Hypotensive Effects of Eugenosedin-A with Serotonin, Alpha- and Beta-Adrenoceptor Antagonistic Activities in Spontaneously Hypertensive and Normotensive Rats

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Abstract

Eugenosedin-A is a newly synthesized compound with special serotonergic, α- and β_1-adrenergic blocking actions. Intravenous injection of eugenosedin-A significantly caused dose-dependent decreases in the mean arterial blood pressure and heart rate in normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). The effects of eugenosedin-A-decreased blood pressure and heart rate in SHR were more potent than in WKY. In in vitro experiments, eugenosedin-A competitively antagonized the serotonin-, norepinephrine- and clonidine-induced vasocontraction in a concentration-dependent manner in isolated thoracic aorta of WKY and SHR. We also observed that eugenosedin-A competitively antagonized the isoproterenol-induced positive inotropic effects in a concentration-dependent manner in the isolated left atrium of WKY and SHR. These findings clearly suggested that eugenosedin-A possesses α_1/α_2, β_1 and 5-HT$_{2A}$ receptor-blocking activities. The order of pA$_2$ values in isolated tissues of WKY was 5-HT$_{2A} > \alpha_1/\alpha_2 > \beta_1$. However, the order of pA$_2$ values in isolated tissues of SHR was $\alpha_1/\alpha_2 > $5-HT$_{2A} > \beta_1$. Similarly, we found that the in vitro functional activity of eugenosedin-A is quite different between WKY and SHR. On the other hand, in the isolated rabbit ear artery sensitized with 16 mmol/l K$^+$, eugenosedin-A antagonized 5-nonyloxytryptamine- and serotonin-induced vasocontractions, indicating that it also blocked 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors. In radioligand binding experiments, eugenosedin-A had significant binding affinities on α_1/α_2, β_1, 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors. Finally, we suggest that the hypotensive effects of eugenosedin-A can be attributed to its multiple actions on the blockade of 5-HT$_{1B}$, 5-HT$_{2A}$, α and β_1 receptors in both WKY and SHR strains.

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Introduction

5-Hydroxytryptamine (5-HT) has many important effects on cardiovascular function, and its antagonists, such as ketanserin, have been used to treat cardiovascular diseases [1]. In addition, 5-HT is a potent vasoconstrictor agent, capable of contracting arterial and venous tissues in vitro and increasing blood pressure [1]. An increase in the response to serotonergic agents has been documented in
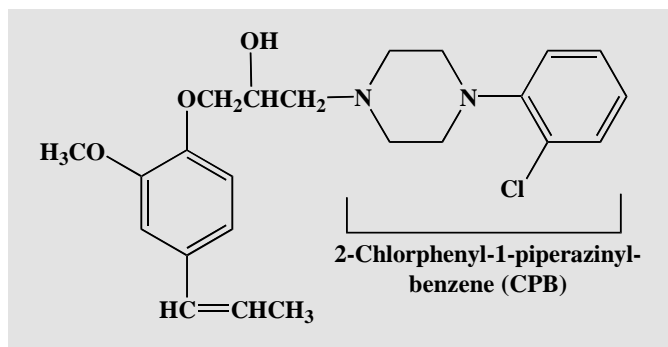
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Fig. 1. Chemical structure of eugenosedin-A.

vessels isolated from animal models of vasospasm or atherosclerosis. These lines of evidence suggest that augmented vasocontraction to serotonin, in relation to atherosclerosis, may principally contribute to the genesis of myocardial ischemia [2]. The increase in sensitivity to 5-HT in hypertension has been demonstrated by showing that aorta rings from spontaneously hypertensive rats (SHR) had a greater sensitivity to 5-HT than rings from Wistar-Kyoto rats (WKY) [3]. Blockade of peripheral vascular serotonergic receptors has been proposed as a useful mechanism for lowering blood pressure in animals and humans. Since the early 1960s there have been reports of interactions between α-adrenergic and serotonergic systems [4–6]. In the past decade, more experimental evidence has appeared indicating that some agonists (5-HT) and antagonists (metitepine, ketanserin, cyproheptadine) of the 5-HT receptor subtypes are able to interact with α-adrenoceptors [6].

In the hypertensive situation, increased contractile responses to agonists have been correlated with an enhanced protein kinase C activity [7], augmented phosphoinositide metabolism [8], and increased Ca^{2+} mobilization [9]. Whether these alterations are related to a specific α_1-adrenoceptor subtype in the vasculature of SHR, different to the one present in WKY, is not exactly known [10]. However, Michel et al. [11] showed an increase in α-adrenoceptor density in kidneys from hypertensive rats. Thereafter, Suzuki et al. [12] also demonstrated an increase in the number of α_1-adrenoceptors along with an increase in the affinity for antagonists in the mesenteric vasculature of deoxycorticosterone-salt hypertensive rats.

Central and peripheral noradrenergic systems are implicated in the regulation of arterial blood pressure. Hypertension partially resembles a hyperadrenergic state, dysfunction of a central mechanism in SHR, leading to an increase in the release of noradrenaline [13]. The release of noradrenaline from noradrenergic nerve terminals is regulated by presynaptic α_2-adrenoceptors, and it is assumed that the hypotensive effect of various centrally acting antihypertensive drugs is due to the stimulation of pre- and/or postsynaptic inhibitory α_2-adrenoceptors. However, the existence of three subtypes of α_2-adrenoceptors, designated as α_{2A}, α_{2B} and α_{2C}, was proposed by Murphy and Bylund [14]. The α_{2A}-adrenergic subtype is located in the CNS and is concentrated in the cardiovascular control center of the brainstem. α_{2B}-Adrenergic receptors are located in arterial vascular smooth muscle cells and cause peripheral vasoconstriction [15, 16]. In addition, Fujimoto and Itoh [17] have shown that the α_2-adrenoceptor agonist activity of clonidine in the thoracic aorta produced contractile responses.

