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ORIGINAL ARTICLE

# Impact of Ginsenoside-Rg3 on Catecholamine Secretion in the Perfused Model of the Rat Adrenal Medullae

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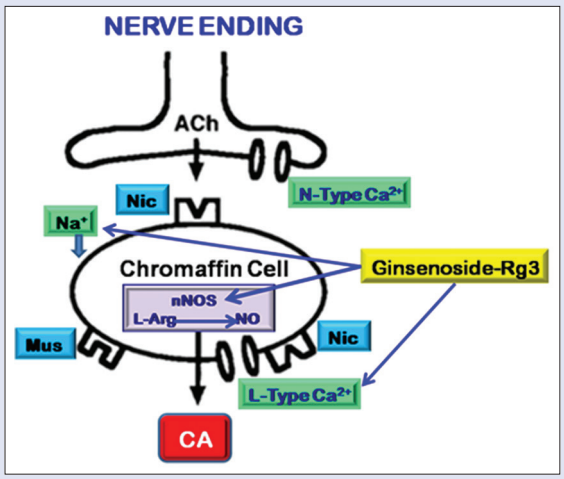
## ABSTRACT

**Background:** The present study was the first attempt to explore the characteristics of ginsenoside-Rg3 (Rg3) on release of catecholamines (CA) in the perfused rat adrenal medullae and also to verify the underlying action mechanism. **Materials and Methods:** The adrenal medulla was separated by some modification of the previous method and perfused with Krebs solution. CA was assayed directly by the fluorometry. **Results:** Rg3 reduced acetylcholine (ACh)-produced CA release in a dose- and time-dependent manner. Rg3 time-dependently depressed CA release produced by 3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride (McN-A-343), 1,1-dimethyl-4-phenyl piperazinium iodide, and angiotensin II. In the presence of Rg3, the CA release produced by high $K^+$, veratridine, cyclopiazonic acid, and methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4- (2-trifluoromethyl-phenyl)-pyridine-5-carboxylate (Bay-K-8644) was also markedly suppressed. However, during the simultaneous perfusion of Rg3 and $N^{\omega}$-nitro-l-arginine methyl ester hydrochloride (l-NAME), the release of CA produced by ACh, angiotensin II, Bay-K-8644, and veratridine was restored closely to the level of each control, in contrast to that of Rg3-treatment alone. The nitric oxide (NO) release was significantly elevated by Rg3-treatment. Furthermore, in the coexistence of Rg3 and fimasartan, ACh-produced CA release was more significantly reduced as compared to that of fimasartan-treatment alone. **Conclusions:** We present the first evidence that Rg3 markedly depresses the CA secretion produced by activation of neuronal cholinergic and angiotensinergic receptors. Rg3-produced inhibition appears to be evoked not only by blocking the inflow of $Na^+$ and $Ca^{2+}$ into adrenomedullary cells but also by preventing the $Ca^{2+}$ release from intracellular storage, partly through enhancement of NO release by NO synthase activation. Coadministration of Rg3 and fimasartan may be clinically beneficial for the treatment of cardiovascular diseases.

**Key words:** Adrenal catecholamine secretion, angiotensinergic receptors, ginsenoside-Rg3, neuronal cholinergic receptors, nitric oxide synthase

## SUMMARY

- Rg3 depressed the CA secretion produced by activation of neuronal cholinergic and angiotensinergic receptors. Rg3-produced inhibition appears to be evoked not only by blocking the inflow of Na+ and Ca2+ into adrenochromaffin cells but also by preventing the Ca2+ release from intracellular storage.
- Rg3 produced the enhancement of NO release by NO synthase activation.
- Co-administration of Rg3 and fimasartan may be clinically beneficial for the treatment of cardiovascular diseases.




