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Anti-hypertensive effects of probenecid *via* inhibition of the α-adrenergic receptor

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Abstract:
Probenecid has long been used in the treatment of gout. Its anti-gout mechanisms consist of uric acid reuptake inhibition and the consequent facilitation of uric acid excretion. In the present study, we investigated whether probenecid could exert an anti-hypertensive effect in spontaneously hypertensive rats (SHR). The noninvasive indirect tail cuff method was employed to measure blood pressure and heart rate. The administration of probenecid (50 mg/kg, *ip*) induced a significant systolic blood pressure (SBP) decrease, from 167 mmHg to 141 mmHg, within 120 min. In contrast, probenecid had little effect on normotensive control Wistar Kyoto rats (WKY). The anti-hypertensive effects of probenecid are almost as potent as those of atenolol. In a further exploration of the anti-hypertensive mechanisms of probenecid, its effects on phenylephrine-induced blood vessel contraction were tested. Our results suggest that probenecid significantly inhibited the contractions of rat aorta. This effect was also observed with endothelium-removed rat aorta, suggesting that probenecid can directly interact with the α-adrenergic receptor. Moreover, probenecid inhibited the α-adrenergic-receptor-mediated activation of ERK I/II in MC3TC-E1 cells. Therefore, our results indicate that probenecid might alleviate high blood pressure in SHR *via* inhibition of the α-adrenergic receptor and ERK I/II.

Key words:
probenecid, hypertension, α-adrenergic receptor, ERK I/II

Introduction

Probenecid has long been used in the treatment of gout. Its anti-gout action inhibits a renal tubular transporter, thereby inhibiting uric acid reuptake and, in turn, stimulating uric acid excretion in urine [2, 10, 14]. The effects of probenecid on anionic transporters are well established: the blockade of cAMP or cGMP release from erythrocytes [4, 8], ATP release from glial cells [1, 3], and dye loss in several cell types [5, 6]. Recently, it has been found that probenecid inhibits pannexin 1 channels [18]. Moreover, probenecid acts as a non-selective inhibitor of multidrug resistance-associated proteins, and strikingly, it has neuroprotective effects in a transgenic animal model of Huntington's disease [21].

A considerable body of evidence indicates that uric acid plays a significant role in the development of hypertension. In rats, mild hyperuricemia causes elevated blood pressure, which can be prevented by administration of uric acid-lowering agents such as allopurinol, a xanthine oxidase inhibitor, and benziodarone, a uricosuric agent [12]. Several mechanisms implicating uric acid in hypertension have been proposed, such as inflammatory and vascular changes in renal microcirculation, the activation of the renin-angiotensin system, and endothelial dysfunction [12, 13, 15, 17]. Endothelial function by itself is important in regulation of blood pressure [23]. In addition, considerable clini-

cal data suggest the potential role of uric acid in systemic hypertension [11]. Therefore, the capacity of probenecid to reduce uric acid might have a blood pressure-lowering functionality. In the present study, we tested the hypothesis that probenecid can reduce blood pressure, and we explored the potential role of the α-adrenergic receptor.

Materials and Methods

Animals and reagents

Fourteen-week-old male spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats were purchased from Hanlim Experimental Animals Co., Korea. The body weights (g) of SHR and WKY rats were 310 ± 9.4 and 363 ± 7.0, respectively. All of the necessary chemicals were purchased from Sigma Chemical Co. Throughout the experiments, the animals were treated according to NIH guidelines for care and use of laboratory animals.

Measurement of vasorelaxant activity

WKY rats were sacrificed by CO_2 administration, after which the thoracic aorta was removed and cleaned of adherent tissues. The aorta was cut into 3–5 mm long rings, from which endothelial cells were mechanically removed by gently rubbing off the lumen with a cotton swab to avoid mediation of endothelium-derived vasodilator substances and endothelial ATP-sensitive K channels [26]. The removal of the endothelial functionality was verified by the lack of relaxation when acetylcholine (3 μM) was applied to rings precontracted with phenylephrine (3 μM). The rings were then suspended on a fixed and flexible stainless steel wire (1.0 g resting tension) in a 10 ml organ bath containing physiological salt solution (PSS; composition in mmol/l: NaCl 118.0, KCl 4.0, $CaCl_2$ H_2O 1.9, $MgSO_4$ $7H_2O$ 0.4, KH_2PO_4 1.0, $NaHCO_3$ 25, glucose 11.1). The wire was connected to a Grass FT 03 force transducer attached to a McLab computerized digital recording system (AD Instruments, Australia) to record contractile responses. After a resting period of 1 h, the rings were incubated in phenylephrine (10^{-7} M) to which probenecid (10^{-3} M) was subsequently added.