β-Adrenoceptor blockers are an important class of drugs in the management of patients with cardiovascular diseases. These drugs have been shown to reduce mortality in hypertension [18] and prolong survival in patients with ischemic heart diseases [19]. β-Blockers retain their position among basic therapies for numerous other cardiovascular and non-cardiovascular conditions, including arrhythmias, hypertropic cardiomyopathy, migraine, glaucoma and thyrotoxicosis. The third-generation β-adrenoceptor blockers, such as labetalol and carvedilol which predominantly have β-adrenoceptor- and also ancillary α-adrenoceptor-blocking activity, have been approved for the treatment of hypertension [20]. Cardiac β-adrenoceptor responsiveness might be reduced in established hypertension in man and rat. Some function studies indicated impaired contractility in response to β-adrenoceptor agonists in the papillary muscle or whole heart of SHR [21, 22].

2-Chloro-1-piperazinyl benzene (CPB) is a basic chemical structure found in trazodone-like antidepressants with α_2-adrenoceptor and 5-HT antagonist activities. Aryloxypropanolamines, especially those that are isoeugenol-based, have been reported to have antioxidizing activities in addition to their β-adrenoceptor-blocking effects [23, 24]. To the best of our knowledge, eugenosedin-A (fig. 1), 4-{2-hydroxy-3-[1-(2-chlorophenyl-piperazinyl)]-propoxy}-3-methoxy-1-propylenyl-benzene, was first synthesized in this laboratory by combining isoeugenol-based oxypropanolamine and CPB. We supposed that eugenosedin-A, structurally with one part CPB base, could possess CPB-related α_2-adrenoceptor and 5-HT receptor antagonist properties, and its β-adrenoceptor blocking actions could be derived from another part of isoeugenol-based oxypropanolamine. This study aimed to look into the

striking differences in the hypotensive effects of eugenosedin-A in both WKY and SHR strains, and these findings might provide more valuable information showing that eugenosedin-A is suitable for use in the treatment of patients with cardiovascular disorders.

Materials and Methods

Animals

WKY, SHR, and New Zealand White rabbits were provided by the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). They were housed under conditions of constant temperature and controlled illumination (lights on between 07.30 and 19.30 h). Food and water were available ad libitum. The study was approved by the Animal Care and Use Committee of Kaohsiung Medical College.

Drugs and Chemicals

5-Nonyloxytryptamine, atenolol, clonidine, isoproterenol, ketanserin, labetalol, norepinephrine, prazosin, propranolol and serotonin were all purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Eugenosedin-A (synthesized in this laboratory) was solubilized in 50% absolute alcohol at 1 mmol/l and further dilutions of it were made in distilled water.

Measurement of Blood Pressure and Heart Rate

The experiments were accomplished as previously described [25]. In brief, WKY and SHR, weighing 250–300 g, were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Following tracheal cannulation, systemic arterial blood pressure and heart rates were recorded from the femoral artery with a pressure transducer (Model P10EZ, Spectramed, Oxnard, Calif., USA). The body temperature was maintained at 37°C by an electric heating pad. The femoral vein was cannulated for intravenous administration of drugs.

In Vitro Study
Isolated Left Atria

WKY and SHR of either sex, weighing 350–500 g, were sacrificed after mild anesthesia with ether and their hearts were quickly excised. Left atria were dissected from the hearts and mounted in a 10-ml organ bath with one end fixed and the other end connected to a force displacement transducer (Grass, Model FT03). The experiments were carried out at 37°C in Krebs solution of the following composition (mmol/l): NaCl 113, KCl 4.8, CaCl$_2$ 2.2, KH$_2$PO$_4$ 1.2, MgCl$_2$ 1.2, NaHCO$_3$ 25, dextrose 11.0; bubbled with a 95% O$_2$ and 5% CO$_2$ mixture. The left atrium was pre-stretched to a baseline tension of 0.5 g and equilibrated for 60 min in an aerated Krebs solution before initiation of the experimental protocols. Atria were driven at 2-second intervals via two platinum electrodes placed on either side of the atrium. An incubation time of 30 min was allowed for the test compound. Data were calculated as a percentage of the maximal contraction.

Isolated Thoracic Aorta

The thoracic aorta of the rat was quickly removed, cleaned of adhering fat and connective tissue and cut into 3- to 4-mm-wide transverse rings, which were then mounted at 1 g resting tension on stainless steel hooks in a 10-ml organ bath; bathed at 37°C in physiological solution (mmol/l: NaCl 118, KCl 4.8, CaCl$_2$ 2.5, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, NaHCO$_3$ 24, glucose 11); and aerated with a 95% O$_2$ and 5% CO$_2$ mixture. The isometric tension of the aortic rings was monitored by a force displacement transducer (Model 7004, Ugo Basile, Comerio, Italy). The tissues were allowed to equilibrate for 1 h in physiological solution. Clonidine, norepinephrine and serotonin (10^{-7}–10^{-4} mol/l) were cumulatively added to the bath to induce contractions or the bath was pretreated with eugenosedin-A and then clonidine, norepinephrine and serotonin were cumulatively added.

#### Isolated Rabbit Ear Artery

This experiment was performed as previously described [26]. Rabbits (2–3 kg) were euthanized by pentobarbital to produce deep anesthesia, then rapidly decapitated. Ear arteries were isolated, cleaned of adhering fat and connective tissue and cut into 3- to 4-mm-wide transverse rings; these rings were mounted for the measurement of isometric contraction in tissue baths containing 95% O$_2$ and 5% CO$_2$-gassed Krebs solution at 37°C. Then mounted at 1.5 g resting tension on stainless steel hooks in a 10-ml organ bath; bathed at 37°C in physiological solution and aerated with a 95% O$_2$ and 5% CO$_2$ mixture. The isometric tension of the aortic rings was monitored by a force displacement transducer (Model 7004, Ugo Basile). The tissues were allowed to equilibrate for 1 h in physiological solution; then incubated with 16 mmol/l K$^+$ for 30 min before 5-nonyloxytryptamine or serotonin (10^{-8}–10^{-4} mol/l) were cumulatively added to the bath to induce contractions, or the bath was pretreated with eugenosedin-A and then 5-nonyloxytryptamine (5-HT$_{1B}$ agonist) or serotonin (5-HT$_{1B}$, 5-HT$_{2A}$ agonist) were cumulatively added.