**Abbreviations used:** Rg3: Ginsenoside-Rg3; VDCC: L-type voltage-dependent $Ca^{2+}$ channels; Bay–K-8644: Methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-carboxylate; ACh: Acetylcholine; McN-A-343: 3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium; DMPP: 1.1-dimethyl-4-phenyl piperazinium; NO: Nitric oxide; L-NAME: $N^{\omega}$-nitro-l-arginine methyl ester; nNOS: Neuronal NO synthase.

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## INTRODUCTION

Previously, we found that ginsenoside-Rb2, one of the panaxadiol-type saponins, reduced the release of CA in the isolated adrenal medulla of the rat.[1] In addition, total ginseng saponin (TGS) has been reported rather to reduce the release of CA from the perfused model of rat adrenal glands[2] as well as spontaneously hypertensive rat adrenal glands.[3] Ginsenoside components isolated from *Panax ginseng* have been reported to reduce the ACh-produced secretion of CA from the cultured bovine adrenochromaffin cells.[4-7]

It also has found that the ginsenosides (1–100 μM) mostly revealed a tendency to suppress ACh-produced secretion of CA.[5] These investigators showed that the potency order of reducing effects (at 10 μM) on the secretion of CA was as followings:

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Rg2>Rf>Re>Rh1>Rb2, Rg1>Rb1>Rc>Rb3, Rd, Ro, $Rs_1$. The inhibitory effect of ginsenoside-Rg2 (10 μM) was 72%. However, ginsenosides-Rb3, -Rd, Ro, and -$Rs_1$ failed to show the inhibitory activity. However, gintonin, which is found as a new G protein-coupled lysophosphatidic acid (LPA) receptor ligand obtained from Korean ginseng, has been reported markedly to elevate the release of CA from the isolated adrenal medulla of the rat.[8] The CA secretory effect of gintonin appears to be relevant to activation of LPA and cholinergic receptors, which are related to the increase of intracellular $Ca^{2+}$ not only by elevation of the $Ca^{2+}$ inflow but also by the reduction of $Ca^{2+}$ uptake into the storage of intracellular $Ca^{2+}$, without the increase of nitric oxide production.[8] Furthermore, previously, all of TGS,[9] panaxadiol-type saponin[10] and panaxatriol-type saponin[11] has been shown to produce the secretion of CA in the perfused adrenal glands of the rabbit in a $Ca^{2+}$-dependent manner, which is revealed by stimulation of acetylcholinergic (AChergic) receptors and in part by the direct effect on adrenochromaffin cells of the rabbit.

Jeon *et al.*[12] have found that Korean Red Ginseng (KRG)-produced liberation of NO in the conscious rats may be partly related to the hypotensive action. In addition, Rg3 has been found to dilate thoracic aorta of the rat through the increased production of NO.[13] Han *et al.*[14] have shown proof that the level of NO in breathing out of human volunteers by KRG was elevated together with decreased heart rate and lowered blood pressure. In some studies, it is reported that ginsenosides decrease blood pressure through elevation of endothelial NO production[15] and that Rg3 is the most powerful among ginsenosides that dynamize endothelial NO synthase (eNOS) in the isolated aorta of the rat.[16] Rg3 has been found to activate eNOS in vasculature of the animal models.[15,17] Furthermore, Rg3 is known to activate eNOS via phosphorylation of eNOS in ECV 304 human endothelial cells and elevates expression of eNOS.[18]

In the previous studies, it has been verified that ginsenoside-Rg1 produces endothelium-dependent vasorelaxation in the isolated aorta of the rat[17] and induces the elevated production of endogenous NO in endothelial cells of human umbilical vein,[19] murine kidney,[20] and in coronary arteries of the pig.[21]

Notwithstanding several studies on many ginseng saponins, little activity of Rg3, one of the panaxadiol-type saponins, on adrenal secretion of CA is still known. Thus, the present study was the first attempt not only to explore whether Rg3 can affect the CA release produced by several secretagogues in the perfused adrenal medullae of the rat but also to verify the underlying action mechanism.

