VINIGROL, A NOVEL ANTIHYPERTENSIVE AND PLATELET AGGREGATION INHIBITORY AGENT PRODUCED BY A FUNGUS, *VIRGARIA NIGRA*

II. PHARMACOLOGICAL CHARACTERISTICS

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Vinigrol, a novel diterpene produced by *Virgaria nigra*, was tested orally in conscious spontaneously hypertensive rats (SHR) to confirm its antihypertensive activity. Vinigrol (2 mg/kg, po) decreased the mean arterial blood pressure of SHR by approximately 15% for at least 6 hours.

Vinigrol induced contraction of rat aortic smooth muscle preparation at 1.5×10^{-7} M, the contraction was blocked by nilvadipine, a Ca^{2+} entry blocker, but was not inhibited by prazosin or yohimbine.

Radio-receptor binding assay of α adrenoceptors of rat brain membrane revealed that vinigrol had no affinity for these receptors.

Vinigrol was isolated as a novel antihypertensive compound produced by *Virgaria nigra*.

In addition it was found that vinigrol inhibited both rabbit and human platelet aggregation induced by epinephrine or platelet activating factor (PAF) specifically. However, vinigrol induced platelet aggregation at higher concentrations as reported in a preceding paper[1].

It is well established that the human platelet aggregation induced by epinephrine is mediated *via* α_2 adrenoceptors of the platelet membrane[2,3].

Arterial blood pressure is mainly controlled by the sympathetic nervous system. The tension of vascular smooth muscle is regulated with α_1 adrenoceptor which is stimulated with norepinephrine released from the terminal of the sympathetic neuron. The release of norepinephrine is controlled *via* presynaptic α_2 adrenoceptors.

In this paper, we describe the antihypertensive activity of vinigrol given orally to spontaneously hypertensive rats (SHR) and the effect of this compound on rat aortic smooth muscle preparations *in vitro* in order to clarify the possible involvement of α adrenoceptors in its antihypertensive activity. Finally, we describe the results of our radio-receptor binding assay of α_1 and α_2 adrenoceptors with vinigrol.

Materials and Methods

Blood Pressure Assay

Male SHR (260~310 g) were anesthetized with pentobarbital (50 mg/kg, ip) and a polyethylene catheter (PE10 connected with PE50) was inserted from the femoral artery into the abdominal aorta. The catheter was held in place by a ligature around the femoral artery. In addition, the catheter was sutured to the surrounding muscle and exteriorized on the dorsal surface of the neck. This surgery was performed 2 days prior to the measurement of blood pressure. The mean arterial blood pressure of conscious SHR was measured from the implanted catheter with a pressure transducer coupled to a

polygraph. Vinigrol was dissolved in a small volume of PEG400 and then diluted with nine volumes of 0.5% methyl cellulose solution. Drug was administered orally with a animal feeding needle at a volume of 5 ml/kg.

Smooth Muscle Assay

The tension of rat aortic smooth muscle was measured isometrically with a force displacement transducer according to the method reported previously[4]. Briefly, male Sprague-Dawley rats (280~330 g) were stunned and killed by bleeding from the carotid artery. The thoracic aorta was removed and placed in Tyrode solution. After removing fatty tissues, spiral strips (2 mm width 40 mm length) were prepared from the aorta and suspended in 30 ml organ baths filled with oxygenated Tyrode solution (37°C) under a resting tension of 1 g. After 1 hour equilibration, each agonist was applied at the concentration that provided additional 500 mg tension. After the contraction reached a plateau, each drug was added by 10-fold dilution into the Tyrode solution to obtain the dose response curve of its relaxation activity. Finally, papaverine (1×10^{-4} M) was applied as standard drug to every preparation to obtain the extent of 100% relaxation.