Receptor-Binding Experiments

Radioligand-binding experiments were carried out as previously published [25]. Briefly, both WKY and SHR brains (for α_1, α_2-adrenoceptor, serotonergic receptor binding), hearts (for β_1-adrenoceptor) and lungs (for β_2-adrenoceptor) were homogenized with a Kinematica polytron in 20 vol of ice-cold TE buffer (10 mmol/l Tris HCl, 1 mmol/l EDTA, 0.1 mmol/l ascorbic acid, pH 7.4). The homogenate was filtered with pressure through muslin. The filtrate was centrifuged at 1,000 g for 10 min. The supernatant was centrifuged again at 10,000 g for 12 min at 4°C. The second supernatant was centrifuged at 30,000 g for 15 min at 4°C, and the final pellet was resuspended in assay buffer (75 mmol/l Tris HCl, 25 mmol/l MgCl$_2$, pH 7.4). The protein content was determined by Bradford's method. Radioligand agents and membranes (200–300 µg) were incubated for 60 min at 25°C with or without the addition of nonspecific binding agents (table 1), in a 75-mmol/l Tris HCl buffer with 25 mmol/l MgCl$_2$, to make a final volume of 500 µl. In competitive-binding experiments, the competing agent was added directly to the incubation mixture. The incubation was terminated by addition of 1 ml of ice-cold assay buffer followed by immediate filtration through Whatman GF/C glass fiber filters supported on a 12-port filter manifold (Millipore). The filters were immediately washed 3 times with 5 ml of ice-cold assay buffer and dried in an oven at 60°C for 2 h before adding 5 ml of Triton-toluene-based scintillation fluid. Membrane-bound radioligand trapped in the filters was counted in a Beckman LS6500 scintillation system (Fullerton, Calif., USA) with an efficiency of 45%. In each experiment, nonspecifically bound radioligand agents were determined by incubating membrane protein. Specific binding was thus obtained by deducting this value from the total binding of radioligand agents for each sample.
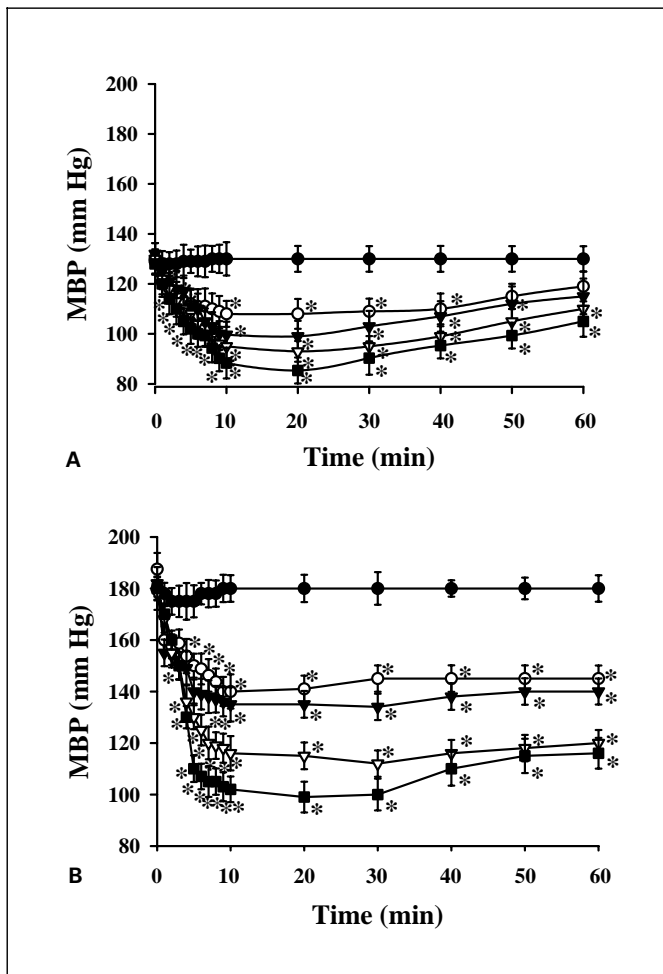
Fig. 2. Effects of eugenosedin-A (○ = 1 mg/kg; ▼ = 3 mg/kg; ▽ = 5 mg/kg; ■ = 10 mg/kg) and its vehicle (●) on mean arterial blood pressure in WKY (**A**) and SHR (**B**), anesthetized with pentobarbital. Each point represents the mean of 8 rats. * Significantly different from control, p < 0.05 (two-way repeated measures analysis of variance (ANOVA) followed by Student-Newman-Keuls test).
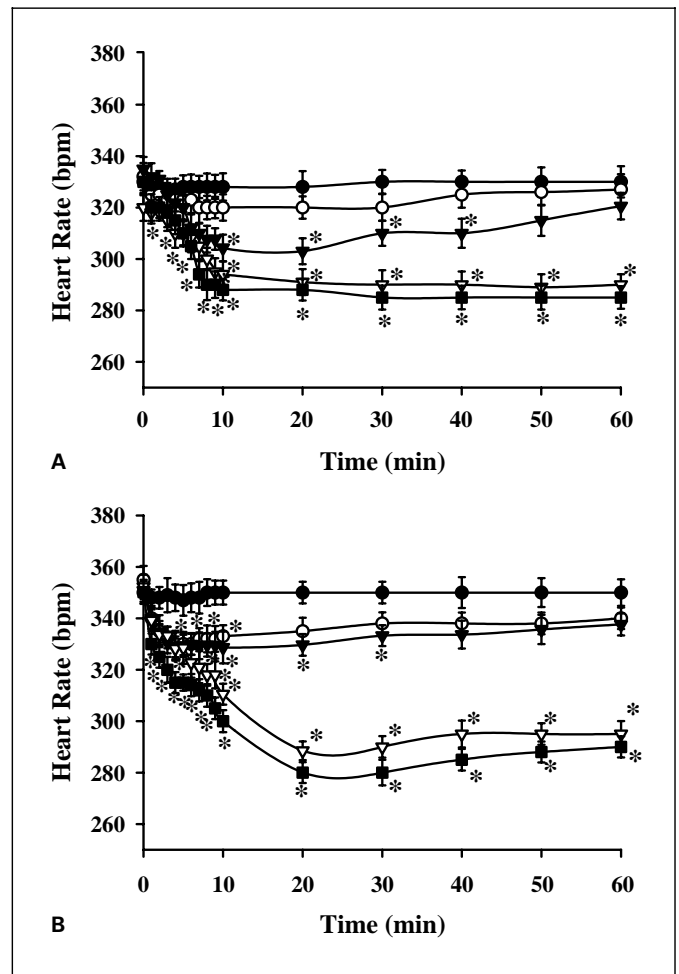
Fig. 3. Effects of eugenosedin-A (○ = 1 mg/kg; ▼ = 3 mg/kg; ▽ = 5 mg/kg; ■ = 10 mg/kg) and its vehicle (●) on the heart rate in WKY (**A**) and SHR (**B**) anesthetized with pentobarbital. Each point represents the mean of 8 rats. * Significantly different from control, p < 0.05 (two-way repeated-measures analysis of variance (ANOVA) followed by Student-Newman-Keuls test).

