The maximum responses of NE were reduced by genistein (3 - 30 μmol·L⁻¹), tyrphostin A₄₇ (1 - 30 μmol·L⁻¹), and quercetin (3 - 100 μmol·L⁻¹) in a concentration-dependent manner (Tab 1, Fig 1).

Tab 1. The effects of tyrosine kinase inhibitor on norepinephrine-induced contractile responses in rat perfused hindlimb. *n* = rats. $\bar{x} \pm s$.

**P* > 0.05, ^b*P* < 0.05, ^c*P* < 0.01 vs control.

μmol·L ⁻¹	<i>n</i>	pD ₂ (-lg mol·L ⁻¹)	Maximum response/kPa
NE-control	12	5.27 ± 0.14	19.3 ± 1.3
Quercetin	3	5.08 ± 0.17 ^a	15.1 ± 2.9 ^c
	10	5.12 ± 0.12 ^a	10.7 ± 1.3 ^c
	30	4.97 ± 0.17 ^a	8.1 ± 2.4 ^c
	100	5.1 ± 0.3 ^a	2.4 ± 0.3 ^c
NE-control	13	5.15 ± 0.14	18.7 ± 2.9
Genistein	3	4.91 ± 0.27 ^a	14.1 ± 2.6 ^c
	10	4.75 ± 0.20 ^a	9.7 ± 3.3 ^c
	30	4.8 ± 0.4 ^a	7.2 ± 2.9 ^c
NE-control	13	5.29 ± 0.18	18.7 ± 1.9
Tyrphostin A ₄₇	1	5.18 ± 0.17 ^a	14.9 ± 3.9 ^c
	3	4.97 ± 0.22 ^b	13.8 ± 2.3 ^c
	10	5.05 ± 0.12 ^b	11.9 ± 1.6 ^c
	30	4.90 ± 0.15 ^b	8.7 ± 1.6 ^c

Effects of Na₃VO₄ on the NE-induced vasopressor responses The basal perfusion pressure was increased from (5.2 ± 0.3) to (8.9 ± 0.9) kPa (*n* = 5, *P* < 0.01) by Na₃VO₄ 100 μmol·L⁻¹. The maximum increase of perfusion pressure induced by NE decreased from (17.6 ± 1.7) and (14.1 ± 1.5) kPa (*n* = 5, *P* < 0.01). However, the peak perfusion pressures were not significantly different between the control and Na₃VO₄ 100 μmol·L⁻¹ [(17.6 ±

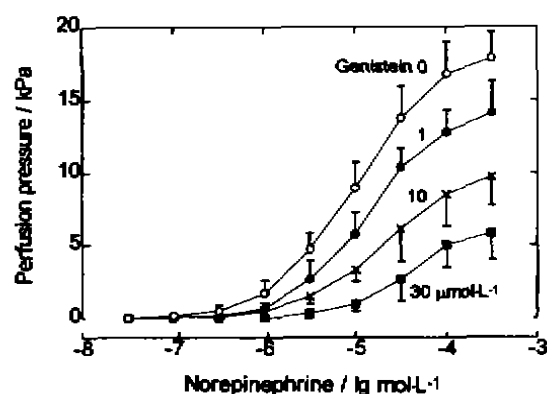


Fig 1. Effects of genistein on the NE-induced vasopressor response in perfused hindlimb. $n = 5 - 13$ rats. $\bar{x} \pm s$.

1.7) to (17.8 ± 2.3) kPa]. The pD_2 values increased from 5.18 ± 0.06 to 5.7 ± 0.4 ($P < 0.05$, Fig 2).

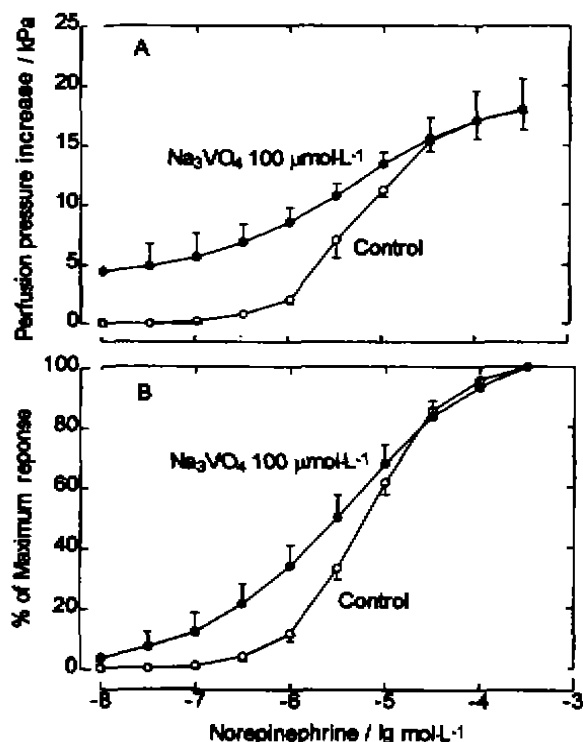


Fig 2. Effect of Na_3VO_4 on the NE-induced vasopressor response in perfused hindlimb ($n = 5$ rats). $\bar{x} \pm s$.