Membrane Preparation

Male Wister rats were decapitated and the brains were obtained and placed in ice-cold buffer (0.25 M sucrose, 5 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.5). The cerebral cortex was carefully dissected and homogenized for ten strokes in 10~20 volumes (w/v) of ice-cold buffer using a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged at $1,000\times g$ for 10 minutes at 4°C and the pellet was discarded. The supernatant was centrifuged at $30,000\times g$ for 20 minutes at 4°C. The pellet obtained was washed by resuspension in 20 ml ice-cold buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5) and recentrifuged at $30,000\times g$ for 20 minutes at 4°C. This final membrane suspension was prepared in the same buffer at a protein concentration of 1 mg/ml and stored at −80°C. Protein concentration was determined by the method of LOWRY *et al.*

Binding Assay[5]

Binding experiments were performed in duplicate using [^{3}H]prazosin for the α_1 receptor or [^{3}H]clonidine for the α_2 receptor. The membrane suspension (0.4~0.5 mg protein/tube) was incubated by constant shaking at 25°C for 20 minutes (with [^{3}H]prazosin, Amersham) or 50 minutes (with [^{3}H]clonidine, Amersham) in a total volume of 500 μl of the buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5). The incubation was terminated by adding 3 ml of ice-cold buffer followed by rapid filtration through Whatman GF/C glass fiber filters under vacuum. Each assay tube was immediately rinsed with 3 ml of ice-cold buffer and the filter was further rinsed with 20 ml of ice-cold buffer. The filtration was carried out using a single manifold with controlled flow rate. The filters were removed into 10 ml of AQUASOL-11 (New England Nuclear) and counted on a Packard Model 4530 scintillation spectrophotometer at approximately 40% efficiency.

Results

Effect of Vinigrol on the Blood Pressure of Conscious SHR

In the preceding paper, we reported that vinigrol decreased mean arterial blood pressure of anesthetized normotensive SD rats by 20% at 100 μg/kg, iv.

In order to confirm the antihypertensive activity of vinigrol, we tested the compound in conscious SHR orally with the results shown in Table 1. Vinigrol decreased the blood pressure of SHR by approximately 15% at 2 mg/kg and the duration of the effect was longer than 6 hours. A slightly greater effect was observed at 20 mg/kg.

Effect of Vinigrol on the Tension of the Smooth Muscle

In our preliminary experiment, we observed that vinigrol induced the contraction of rat aortic strips remarkably. Clonidine (α_2 agonist) also revealed the similar activity on the same preparation.

Table 1. Effect of orally administered vinigrol on blood pressure in conscious SHR.

		Before	Time after administration (hours)				
			0.5	1	2	3	6
Vehicle	MBP (mmHg)	188±3.3[†]	186±2.4	182±1.3	185.5±1.6	186±2.0	184±2.1
(5 ml/kg, po, n=8)	Change (%)	0	−0.1±4.1	−3.0±1.8	−1.0±2.1	−0.6±2.1	−1.6±2.8
Vinigrol	MBP (mmHg)	184±2.6	157±4.6[a]***	158±4.3***	156±5.3**	158±2.5***	159±6.3**
(2 mg/kg, po, n=5)	Change (%)	0	−15.1±1.5[b]*	−14.3±1.3**	−15.2±2.5**	−14.4±1.5***	−13.5±2.8**
Vinigrol	MBP (mmHg)	182±2.3	137±3.7[a]**	151±5.5*	142±4.2**	145±7.9*	163±3.7**
(20 mg/kg, po, n=3)	Change (%)	0	−25.3±1.6[b]**	−17.1±2.6**	−22.0±1.3***	−20.1±3.4***	−11.4±0.9*

[†] Mean±SE.

Student's t-test; * $P<0.05$, ** $P<0.01$, *** $P<0.001$. [a] Against before (paired), [b] against vehicle (unpaired).

Vehicle; 10% PEG400 in 0.5% methyl cellulose solution.

Table 2. Effect of vasodilators on rat aortic strips contracted with various agonists.

Antagonist	ED_{50} (M)			
	Vinigrol (1.5×10^{-7} M)	Clonidine (4×10^{-7} M)	Phenylephrine (5×10^{-7} M)	KCl (50 mM)
Nilvadipine	1.7×10^{-9}	8.8×10^{-10}	3.9×10^{-7}	9.3×10^{-10}
Prazosin	$>1\times10^{-4}$	8.4×10^{-10}	9.7×10^{-10}	$>3\times10^{-5}$
Yohimbine	$>3\times10^{-5}$	5.6×10^{-7}	4.9×10^{-7}	$>3\times10^{-5}$

$n=3\sim5$.