# MATERIALS AND METHODS

## Materials

The following drugs were used: Ginsenoside-Rg3 (Rg3) (provided by the Society of Korean Ginseng, Seoul, Korea), methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-carboxylate [Bay–K-8644], ACh chloride, 3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride (McN-A-343) (RBI, USA), cyclopiazonic acid, norepinephrine bitartrate, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), veratridine hydrochloride, potassium chloride (KCl), calcium chloride, sodium bicarbonate, $N^{\omega}$-nitro-larginine methyl ester hydrochloride (l-NAME), sodium chloride, potassium phosphate, angiotensin II, ascorbic acid, glucose, disodium EDTA, magnesium chloride (Sigma Chemical Co., USA), fimasartan (a gift donated from Boryung Pharmaceutical Company, Seoul, Korea). Exceptionally, Bay-K-8644 was dissolved in 99.5% (stock) ethanol and then diluted adequately with Krebs-bicarbonate solution (final concentration of ethanol was less than 0.1%). Usually, drugs were dissolved in distilled water (stock) and added to the normal Krebs-bicarbonate solution; concentrations of all drugs employed in this study are depicted in terms of their molar base.

## Experimental procedure

All operating procedures including the animal experiments were performed strictly in accordance with the guidance for the care and employment of Laboratory Animals of the National Institutes of Health (NIH Publications No. 80-23; revised in 1996) and approved by the Committee of Experimental Animals, School of Medicine, Chosun University.

Mature Sprague Dawley male rats (DAMOOL SCIENCE, Daejeon, Korea), weighing 180–250 g, were utilized in this study. The experimental rat was individually raised in separate cage, and assorted feed (DAMOOL SCIENCE, Daejeon, Korea) and faucet water were permitted freely for 1 week before the experiment started. On the day of the animal experiment, anesthesia of the rat was performed with thiopental sodium given into peritoneum (50 mg/kg) and fixed in supine position on the operating table.

### *Isolation of adrenal medulla*

The adrenal medulla was separated by the method of Wakade with some modifications.[22] The abdominal cavity was exposed by a midline incision, and the left suprarenal gland and surrounding area were opened by hanging placement of three-hook retractors on both sides of abdominal muscle. The stomach, portion of the liver, and the intestine were not subduct but were shoved over to the right side and pulled up by saline-steeped cotton, and urine in bladder was drained to consolidate enough working area for ligating blood vessels and cannulations. Prior to tying vessels and cannulations, injection of heparin (400 IU/mL) was administered into the vena cava to avoid blood clotting. A cannula, employed for infusion of the adrenal medulla, was placed into the terminal of the renal vein with all branches of adrenal vein (if any), aorta vena, and cava tied. The bark of the adrenal gland was cut so as to make a small slit into just opposing side of the adrenal vein. When the adrenal medulla was initiated to perfuse, checking up there is no leak, and the infusion fluid flowed out only from the cleft made in the bark of adrenal gland. Then, the adrenal medulla, including its tied blood vessels and the cannula, was prudentially separated from the rat and put on a flat panel of a leucite chamber. The chamber was constantly cycled with water heated at 37°C ± 1°C.

## Perfusion of adrenal medulla

The perfusion of the isolated adrenal glands was made by peristaltic pump (Isco, St. Lincoln, NE, USA) with a rate of 0.31 mL/min. The perfusion of the adrenal medulla was performed with a Krebs-bicarbonate solution containing the next composition (mM): Glucose, 11.7; $CaCl_2$, 2.5; NaCl, 118.4; $KH_2PO_4$, 1.2; $NaHCO_3$, 25; $MgCl_2$, 1.18; and KCl, 4.7. The solution of perfusion was steadily bubbled with 95% $O_2$ and 5% $CO_2$ and the last pH of the Krebs-bicarbonate solution was kept at 7.4–7.5. Ascorbic acid (100 μg/mL) and disodium EDTA (10 μg/mL) were added into the solution of perfusion to block the CA oxidation.