By Na_3VO_4 $30 \mu\text{mol}\cdot\text{L}^{-1}$, although both the basal perfusion pressures [(5.1 ± 0.6) and (5.6 ± 0.3) kPa, $n = 5$, $P > 0.05$] and maximum responses [(18.8 ± 0.6) and (18.4 ± 0.6) kPa, $n = 5$, $P > 0.05$] induced by NE were not affected, the pD_5 values increased from $(5.78 \pm$

$0.10)$ to (6.07 ± 0.25) ($n = 6$, $P < 0.05$].

Effects of genistein, tyrophostin A_{47} , and Na_3VO_4 on KCl-induced vasoconstriction The genistein ($10 \mu\text{mol}\cdot\text{L}^{-1}$), tyrophostin A_{47} ($10 \mu\text{mol}\cdot\text{L}^{-1}$), and Na_3VO_4 (30 and $100 \mu\text{mol}\cdot\text{L}^{-1}$) had no effects on contractile response induced by KCl (Tab 2).

Tab 2. The effects of genistein and tyrophostin- A_{47} on contractile agent-induced contractile responses in rat perfused hindlimb. $n = 5$ rats. $\bar{x} \pm s$. $^c P < 0.01$ vs control.

$\mu\text{mol}\cdot\text{L}^{-1}$	pD_2 ($-\lg \text{mol}\cdot\text{L}^{-1}$)	Maximum response/kPa
KCl-control	1.4 ± 0.5	7.8 ± 1.7
Genistein 10	1.17 ± 0.07	8.0 ± 1.9
30	1.17 ± 0.06	6.0 ± 1.1
KCl-control	1.31 ± 0.11	8.1 ± 2.4
Tyrophostin A_{47} 10	1.34 ± 0.04	8.6 ± 2.3
30	1.38 ± 0.04	6.3 ± 1.4
KCl-control	1.30 ± 0.12	8.0 ± 1.2
Na_3VO_4 10	1.34 ± 0.02	7.7 ± 1.7
30	1.31 ± 0.01	6.5 ± 1.9
PMA-control	7.1 ± 0.7	5.5 ± 2.6
Genistein 10	7.0 ± 0.5	5.5 ± 2.9
PMA-control	7.04 ± 0.04	5.5 ± 2.4
Tyrophostin A_{47} 10	7.06 ± 0.23	5.7 ± 2.1
NaF-control	2.1 ± 0.5	21.4 ± 2.9
Genistein 10	2.30 ± 0.17	14.3 ± 1.7^c
NaF-control	1.8 ± 0.4	24.1 ± 2.8
Tyrophostin A_{47} 10	1.57 ± 0.13	18.1 ± 2.3^c

Effects of genistein and tyrophostin A_{47} on the PMA-induced vasopressor responses

Both genistein ($10 \mu\text{mol}\cdot\text{L}^{-1}$) and tyrophostin A_{47} ($10 \mu\text{mol}\cdot\text{L}^{-1}$) did not show any significant effect on the PMA-induced vasopressor response in rat hindlimb vasculature (Tab 2).

Effects of genistein and tyrophostin A_{47} on the NaF-induced vasopressor responses The maximum response induced by NaF were inhibited by genistein ($10 \mu\text{mol}\cdot\text{L}^{-1}$) and tyrophostin A_{47} ($10 \mu\text{mol}\cdot\text{L}^{-1}$), and the pD_2 had no significant change (Fig 3, Tab 2).

DISCUSSION

In present study, we demonstrated that the putative tyrosine kinase inhibitors, genistein and tyrophostin, attenuated the NE-induced vasopressor responses in perfused rat hindlimb.

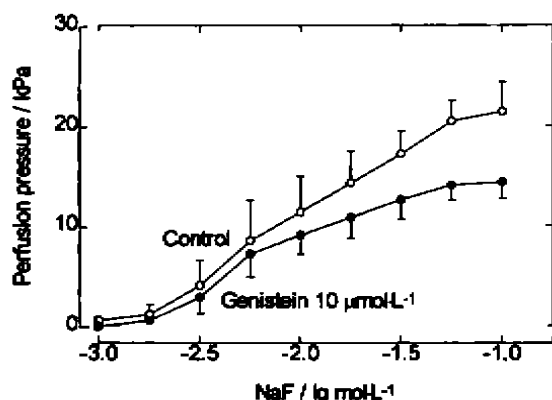


Fig 3. Effect of genistein on the NE-induced vasopressor response in perfused hindlimb. $n = 5 - 13$ rats. $\bar{x} \pm s$.