Table 3. *pKi* values of the radio-ligand binding assay of α adrenoceptors.

Ligand	*pKi*				
	Vinigrol	Clonidine	Phenylephrine	Prazosin	Yohimbine
[^{3}H]Prazosin	<4.0	6.3	5.2	9.8	6.3
[^{3}H]Clonidine	<4.0	9.1	7.1	5.4	7.0

$pKi=-\log Ki$ (M). $n=3\sim4$.

So, we employed vinigrol, clonidine, phenylephrine (α_1 and α_2 agonist) and KCl as agonists to induce the contraction of the rat aortic strips and compared their sensitivity to the inhibitory effect of the vasodilators such as nilvadipine (Ca^{2+} blocker), prazosin (α_1 blocker) and yohimbine (α_2 blocker). The results are shown in Table 2 as ED_{50} values of each drug to the four agonists.

These results clearly reveal that the sensitivities of vinigrol to nilvadipine, prazosin and yohimbine resemble those of KCl and imply that vinigrol exhibits Ca^{2+} agonistic activity on smooth muscle preparations.

Effect of Vinigrol on Radio-receptor Binding Assay

Effect of vinigrol on α adrenoceptors was estimated by radio-ligand binding assay of the rat brain membrane as a receptor source. Non-specific binding was determined by adding unlabeled prazosin (1 μM) or clonidine (1 μM) to the incubates. Specific binding, defined as the difference between total and non-specific binding, represented $93\pm0.2\%$ and $96\pm0.1\%$ of the total binding at 5×10^{-10} M for [^{3}H]prazosin and 1×10^{-9} M for [^{3}H]clonidine, in the range of the *Kd* values, respectively. For competition studies, the membranes were incubated with either [^{3}H]prazosin (0.5 nM) or [^{3}H]clonidine (1 nM). *pKi* values obtained from these experiments are summarized in Table 3. These results clearly show that vinigrol has no affinity for either α_1 nor α_2 adrenoceptors of rat brain membrane.

Discussion

Although originally discovered as an antihypertensive compound we showed that vinigrol inhibited rabbit and human platelet aggregation induced by epinephrine or PAF specifically[1]. Vinigrol did not reveal any inhibitory effect on other aggregation agents such as ADP, thrombin and collagen.

We speculated that vinigrol exerts its inhibitory effect on platelets by interacting with α_2 adrenoceptors on the platelet membrane. Furthermore, we speculated that the antihypertensive activity of vinigrol depends on the interaction of vinigrol with α adrenoceptors in the cardiovascular system.

In order to evaluate these suggested mechanisms, we compared the effect of vinigrol on rat aortic smooth muscle strips with those of clonidine, phenylephrine and KCl as agonists and also compared the sensitivity of the four agonists to nilvadipine, prazosin and yohimbine. The results of these experiments (Table 2) clearly showed that vinigrol provoked the contraction of the rat aortic smooth muscle preparation in a manner similar to clonidine. However, contraction by vinigrol was only

inhibited by nilvadipine, but not by prazosin and yohimbine. Despite the similarity in the contractile activity of vinigrol and clonidine, significant differences were observed in the sensitivities of these compounds to vasodilators. So, it is quite unlikely that vinigrol exhibits its antihypertensive activity by interacting with α adrenoceptors of the vascular system.

The assay results from radio-receptor binding of vinigrol to α adrenoceptors of rat cerebral membrane (Table 3) also support our finding.

Finally, we conclude that vinigrol is a Ca^{2+} agonist showing inhibitory effects on Ca^{2+} movement at lower concentrations. However, vinigrol is not a Ca^{2+} facilitator such as CGP28,392[6], because unlike CGP28,392, vinigrol relaxes the rabbit aortic smooth muscle preparation contracted by norepinephrine (data not shown). More study is required in order to conclusively define the mode of action of vinigrol on calcium channels and also to clarify the mechanism of its antihypertensive activity.

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