## Administration of the drug

An injection of ACh (5.32 mM) with a volume of 50 μL and KCl (56 mM) with a volume of 100 μL and/or infusion of angiotensin II (Ang II, 100 nM) for 1 min and DMPP (100 μM) for 2 min were given into adrenal vein through a three-way stopcock, respectively. Veratridine (50 μM), McN-A-343 (100 μM), cyclopiazonic acid (10

μM) and Bay-K-8644 (10 μM) were administered by perfusion for 4 min.

In preliminary studies, upon injection or infusion of these secretagogues, the CA secretory responses to ACh, McN-A-343, Ang II, KCl, veratridine, cyclopiazonic acid, and Bay-K-8644 turned back to the level of preinjection within 4 min, but the responses to DMPP lasted about 8 min.

## Collection of the perfusate

Before activation with various secretagogues, the perfusate was collection for 4 min to determine the basal release of CA (background sample). Immediately following collecting the basal sample, the collection of perfusates was performed continuously in another tube as soon as the solution of perfusion including the secretagogue got to the adrenal medulla. The perfusate collection of each stimulated sample was performed for 4 or 8 min. The quantity of CA in the basal sample was subtracted from that in the stimulated sample to get the net release of CA, which is described in all figures.

Before exploring the effects of Rg3 on the basal and the produced CA release, the adrenal medulla was perfused with normal Krebs solution for 1.5 h and the perfusate was collected for a certain period (background sample). Then, the solution was displaced by the one including the secretagogue, either alone or in combination with Rg3, and the collection of perfusates was made for the same time as that for the basal sample. The perfusate was collected in chilled tubes.

## Measurement of catecholamines

The level of CA in perfusate, including epinephrine, norepinephrine, and dopamine, was assayed directly by the fluorometry of Anton and Sayre[23] without intermediate purification with alumina for the reasons described previously[22] using a fluorospectrophotometer (Kontron Co., Milano, Italy).

A 0.2 mL of the perfusate was employed for the measurement of CA. In this study, the CA level in the perfusate of adrenal medulla stimulated by secretagogues given gave readings several times greater than the reading of unstimulated samples (control). The level of CA in the perfusate was illustrated in terms of norepinephrine (base) equivalents.

## Measurement of nitric oxide release

An amplifier (inNo meter, Innovative Instruments Inc., USA) and the NO-selective microelectrode (ami700, Innovative Instruments Inc., USA) were employed for assay of NO liberated in the perfused rat adrenal medulla. The amount of NO liberated in adrenal medulla was calculated as the integrated signal sensed by the microelectrode following loading of Rg3 into the adrenal medulla, as described previously.[24] The electrode was calibrated by establishing standardized levels of NO in 0.5% (wt/vol) KI in 0.1 Mol/L $H_2SO_4$ from standards of $NaNO_2$ standards. Release of NO was measured as the current sensed at the electrode following perfusion of Rg3 into the adrenal vein. The net release of NO was described as picomoles.

## Analysis of experimental data

Statistically, difference between the drug-treated group and the control group was analyzed using the Student's *t*-test and ANOVA. Unless specifically described in the text, $P < 0.05$ was regarded to express statistically significant difference. Values described in the text refer to means ± the standard errors of the mean. The experimental data were statistically assayed using the software of Tallarida and Murray.[25]

# RESULTS

## Inhibitory effects of ginsenoside-Rg3 on the catecholamines release produced by 1.1-dimethyl-4-phenyl piperazinium, acetylcholine, 3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride, and angiotensin II in the perfused rat adrenal medullae

After loading of oxygenated Krebs-bicarbonate solution for 60 min, the basal secretion of CA from the perfused adrenal medulla of the rat was 23 ± 3 ng for 2 min ($n$ = 10). Since it has been previously found that ginsenosides (1–100 μM) tends to reduce ACh-produced CA secretory response,[5] we initially detected the influence of Rg3 itself on the release of CA. However, in our study, Rg3 itself failed to influence the basal CA release (data not shown). Thus, it was attempted to analyze effects of Rg3 on the release of CA produced by the activation of cholinergic as well as Ang II receptors. Secretagogues were given into an adrenal vein at 15–20 min intervals. Rg3 was