Table 1. Assay conditions for ^{3}H-ligand displacement studies

Receptor	^{3}H-ligand nmol/l	Kd in WKY nmol/l	Kd in SHR nmol/l	Nonspecific ligand μmol/l
5-HT$_{1A}$	WAY1000635 (1)	0.12 ± 0.01	0.26 ± 0.06	Serotonin (10)
5-HT$_{1B}$	GR125743 (3)	0.79 ± 0.03	1.05 ± 0.02	Serotonin (10)
5-HT$_{2A}$	Ketanserin (0.5)	0.52 ± 0.04	0.39 ± 0.01	Serotonin (10)
α$_1$	Prazosin (0.2)	0.25 ± 0.05	0.31 ± 0.02	Phentolamine (10)
α$_2$	Yohimbine (2)	5.34 ± 0.06	6.76 ± 0.08	Phentolamine (10)
β$_1$	CGP12177 (1)	0.16 ± 0.01	0.37 ± 0.03	Propanolol (10)
β$_2$	CGP12177 (3)	1.21 ± 0.03	1.14 ± 0.05	Propanolol (10)

Kd denotes the equilibrium dissociation constant obtained from Scatchard analysis.

Fig. 4. Antagonism of the vasocontractile effects of serotonin (**A**), norepinephrine (**B**) and clonidine (**C**), and the atrial contractility of isoproterenol (**D**) in the absence (●) or presence of eugenosedin-A ($\bigcirc$ = 10^{-8} mol/l; ▼ = 10^{-7} mol/l; ▽ = 10^{-6} mol/l). Cumulative concentration-response curves were determined in isolated WKY thoracic aortic rings. Each value represents the mean ± SE of 8 rats.
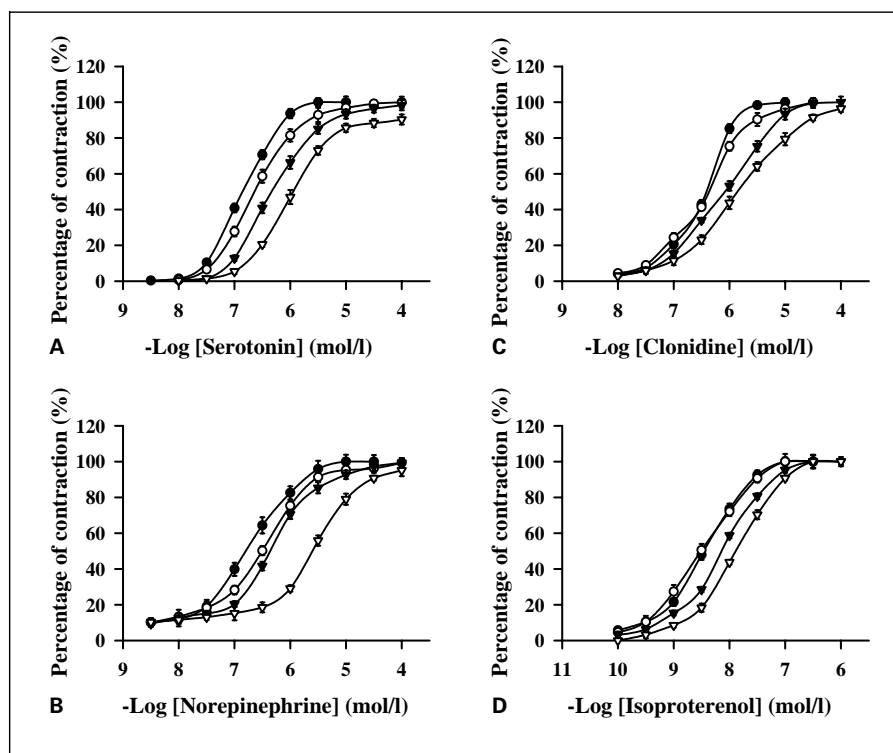
Statistical Evaluation of Data

The results are expressed as mean ± SE. Statistical differences were determined by independent and paired Student's t test in unpaired and paired samples, respectively. Whenever a control group was compared with more than 1 treated group, the one-way ANOVA or two-way repeated measures ANOVA was used. When the ANOVA manifested a statistical difference, the Dunnett's or Student-Newman-Keuls test was applied. A p value of <0.05 was considered to be significant in all experiments. Analysis of the data and plotting of the figures were done with the aid of software (SigmaPlot Version 8.0 and SigmaStat Version 2.03, Chicago, Ill., USA) run on an IBM-compatible computer.

Results

Effects of Eugenosedin-A on Blood Pressure and Heart Rate

Acute intravenous injection of eugenosedin-A (1.0, 3.0, 5.0, 10.0 mg/kg) produced long-lasting dose-dependent hypotensive effects on blood pressure and decreases in heart rate in pentobarbital-anesthetized WKY and SHR (fig. 2, 3). The decreases in blood pressure and heart rate by eugenosedin-A were more significant in SHR than in WKY.

Vasorelaxant Effects of Eugenosedin-A

Eugenosedin-A (10^{-8}, 10^{-7}, 10^{-6} mol/l) concentration-dependently inhibited the cumulative serotonin-, norepinephrine- and clonidine-induced contractile effects in isolated WKY and SHR thoracic aorta. Serotonin, norepinephrine and clonidine concentration-response curves caused a dose-dependent parallel shift to the right by eugenosedin-A (fig. 4, 5). The pA_2 values and slopes of regression lines are indicated in table 2.

Electrically Stimulated Left Atria

Eugenosedin-A (10^{-8}, 10^{-7}, 10^{-6} mol/l) also concentration-dependently antagonized the cumulative isoproterenol-induced positive inotropic effects in isolated WKY and SHR left atria. Eugenosedin-A produced a dose-dependent parallel shift to the right of the isoproterenol concentration-response curves (fig. 4, 5). The pA_2 values and slopes of regression lines are indicated in table 2.