Additional support for the involvement of tyrosine kinases in the NE-induced contraction of rat hindlimb vasculature was provided by the ability of Na_3VO_4 to potentiate the effect of the agonist. Na_3VO_4 , by inhibiting the activity of tyrosine-specific phosphatases^[8], has been shown to enhance tyrosine kinase-mediated response. These results suggest that at least part of the NE response of this tissue is mediated by activation of tyrosine kinases. Furthermore, the lack of the tyrosine kinase- and phosphatase-inhibitors effect on contractile responses generated by KCl provides evidence that the inhibitors did not produce their effects nonspecifically by inhibiting the contractile machinery or protein kinases directly involved in the contractile processes or by depolarization-associated mechanisms.

With regard to the specificity of these inhibitors used in this study, other investigators also reported that although both genistein and tyrphostin in the concentrations used in the current study could inhibit the activity of tyrosine kinases, they had no significant effect on other enzymes, including myosin light chain kinase, protein kinase A, or protein kinase C (PKC)^[9,10]. In addition, Na_3VO_4 in the concentration used in the current study has been shown to inhibit protein tyrosine phosphatases involved in agonist-induced responses selectively^[8]. Therefore, the effects of the inhibitors observed in the current study were result from selective inhibition of tyrosine kinases or tyrosine phosphatases.

It is not clear which is the target of tyrosine kinase in the signaling pathway of α_1 -

adrenoceptors. The present study show that the tyrosine kinase inhibitors noncompetitively inhibit the G protein activator (NaF)-induced vasopressor response, while it had no effect on the vasopressor response induced by PKC activator (PMA). Since NaF produces contraction of vascular smooth muscle by directly activating G-protein^[11], those results suggest that the target of tyrosine kinases may lie after the receptor activation and beyond the PKC activation. Recently Umemori *et al*^[12] reported that the G-protein $G_{q/11}$ was activated by tyrosine phosphorylation of the α subunit in several G protein-coupled receptors. Since the α_1 -adrenoceptor is coupled with $G_{q/11}$ protein, the tyrosine kinase inhibitors may block the signaling pathway of α_1 -adrenoceptor by phosphorylation of its coupled G protein. Jayarman *et al*^[13] reported that activation of the IP_3 -receptor by tyrosine phosphorylation may play an important role in mobilization of intracellular calcium store in mice T cells. Therefore, the IP_3 -receptor may be another target of tyrosine kinase in the signaling pathway of α_1 -adrenoceptors.

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酪氨酸激酶参与 α_{1A} -肾上腺素受体介导的
灌流大鼠后肢血管床收缩反应¹

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关键词 α_1 -肾上腺素受体; 酪氨酸激酶;

后肢; erbstatin; 槲皮素; 氟化钠; 钒酸盐; 血 $\frac{2}{13}$ 床收
十四酰乙酸盐

目的: 研究酪氨酸激酶是否参与 α_{1A} -肾上腺素受体引起血管平滑肌收缩的信号传导. 方法: 灌流大鼠后肢血管床标本, 观察酪氨酸激酶抑制剂对去甲肾上腺素(NE)引起收缩反应的影响. 结果: 酪氨酸激酶抑制剂 tyrphostin 和 genistein 均显著抑制 NE 引起的收缩反应, 但对 KCl 引起的收缩反应无影响; 酪氨酸磷酸酶抑制剂 Na_3VO_4 显著加强 NE 引起的收缩反应; tyrphostin 和 genistein 对蛋白激酶 C 激动剂 phorbol 12-myristate 13-acetate 引起的收缩反应均无影响, 但均抑制 G 蛋白激动剂 NaF 引起的收缩反应. 结论: Tyrphostin 和 genistein 敏感的酪氨酸激酶参与 α_{1A} -肾上腺素受体介导的大鼠后肢血管床收缩反应.

Mediation of calcitonin gene-related peptide in protection of ischemic preconditioning in rat hindlimbs¹

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KEY WORDS calcitonin gene-related peptide; capsaicin; acetylcholine; phenylephrine; norepinephrine; vasodilation; reperfusion injury

AIM: To study modulation of calcitonin gene-related peptide (CGRP) in the protective effect of ischemic preconditioning on endothelial cells. **METHODS:** Rat hindlimbs were subjected to ischemia for 2 h, and endothelium-dependent vasorelaxation to acetylcholine (ACh) was examined in rat hindlimbs. **RESULTS:** Two hours of ischemia elicited no effect on vasoconstrictor responses to norepinephrine, but markedly impaired vasodilator responses to ACh.

Ischemic preconditioning induced by 5-min aortic occlusion and 10-min blood reperfusion prevented the impairment of vasorelaxation to ACh due to long-term ischemia. The protection of ischemic preconditioning was abolished by repeated pretreatments with capsaicin to deplete CGRP. Acute application of capsaicin to evoke CGRP release or CGRP caused an ischemic preconditioning-like protection. **CONCLUSION:** Capsaicin-sensitive sensory nerves are involved in the protective effect of ischemic preconditioning on endothelial cells in the rat hindlimbs, and CGRP can mimic the protective effect of ischemic preconditioning in blood vessels.

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Ischemic preconditioning showed protection on not only the ischemic myocardium, but also endothelial cells. It was postulated the cardioprotection of ischemic preconditioning might be