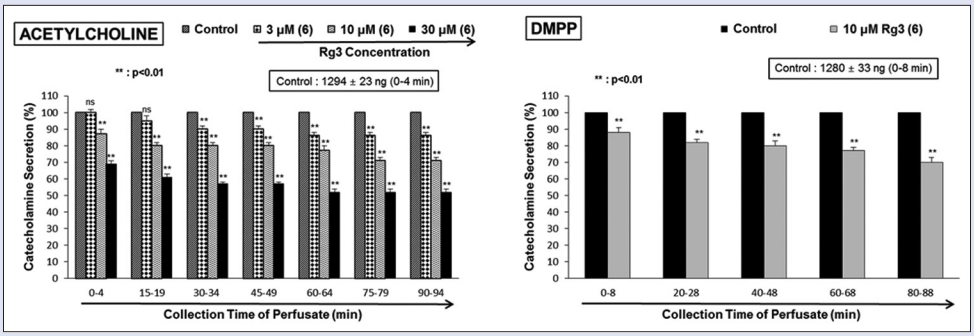


**Figure 1:** Inhibitory responses of Rg3 on release of CA produced by acetylcholine (left) and DMPP (right) in the perfused adrenal medulla of the rat. The release of CA evoked by an injection of ACh (5.32 mM) with a volume of 50 μL at 15-min intervals or by infusion of DMPP (100 μM) for 2 min at 20-min intervals was produced during perfusion of 3, 10, and 30 μM of Rg3 for 1.5 h as designated by bolt marks, respectively. The number in each parenthesis displays number of adrenal medulla. T-type bar on each column indicates SEM. Ordinate: The quantities of CA released in adrenal medulla (% of control). Abscissa: Collecting time of the perfusate (min). Significant difference was statistically analyzed by comparing the value of the control with individual dose-treatment group of Rg3. The collection of perfusates produced by ACh and DMPP was performed for 4 and 8 min, respectively. **$P < 0.01$. Rg3: Ginsenoside-Rg3; ns: Statistically nonsignificant; CA: Catecholamines; DMPP: 1,1-dimethyl-4-phenylpiperazinium; ACh: Acetylcholine; SEM: Standard errors of the mean

infused for 1.5 h following the corroboration of the corresponding control secretion level.

When ACh (5.32 mM) with a volume of 50 μL was given into an adrenal vein, the CA secretory response was sharply increased (1294 ± 23 ng/0–4 min). However, during perfusion of Rg3 with the range of 3–30 μM for 1.5 h, ACh-produced secretion of CA was time-and concentration-dependently suppressed. As in Figure 1 (upper), under the existence of Rg3, the CA secretion of ACh was reduced maximally to 52% of the control secretion (100%).

DMPP (100 μM), a selective agonist of neuronal nicotinic ACh receptor agonist in autonomic sympathetic ganglia, produced a rapid and sharp increment in the release of CA (1280 ± 33 ng/0–8 min). However, as shown in Figure 1 (lower), during perfusion of Rg3 (10 μM) for 1.5 h DMPP-produced release of CA was vastly diminished (to 73% of the control secretion).

McN-A-343 (100 μM), a selective AChergic muscarinic $M_1$-receptor agonist,[26] administered intravenously for 4 min, elevated the release of CA (640 ± 33 ng/0–4 min). But, under the influence of Rg3 (10 μM), McN-A-343-produced secretion of CA was greatly suppressed to 73% of the control level [Figure 2-upper].