5-HT_{1B}/5-HT_{2A} Receptor Antagonist Activity

In isolated rabbit ear arteries sensitized with 16 mmol/l K^+, 5-nonyloxytryptamine was subsequently added to induce vasoconstriction. Eugenosedin-A (10^{-6}, 10^{-5}, 10^{-4} mol/l) caused the concentration-response curves of 5-

Fig. 5. Antagonism of vasocontractile effects of serotonin (**A**), norepinephrine (**B**) and clonidine (**C**), and the atrial contractility of isoproterenol (**D**) in the absence (●) or presence of eugenosedin-A (○ = 10^{-8} mol/l; ▼ = 10^{-7} mol/l; ▽ = 10^{-6} mol/l). Cumulative concentration-response curves were determined in isolated SHR thoracic aortic rings. Each value represents the mean ± SE of 8 rats.
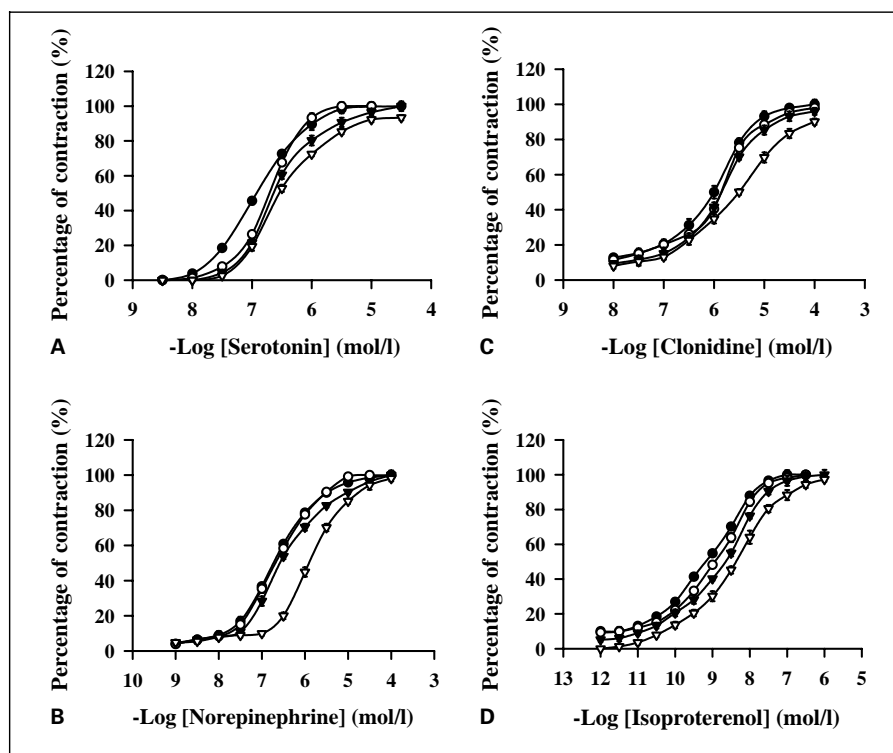
Table 2. The pA_2 values for eugenosedin-A in isolated tissues of Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR)

	WKY (slope)	SHR (slope)
Thoracic aorta (5-HT$_{2A}$)	8.86 ± 0.32 (0.89 ± 0.12)	7.08 ± 0.24 (0.95 ± 0.10)
Thoracic aorta (α_1)	7.88 ± 0.13 (0.85 ± 0.07)	7.71 ± 0.32 (0.84 ± 0.09)
Thoracic aorta (α_1/α_2)	7.49 ± 0.27 (0.60 ± 0.10)[a]	7.68 ± 0.15 (0.65 ± 0.08)[a]
Atrium (β_1)	6.55 ± 0.24 (1.14 ± 0.13)	6.34 ± 0.22 (0.88 ± 0.12)

The pA_2 values were calculated from individual Schild plots by regression analysis. Each pA_2 value is the mean ± SE of 6–8 experimental results.
[a] The slope of the Schild plot was significantly less than unity.

nonyloxytryptamine or serotonin to shift to the right. The pA_2 values of eugenosedin-A for 5-HT$_{1B}$ and 5-HT$_{1B/2A}$ receptors were 5.67 ± 0.18 and 6.41 ± 0.74, respectively (fig. 6).

Radioligand-Binding Study

As shown in figure 7, eugenosedin-A was competitively bound to the α- and β-adrenoceptors and serotonin receptors. The Ki values of eugenosedin-A for each [^{3}H]-ligand binding site in the various tissue membranes of WKY and SHR are indicated in tables 3 and 4. Eugenosedin-A was mainly bound to α_1, α_2, β_1, 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors;