Measurement of blood pressure and heart rate

Rats were kept in a warm environment (30–32°C) for 15–20 min, after which their systolic blood pressure (SBP) was measured by the indirect tail cuff method [22, 25]. According to this protocol, constant pressure was applied to a programmed electrosphygmomanometer (Narco Biosystems PE-300) connected to an occlusion cuff, and while the pressure decreased, a Korotkoff sound microphone and physiograph (Narco Trace TM-80) recorded the first point of the emerging pulse, which was regarded as the SBP. Heart rates were measured using a MacLab data acquisition system to count the beats per min.

Western blot analysis

Proteins (50 μg/lane) were electrophoretically separated in 10% polyacrylamide gels containing SDS and then transferred to nitrocellulose membranes (Schleicher & Schuell) for 1 h at 100 V (constant), as described by Towbin et al. [19]. The membranes were preincubated (for 1 h at 23°C) with PBS containing 0.1% Tween 20 and 3% bovine serum albumin. Afterwards, they were washed with PBS containing 0.1% Tween 20, three times for 10 min each. Blots were probed with primary antibodies against ERK and pERK (1:500) for 2 h at room temperature or overnight at 4°C diluted in blocking buffer. Blots were then incubated with HRP-conjugated anti-rabbit IgG for 1 h at room temperature and washed with PBS containing Tween 20, three times for 10 min each. An ECL (NEN) was carried out to detect immobilized specific antigens. Images were analyzed using Image J software.

Statistical analyses

All data were expressed as the mean ± SEM. Statistical analyses were performed using a one-way ANOVA followed by a Turkey's multiple comparison test; $p < 0.05$ was considered to be significant.

Results

SHR have been widely used as an animal model in the study of human essential hypertension. Here, SHR and sex- and age-matched WKY were tested to ex-

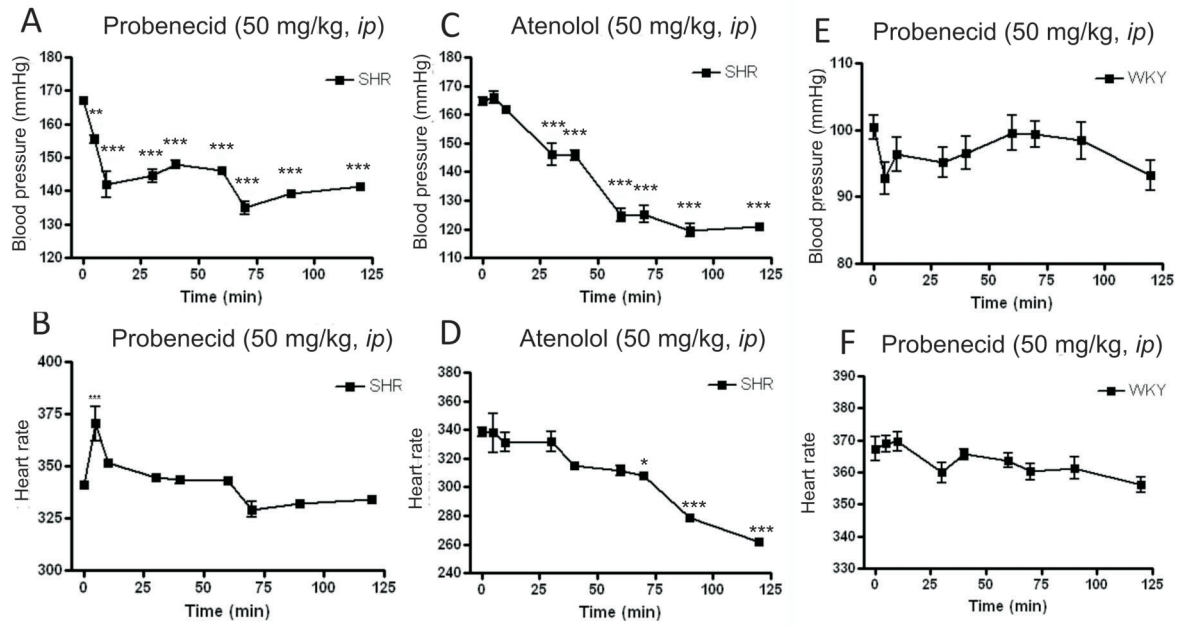

Fig. 1. Effects of probenecid on blood pressure and heart rate in SHR. Following administration of probenecid or atenolol (50 mg/kg, *ip*) to SHR, blood pressure (**A** and **C**) and heart rate (**B** and **D**) were measured as described in the Materials and Methods section. Following administration of probenecid (50 mg/kg, *ip*) to WKY rats, blood pressure (**E**) and heart rate (**F**) were measured. All data are expressed as the mean ± SEM (n = 5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; differences are compared with a control group