Since Ang II has been shown to elevate secretion of epinephrine from the adrenal gland through activation of the $AT_1$ receptors,[27] it was attempted to assess the influence of Rg3 on Ang II-produced secretion of CA. Ang II (100 nM) increased the CA secretion (666 ± 17 ng/0–4 min), while during loading of Rg3 (10 μM) for 1.5 h, Ang II-produced secretion of CA was reduced to 65% of the level of control [Figure 2-lower].

## Inhibitory effect of ginsenoside-Rg3 on the catecholamines release produced by high $K^+$, Bay-K-8644, veratridine, and cyclopiazonic acid in the perfused rat adrenal medullae

Furthermore, high $K^+$ (a direct membrane-depolarizing agent) greatly raised the release of CA (832 ± 24 ng/0–4 min). During perfusion of Rg3 (10 μM) for 1.5 h, high $K^+$ (56 mM)-produced CA release was significantly depressed to 69% of control level at 75–94 min periods, as shown in Figure 3 (upper).

Since Bay-K-8644 has been found to be an activator of L-type voltage-dependent $Ca^{2+}$ channels (VDCC), which elevates the uptake of basal $Ca^{2+}$,[28] and the release of CA,[29] effect of Rg3 on Bay-K-8644-produced adrenal release of CA was tested. During loading of Rg3 (10 μM) for 1.5 h, Bay-K-8644 (10 μM)-produced release of CA was clearly diminished to 66% of the control secretion barring no change for the early 0–4 min period in comparison to the control secretion (683 ± 27 ng/0–4 min) in 6 rat adrenal medulla [Figure 3-lower].

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, is known as an exceptionally selective $Ca^{2+}$-ATPase inhibitor in

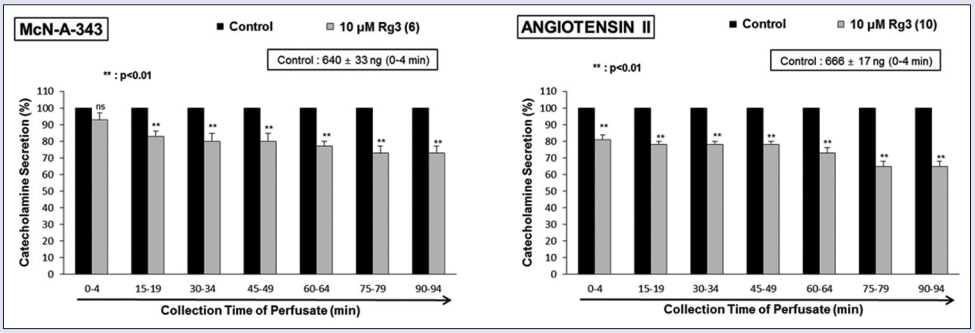


**Figure 2:** Time-dependent effects of Rg3 on the secretion of CA produced by McN-A-343 (left) and Ang II (right) in the isolated adrenal medulla of the rat. The release of CA evoked by infusion of McN-A-343 (100 μM) for 4 min and Ang II (100 nM) for 1 min was produced at 15-min intervals during perfusion of Rg3 (10 μM) for 1.5 h. The collection of perfusates produced by McN-A-343 and Ang II was performed for 4 min. Other legends are identical to those of Figure 1. $^{**}P < 0.01$. Rg3: Ginsenoside-Rg3; ns: Statistically nonsignificant; CA: Catecholamines; Ang II: Angiotensin II; McN-A-343: 3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride

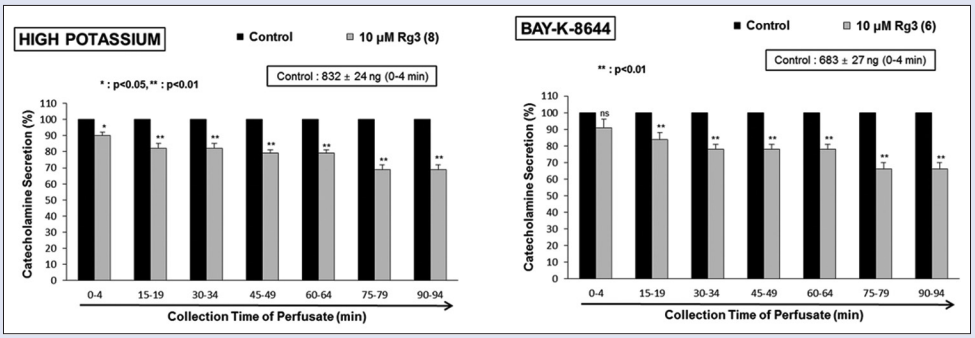


**Figure 3:** Time-dependent effects of Rg3 on the secretion of CA produced by high $K^+$ (left) and Bay-K-8644 (right) in the isolated adrenal medulla of the rat. The release of CA evoked by an injection of $K^+$ (56 mM) with a volume of 100 μL and infusion of Bay-K-8644 (100 μM) for 4 min was produced at 15-min intervals during perfusion of Rg3 (10 μM) for 1.5 h as designated by the bolt marks. The collection of perfusates produced by high $K^+$ or Bay-K-8644 was performed for 4 min. Other legends are identical to those of Figure 1. $^{**}P < 0.01$. Rg3: Ginsenoside-Rg3; ns: Statistically nonsignificant; CA: Catecholamines

sarcoplasmic reticulum of the skeletal muscle.[30,31] The inhibitory activity of Rg3 to cyclopiazonic acid-produced secretion of CA was achieved [Figure 4-upper]. During perfusion of Rg3 (10 μM) for 1.5 h in 6 rat adrenal medulla, cyclopiazonic acid ($10^{-5}$ M)-produced secretion of CA was depressed to 69% of the corresponding control release (619 ± 21 ng/0–4 min), excepting there was no change only for the first period (0–4 min).

It is found that veratridine-produced $Na^+$ inflow induced through voltage-sensitive $Na^+$ channels elevated $Ca^{2+}$ inflow through the activation of VDCC and evoked the exocytotic release of CA from cultured bovine adrenochromaffin cells.[32] Veratridine (50 μM) steeply elevated the CA secretion (832 ± 29 ng/0–4 min). However, in 6 rat adrenal medulla, during perfusion of Rg3 (10 μM) for 1.5 h, veratridine-produced secretion of CA was also attenuated to 67% of the control level [Figure 4-lower].

## Influence of ginsenoside-Rg3 plus Nω-nitro-L-arginine methyl ester on the catecholamines release produced by acetylcholine, Angiotensin II, Bay-K-8644, and veratridine in the perfused rat adrenal medullae

It was found that Rg3 significantly depressed the release of CA produced by stimulation of cholinergic and Ang II receptors in the perfused rat adrenal medullae. Thus, to examine the interrelationship between NO and Rg3-produced inhibitory effect on adrenal release of CA, the influence of L-NAME, an NO synthase inhibitor, on Rg3-produced reduction of CA releasing responses produced by ACh, Bay-K-8644, Ang II, and veratridine was determined.

In 10 rat adrenal medulla, during simultaneous loading of L-NAME (30 μM) and Rg3 (10 μM) for 1.5 h, ACh (5.32 mM)-produced secretion of CA was vastly restored to 100%–92% of the control level (1291 ± 24 ng/0–4 min) in contrast to that of Rg3 (10 μM)-treatment alone, as depicted in Figure 5 (upper).

In addition, during the concurrent loading of L-NAME (30 μM) and Rg3 (10 μM) for 1.5 h in 10 rat adrenal medullae, Ang II (100 nM)-produced CA release was recovered to 100%–89% of the control secretion (628 ± 19 ng/0–4 min) in contrast to that of Rg3-treatment alone [Figure 5-lower].