however, it had no significant binding affinities on β_2 and 5-HT$_{2A}$ receptors. Eugenosedin-A had a greater selectivity in WKY to bind to the 5-HT$_{1B}$ receptor in comparison with SHR. Serotonin was the only one that bound to the 5-HT$_{1A}$ receptor and showed that the binding affinity in SHR was higher than in WKY. We also found that serotonin had α_1-adrenoceptor binding affinity exclusively in SHR, but not in WKY. Ketanserin was greatly selective for the 5-HT$_{2A}$ receptor and also showed significant binding affinities for α_1 and α_2. Prazosin was extremely selective for the 5-HT$_{2A}$ receptor whereas it still had significant binding affinities for α_2 and 5-HT$_{2A}$. The nonse-
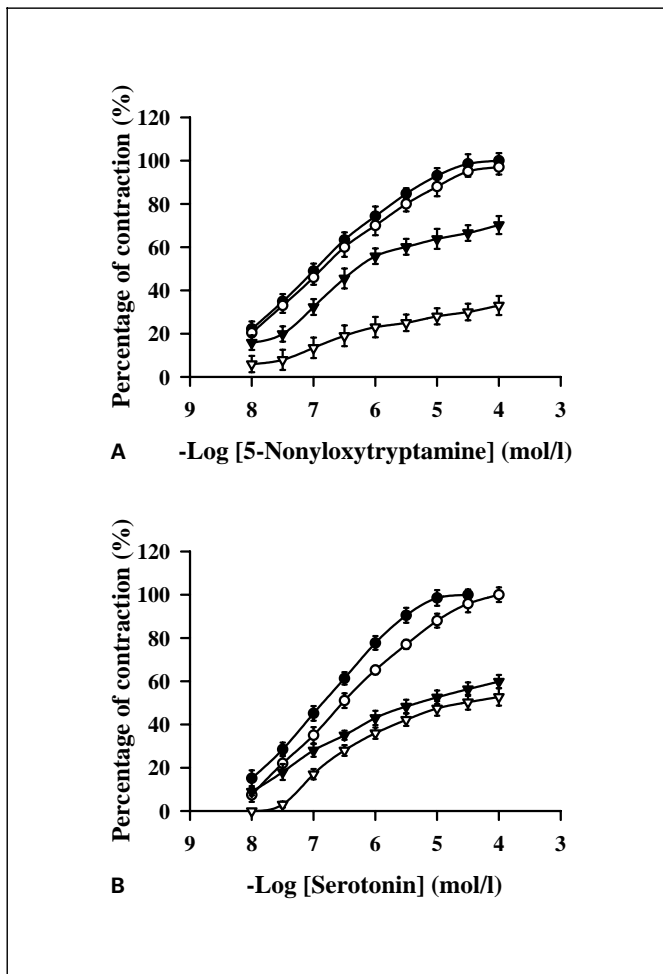
Fig. 6. Antagonism of vasocontractile effects of 5-nonyloxytrypt-amine (**A**) and serotonin (**B**) in the absence (●) or presence of euge-nosedin-A (○ = 10^{-6} mol/l; ▼ = 10^{-5} mol/l; ▽ = 10^{-4} mol/l). Cumula-tive concentration-response curves were determined in isolated rab-bit ear arteries rings. Each value represents the mean ± SE of 8 rats.
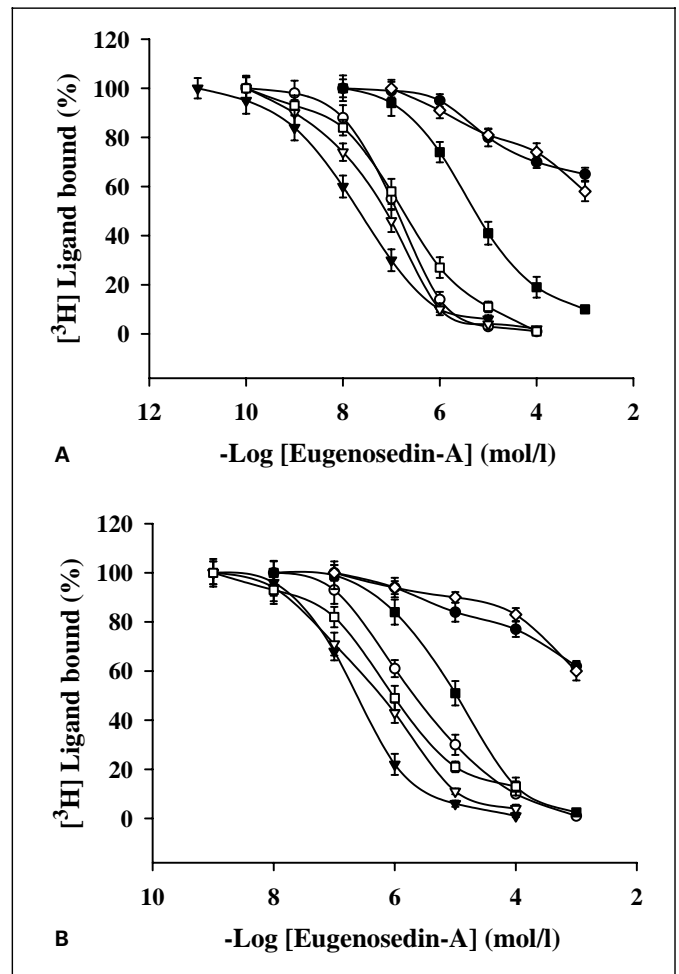
Fig. 7. Inhibition of [^{3}H]-ligand specific binding to serotonin, α- and β-receptor subtypes on various tissue membranes by eugenosedin-A in WKY (**A**) and SHR (**B**). The individual symbol indicated 5-HT$_{1A}$ (●), 5-HT$_{1B}$ (○), 5-HT$_{2A}$ (▼), α_1 (▽), α_2 (■), β_1 (□) and β_2 (◇) recep-tors. Data shown are the means of 3 independent triplicate determi-nations. Each value represents the mean ± SE.

Table 3. Affinity constants for eugenosedin-A and other reference compounds in Wistar-Kyoto rats

Agents	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{2A}$	α_1	α_2	β_1	β_2
Eugenosedin-A	NS	114.41	47.79	151.03	1,746.28	90.06	NS
Atenolol	NS	NS	NS	NS	NS	262.76	8,511.45
Labetalol	NS	NS	612.13	52.48	3,542.91	4.17	52.48
Propranolol	NS	NS	291.63	NS	NS	0.37	0.71
Prazosin	NS	NS	2,298.62	3.31	318.44	NS	NS
Ketanserin	NS	NS	0.047	30.65	2,893.31	NS	NS
Serotonin	0.04	50.64	163.81	NS	NS	NS	NS

Ki values (nmol/l) were calculated from the equation Ki = IC$_{50}$/(1 + (^{3}H)-ligand/Kd). Kd and (^{3}H)ligand denote the dissociation constant and the free concentration of the radiolabel, respectively.

NS = Nonsignificant.

Table 4. Affinity constants for eugenosedin-A and other reference compounds in spontaneously hypertensive rats

Agents	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{2A}$	α_1	α_2	β_1	β_2
Eugenosedin-A	NS	714.16	78.49	184.34	2,336.79	96.15	NS
Atenolol	NS	NS	NS	NS	NS	450.81	5,168.64
Labetalol	NS	NS	722.73	43.25	2,579.5	11.37	10.43
Propranolol	NS	NS	265.04	NS	NS	5.97	1.98
Prazosin	NS	NS	3,086.39	0.003	66.17	NS	NS
Ketanserin	NS	NS	0.01	0.95	2,226.81	NS	NS
Serotonin	0.01	20.33	52.91	185.89	NS	NS	NS

Ki values (nmol/l) were calculated from the equation Ki = IC$_{50}$/(1 + (^{3}H)-ligand/Kd). Kd and (^{3}H)ligand denote the dissociation constant and the free concentration of the radiolabel, respectively.