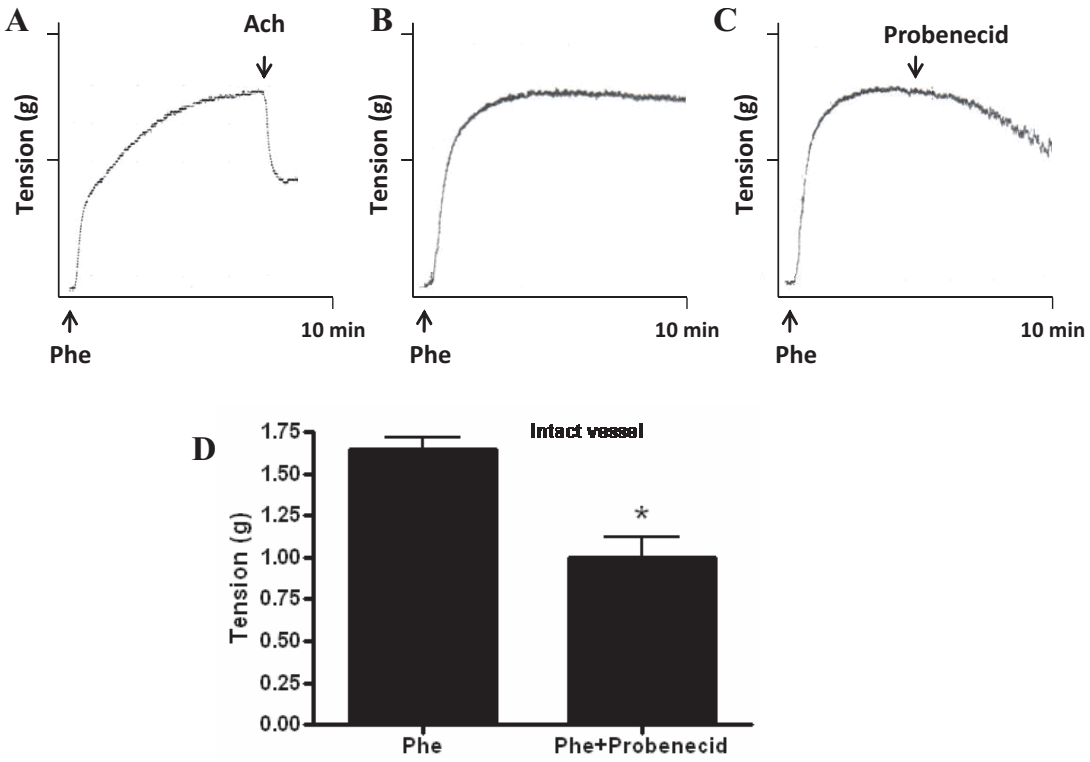

Fig. 2. Effects of probenecid on phenylephrine-induced blood vessel contraction. The thoracic aorta was isolated from WKY rats and subjected to blood vessel contraction experiments, as described in the Materials and Methods section. Blood vessel contraction was measured following phenylephrine treatment (**B**). To verify the integrity of endothelium, acetylcholine was added after the contraction of blood vessel was induced by phenylephrine (**A**). The effect of probenecid on the phenylephrine-induced blood vessel contraction was measured (**C**). Quatitation of the blood vessel contraction by phenylephrine with or without probenecid was measured (**D**). All data are expressed as the mean ± SEM (n = 5). * $p < 0.05$; differences are compared with a control group