NS = Nonsignificant.

lective β-blocker propranolol and the α/β-blocker labetalol both showed significant binding affinities for the 5-HT$_{2A}$ receptor, but in striking contrast to the selective β$_1$-blocker atenolol.

Discussion

Eugenosedin-A is able to antagonize α$_1$-, α$_2$-adrenergic and serotonergic agonists which evoke aortic contractions from WKY and SHR. In the isolated atria, eugenosedin-A concentration-dependently inhibited the positive inotropic effects induced by isoproterenol. Intravenous administration of eugenosedin-A produced dose-dependent decreases in mean blood pressure and heart rate in pentobarbital-anesthetized WKY and SHR, whereas the depressor effect in SHR is more obvious than in WKY. From the functional pA$_2$ results, we demonstrated that eugenosedin-A is more potent on the 5-HT$_{2A}$ receptor in WKY than in SHR. In this report, we clarified that eugenosedin-A is a 5-HT receptor, predominant for 5-HT$_{1B}$ and 5-HT$_{2A}$, and α$_1$/α$_2$-adrenoceptor-blocking agents with additional blockade of the β-adrenoceptor but not β$_2$. Eugenosedin-A combined with 5-HT and α-receptor blockade could contribute to the rapid onset of its hypotensive effects. Its additional β$_1$-adrenoceptor-blocking activity could prevent reflex tachycardia. In this study, we observed that eugenosedin-A caused a significant reduction in heart rate. Particularly, it did not have any significant blockade on β$_2$-adrenoceptor and this could be of clinical relevance because it may reduce the risk of asthmatic attack in patients with bronchospastic disease. On the other hand, Ishida et al. [2] demonstrated that the 5-HT$_{1B}$ receptor was activated by atherosclerosis and augmented vasocontraction to serotonin, in relation to atherosclerosis. Eugenosedin-A also blocked 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors, and thus suggested that it could provide more benefit in the treatment of cardiovascular disorders including hypertension and/or atherosclerosis.

Some reports demonstrated that ketanserin antagonizes contractions of vascular smooth muscle cells occurring in the presence of 5-HT [27]. Van Nueten et al. [28] also pointed out that ketanserin is due to activation of serotonergic receptors, to stimulation of the α-adrenoceptor or to interaction between serotonergic and α-adrenergic activation. Ketanserin caused a dose-dependent reduction in arterial blood pressure in SHR. It could be assumed that the hypotensive effect of ketanserin was also related to its α-adrenolytic properties [28]. In recent years, numerous studies have documented several interactions between noradrenaline and 5-HT systems. Haddjeri et al. [29] reported that pindolol interacts with 5-HT$_{1A}$ receptors with nanomolar affinity and prevents some biologic responses mediated via 5-HT$_{1A}$-receptor activation. It is well established that noradrenaline neurons modulate the activity of the 5-HT system, and there are several lines of evidence supporting the notion that the 5-HT system also influences brain noradrenaline neurons [29].

Seven receptor subtypes of 5-HT are known to exist and, of them, the 5-HT$_1$ and 5-HT$_2$ receptors are known to mediate contractile responses in blood vessels, and the 5-HT$_{1A}$ receptor mediates the central regulation on blood pressure. The 5-HT$_2$ receptor seems to be involved in the vascular response to serotonin [30, 31]. Several pharmacological studies support the involvement of the 5-HT$_{2A}$ receptor in mediating the contractile response in rat pulmonary arteries [32, 33]. In our receptor-binding results, we observed that eugenosedin-A had 5-HT$_2$ receptor-

binding affinity. Meanwhile, in experiments on isolated rat aortas, we also found that eugenosedin-A produced a dose-dependent parallel shift to the right of the serotonin concentration-response curves. Therefore, we suggest that eugenosedin-A has 5-HT$_2$ receptor-blocking activity.

Åhlund et al. [34] reported a 10-fold greater sensitivity to 5-HT on aortic strips from SHR compared to normotensive rats of nonspecified origin. Webb and Vanhoutte [35] also showed that 5-HT constrictor responses are augmented in rat models of genetic hypertension, in vivo and in vitro. In this kind of rat model, pressor responses to 5-HT are enhanced to a much greater extent than those to noradrenaline and angiotensin II. The cellular mechanisms that may contribute to the increased sensitivity to 5-HT in hypertensive rats could include changes in the affinity of the receptors for 5-HT [36]. Thus, we could not ignore that 5-HT also notably affects the cardiovascular system. From our isolated experiments, we found that eugenosedin-A acts on 5-HT receptors and its response is easier to reverse by serotonin in SHR aortic rings than in WKY. Taken together, it could explain our aortic data showing that the antagonistic effect of eugenosedin-A for 5-HT receptors in SHR was lower than that of WKY.

The decreased Kd value of the α_1-adrenoceptor in the myocardium of SHR suggested an increase in the sensitivity of this receptor to the catecholamine [37]. However, Watanabe et al. [38] showed that the B$_{max}$ and Kd values of the α_1-adrenoceptor in WKY and SHR were not significantly different. In our isolated aortic results, we showed that the pA$_2$ and Ki values of eugenosedin-A in SHR and WKY were similar. In SHR, the density of the α_2-adrenoceptor was reduced in the cerebral cortex, hypothalamus and medulla oblongata [39]. The reduced density of the α_2-adrenoceptor in the brain of SHR is in contrast to the higher density found in the kidney, which has been linked to the pathogenesis of some models of genetic hypertension [40]. However, other models of experimental hypertension have been associated with downregulation of the α_2-adrenoceptor in various peripheral tissues including the kidney [41]. Our functional results show that the pA$_2$ value of eugenosedin-A in the thoracic aorta of SHR for the α_2-adrenoceptor was not significantly different from WKY. Therefore, we suggest that the function of the α_2-adrenoceptor in the peripheral aortic tissues of SHR is probably similar to WKY. In addition, the Ki values of eugenosedin-A in WKY showed effects similar to those of SHR. The results indicated that eugenosedin-A might still possess the same binding affinities between SHR and WKY, although the density of the α_2-adrenoceptor is reduced in the brain of SHR as previously reported [40].