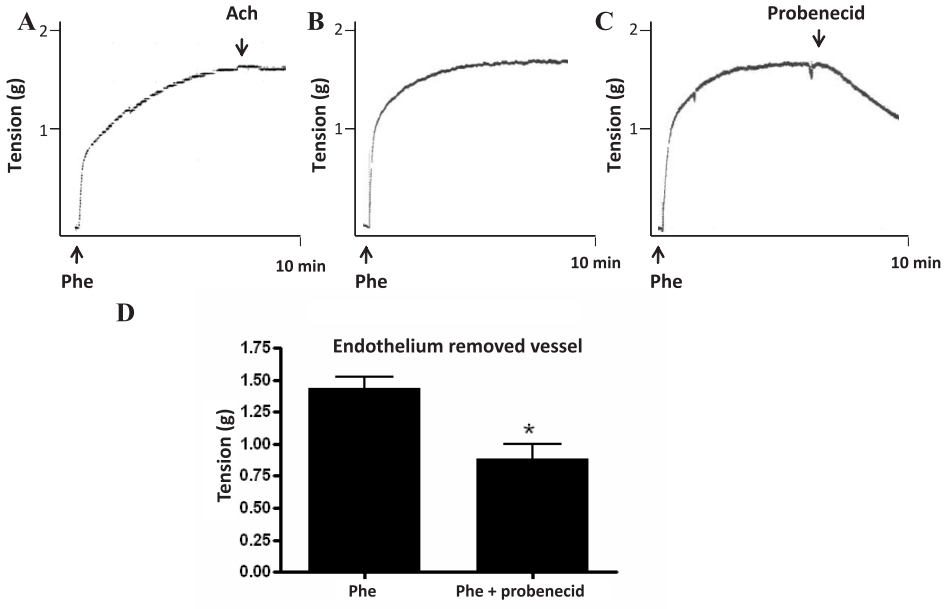

Fig. 3. Effect of probenecid on endothelium-removed blood vessel contraction induced by phenylephrine. The thoracic aorta was isolated from WKY rats and subjected to blood vessel contraction experiments following endothelium removal, as described in Materials and Methods. Blood vessel contraction was measured following phenylephrine treatment (**B**). To verify the integrity of endothelium, acetylcholine was added after the contraction of blood vessel was induced by phenylephrine (**A**). The effect of probenecid on the phenylephrine-induced blood vessel contraction was measured (**C**). Quatitation of the blood vessel contraction by phenylephrine with or without probenecid was measured (**D**). All of the data are expressed as mean ± SEM. (n=5). *p < 0.05, differences are compared with a control group

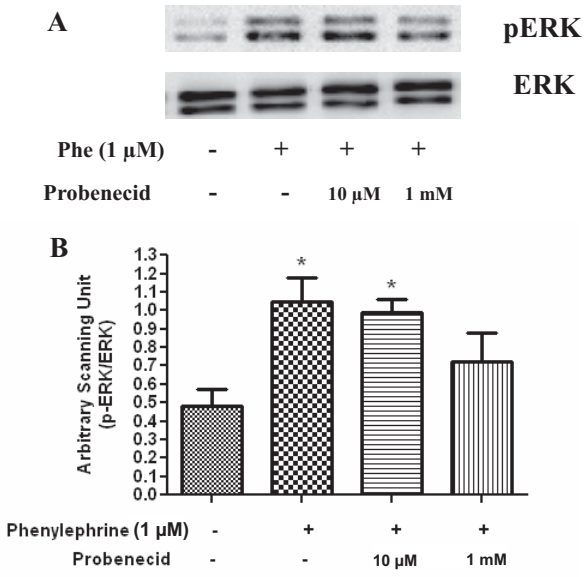

Fig. 4. Effects of probenecid on phenylephrine-induced activation of ERK I/II. Cultured MC3T3-E1 cells were treated with phenylephrine (10 μM) in the absence or presence of probenecid (10 μM or 1 mM) and subjected to cell lysis followed by western blot analysis, as described in the Materials and Methods section. Blots were probed with anti-ERK and anti-pERK antibodies and subjected to ECL detection (**A**). The densities of the ERK and pERK bands were measured by scanning densitometry, and the ratio of pERK to ERK was determined (**B**). Data are expressed as the mean ± SEM (n = 3). * p < 0.05, differences are compared with a control group

plore any possible anti-hypertensive action of probenecid. Following probenecid administration, the noninvasive indirect tail cuff method was employed to measure SBP and heart rate. When 50 mg/kg of probenecid was administered intraperitoneally to SHR, the SBP significantly decreased, from 167 mm Hg to 155 mm Hg in 5 min, and then slowly decreased to 141 mm Hg in 120 min (Fig. 1A). Heart rate changes in response to the same administration were almost normal except for a transient sharp increase after 5 min (Fig. 1B). Next, we compared the anti-hypertensive effects of probenecid with those of atenolol, a standard anti-hypertensive drug. Atenolol showed a capacity to decrease the high blood pressure of SHR to almost normal values (120 mm Hg; Fig. 1C). Similarly, heart rates steadily fell in response to atenolol (Fig. 1D). The effect of probenecid on SBP and heart rates of normotensive WKY rats was almost negligible (Figs. 1E and 1F).

To explore the potential anti-hypertensive mechanism of probenecid in relation to the α-adrenergic receptor, thoracic aorta rings were employed. Specifically, we tested whether probenecid had any effect on phenylephrine-induced vasoconstriction. To verify the integrity of the endothelium, acetylcholine (3 μM)

was added, causing a significant relaxation in rings precontracted with phenylephrine (10^{-7} M; Fig. 2A). A phenylephrine treatment of intact aortas at the concentration of 10^{-7} M caused a clear contraction of vessels; a subsequent probenecid treatment (10^{-3} M), after full vessel contraction, caused a 40% reduction in the response (Figs. 2B through D). Next, we sought to determine if the endothelium was involved in a probenecid-induced reduction of aortic vessel contraction. The endothelium removal was confirmed by the fact that acetylcholine (3 μM) failed to cause relaxation of rings precontracted with phenylephrine (10^{-7} M; Fig. 3A). With endothelium-removed aorta, probenecid (10^{-3} M) could reduce phenylephrine-induced aortic vessel contraction by 38% (Figs. 3 B through D). To test the hypothesis that probenecid regulates the α-adrenergic receptor-mediated blood pressure, we employed MC3T3-E1 cells expressing functional α-adrenergic receptors to determine if probenecid could inhibit α-adrenergic-receptor-activated ERK I/II. Our results showed that while phenylephrine (1 μM) stimulated the ERK I/II activity by 2.19-fold compared with the control, probenecid effectively decreased the phenylephrine-induced ERK I/II activity by 11% and 57% at 10 μM and 1 mM concentrations, respectively (Fig. 4).

Discussion

A significant body of evidence indicates that hyperuricemia is associated with the development of hypertension. Accordingly, a number of uricosuric agents have been used to ameliorate hypertension. Thus, we investigated whether probenecid could exert anti-hypertensive effects on SHR. Indeed, probenecid had remarkable anti-hypertensive properties, which might be related to the inhibition of the α-adrenergic receptor in blood vessels.

Hypertension is one of the most devastating health problems in the world, affecting more than 26% of the adult population. Recently, it has been suggested that hyperuricemia is strongly associated with hypertension and cardiovascular mortality. A xanthine oxidase inhibitor, allopurinol, showed a significant blood pressure-reducing action in a short-term study [7]. Taking these facts into consideration, we hypothesized that probenecid, through its uric acid-lowering effect, can have an anti-hypertensive function. We studied an animal model widely used in studies on essential hypertension, SHR. Our data showed that probenecid (50 mg/kg, *ip*) significantly reduced the high blood pressure of SHR to almost normal levels. Indeed, the anti-hypertensive effects of probenecid were almost equal to those of atenolol. Unlike atenolol, which reduces heart rate in a time-dependent manner, probenecid did not significantly lower the heart rate; this feature could be useful in clinical situations.

To unravel the anti-hypertensive mechanisms of probenecid, we investigated whether it could affect blood vessel contraction. Interestingly, we found that probenecid caused the relaxation of phenylephrine-induced contraction of the thoracic aorta. Moreover, this effect was due to a direct interaction with the smooth muscle of the thoracic aorta because endothelium-removed vessels showed the same effect. Unlike probenecid, which had no effect on the endothelial function, it has been recently suggested that allopurinol, in addition to its uric acid-lowering effect, improved the endothelial function by reducing vascular oxidative stress [9]. Altogether, these results strongly suggest that the vasorelaxation effect of probenecid did not occur *via* endothelium but rather through the α-adrenergic receptor inhibition. Further studies are required to delineate the selectivity of α-adrenergic receptor subtypes for probenecid. However, the relaxation of aortic blood vessels by probenecid might be accomplished mainly through the α1-adrenergic receptor because the major α-adrenergic receptor present in the aorta is the α1 subtype [24]. To obtain additional evidence of the α-adrenergic-receptor-mediated anti-hypertensive action of probenecid, its effects on ERK I/II were investigated. It has already been established that α-adrenergic receptors are present in osteoblastic cells such as SaOS-2, HOS, MG-63, and MC3T3-E1. The latter type of cells expresses the α1-adrenergic receptor, and the receptor activation by phenylephrine could lead to ERK I/II activation [16]. Therefore, we employed MC3T3-E1 cells to test whether probenecid interacts with the α1-adrenergic receptor through its inhibitory effect on ERK I/II. Our results clearly showed that probenecid inhibited phenylephrine-induced activation of ERK I/II, suggesting an interaction with the α1-adrenergic receptor. Considering that uric acid levels in blood of SHR are typically almost normal [20], it is reasonable to suggest that probenecid has an anti-hypertensive effect partly *via* interaction with the α1-adrenergic receptor in blood vessels rather than through uric acid-reducing action.

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