Post-junctional α-adrenoceptors in the vasculature have been subdivided into α_1-and α_2-subtypes. McGrath [42] defined responses mediated by post-junctional α_2-adrenoceptors as those which are insensitive to the α_1-adrenoceptor antagonist prazosin, but which are more sensitive to the α_2-adrenoceptor antagonist rauwolscine. Three subtypes of α_2-adrenoceptors, designated α_{2A}, α_{2B} and α_{2C}, were proposed by Murphy and Bylund [14]. The α_2-adrenergic subtype is located in the CNS and is concentrated in the cardiovascular control center of the brainstem. α_{2B}-Adrenergic receptors are located in arterial vascular smooth muscle cells and cause peripheral vasoconstriction [15, 16]. Since α_2-adrenoceptors may be involved in contractions of rat aorta, Fujimoto and Itoh [17] pointed out that the α_2-adrenoceptor agonist clonidine in the thoracic aorta produces contractile activity. But in some studies, clonidine was described as being able to activate the α_1-adrenoceptor to contract blood vessels [43]. However, the possibility that the rat aorta contains a heterogeneous population of α-adrenoceptors should not be excluded [17]. In view of the fact that the slope of the Schild plot for eugenosedin-A deviated from unity in clonidine-pre-contracted aorta, we therefore propose that eugenosedin-A-mediated inhibition of the clonidine-induced contraction is caused by antagonism on α_1/α_2-adrenoceptors.

The B$_{max}$ value in the β_1-adrenoceptors of 16-week-old SHR was higher than that of WKY [38]. However, Doggrell and Surman [44] have shown that functional β-adrenoceptors in the left atrium of hypertensive rats were not altered. In our left atrial strips, eugenosedin-A antagonized the isoproterenol-induced positive inotropic effects in WKY and SHR. The pA$_2$ value of eugenosedin-A in atria of SHR was similar to WKY and the Ki value was also not significantly different between both strains. Here, we suggest that eugenosedin-A produced no significant changes in the functional and binding affinity of β_1-adrenoceptors in SHR in comparison with WKY, even the density of β_1-adrenoceptor is enhanced in the heart of SHR [38].

In our rabbit ear artery experiments, we used 16 mmol/l K$^+$ on vessel rings to elicit the changes in membrane potential. This elevation in external K$^+$ decreases the concentration gradient for K$^+$ across the cell membrane, and causes a modest depolarization in the muscle cells of isolated rabbit ear artery [26]. In turn, this depolarization is thought to cause contraction by the opening of voltage-dependent calcium channels [45]. Slightly elevated external K$^+$ concentrations increased the sensitivity of both the aorta and ear artery. In the rabbit aorta, the contractile response to serotonin is mediated through 5-HT$_{2A}$ recep-

tors, but serotonin does not act as a full agonist [26]. It was proposed that pre-contraction with either slightly elevated K^+ or the receptor agonist enables or unmasks only 5-HT_{1-like} receptors [47]. Many studies have presented evidence for a 5-HT_{1-like} receptor in several different vascular beds [46, 47]. Moreover, a consensus is building in the literature that the vascular 5-HT_{1-like} receptor belongs to 5-HT_{1B} receptor subtype [48]. Pre-sensitization with K^+ on the rabbit ear artery enables activation of both 5-HT_{2A} and 5-HT_{1B} receptors. Recently, many scientific reports suggested that both the 5-HT_{2A} and 5-HT_{1B} receptors were involved in vascular contraction [26]. Eugenosedin-A not only inhibited serotonin-induced vasocontractions in rat 5-HT_{2A} receptors, but also antagonized serotonin- or 5-nonyloxytryptamine (selective for 5-HT_{1B} receptors)-induced contractions in the rabbit ear artery. However, eugenosedin-A may be shown to have nonspecific inhibition on 5-HT_{2A} and 5-HT_{1B} receptors at high concentrations. It is generally accepted that at high concentrations most agents should activate nonspecific receptors, and this could be happening to eugenosedin-A. At concentrations higher than 10^{-5} mol/l, eugenosedin-A appears to suppress the 5-nonyloxytryptamine- and serotonin-induced rabbit ear artery contractions noncompetitively; thus, we suggest that eugenosedin-A might block additional receptors and not only 5-HT. Indeed, in the isolated thoracic aorta of WKY and SHR we observed that eugenosedin-A competitively antagonized the serotonin-induced vasocontractions in a concentration-dependent manner. Furthermore, in a radioligand-binding assay, we also found that eugenosedin-A in competing with [³H]GR125743 and [³H]ketanserin for 5-HT_{1B} and 5-HT_{2A} receptors, respectively, is reversible displaced and has good affinity as shown in figure 7. Taken together, we still suggest that eugenosedin-A possesses serotonin antagonistic activities and probably has some extent of selectivity for 5-HT_{2A} and 5-HT_{1B} receptors.

In recent years, drugs that combined β-adrenoceptor blocking with vasodilating or α-adrenoceptor-blocking properties have been introduced as more efficacious anti-hypertensive and anti-atherosclerotic agents, since such drugs reduce blood pressure and plasma lipids by two complementary mechanisms [49, 50]. As we know, carvedilol and labetalol are both principal representatives among these agents. However, the antagonistic actions on 5-HT receptors of both agents were little described. It must be emphasized that the actions of eugenosedin-A appear similar to those of carvedilol and labetalol, but in particular with additional 5-HT receptor-blocking activities. It is widely accepted that serotonin-evoked contractions of human large coronary arteries were mediated by both 5-HT_{1B} and 5-HT_{2A} receptors. In addition, an increase in the response to serotonergic agents has been documented in vessels isolated from animal models of vasospasm or atherosclerosis. Serotonin concentrations were elevated in the coronary sinus in patients with coronary artery disease [2]. Augmented constrictor responses to intracoronary administration of serotonin have been demonstrated in patients with coronary atherosclerosis [51]. In this study, we have demonstrated the diverse activities of eugenosedin-A in WKY and SHR, its various receptor-binding affinities between the two strains, and shown that eugenosedin-A not only antagonizes the α_1/α_2- and β_1-adrenoceptors but also blocks the 5-HT_{1B} and 5-HT_{2A} receptors. In conclusion, we suggest that, due to its multiple functional activities, eugenosedin-A might be a development in the treatment of hypertension and/or in preventing the atherosclerosis.

Acknowledgement

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