During concurrent loading of Rg3 (10 μM) and L-NAME (30 μM) for 1.5 h, release of CA produced by Bay-K-8644 (10 μM) and veratridine (50 μM) were recovered mostly to 100%–89% (Bay-K-8644) and 100%–91% (veratridine) of each control secretory response (678 ± 24 ng/0–4 min for Bay-K-8644; 839 ± 25 ng/0–4 min for veratridine), in contrast to the reduction by Rg3-treatment only [Figure 6].

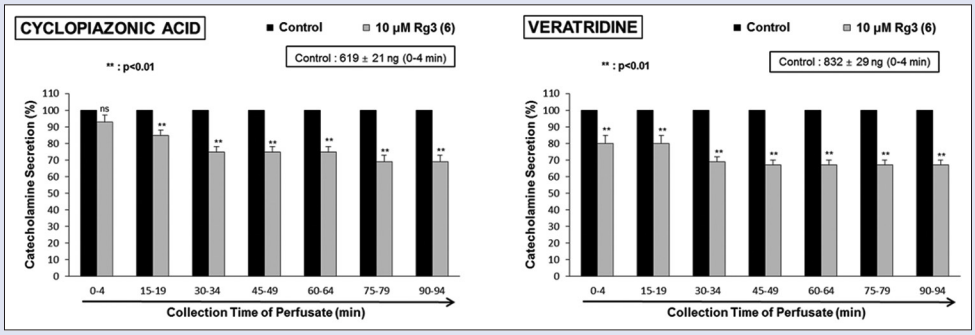


**Figure 4:** Time-dependent effects of Rg3 on the secretion of CA produced by cyclopiazonic acid (left) and veratridine (right) in the isolated rat adrenal medullae. The release of CA evoked by cyclopiazonic acid (10 μM) and veratridine (50 μM) was infused into the perfusion stream for 4 min at 15-min intervals during infusion of Rg3 (10 μM) for 1.5 h. The collection of perfusates produced by cyclopiazonic acid and veratridine was performed for 4 min. Other legends are identical to those of Figure 1. $^{**}P < 0.01$. Rg3: Ginsenoside-Rg3; ns: Statistically nonsignificant; CA: Catecholamines

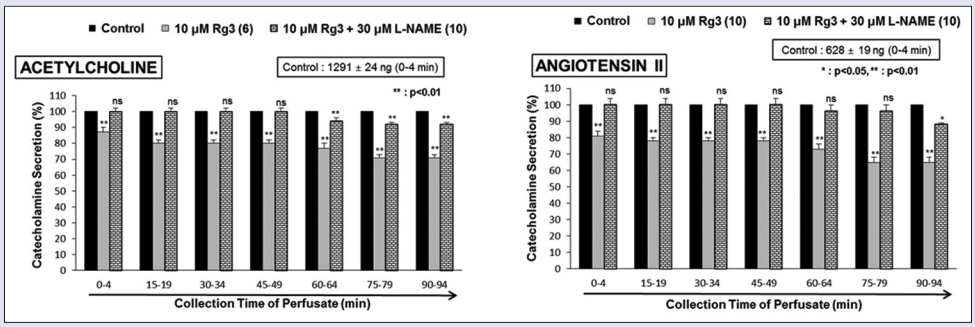


**Figure 5:** Effects of Rg3 plus L-NAME on the secretion of CA produced by acetylcholine (left) and Ang II (right) in the isolated rat adrenal medullae. The release of CA evoked by an injection of ACh (5.32 mM) with a volume of 50 μL and Ang II (100 nM) for 1 min was produced at 15-min intervals during concurrent perfusion of Rg3 (10 μM) plus L-NAME (30 μM) for 1.5 h. Significant difference was statistically analyzed by comparing the value of the control with Rg3-treatment only group or treatment group with Rg3+L-NAME. Other legends are identical to those of Figure 1. $^{**}P < 0.01$. Rg3: Ginsenoside-Rg3; ns: Statistically nonsignificant; CA: Catecholamines; Ang II: Angiotensin II; L-NAME: Nω-nitro-l-arginine methyl ester; ACh: Acetylcholine

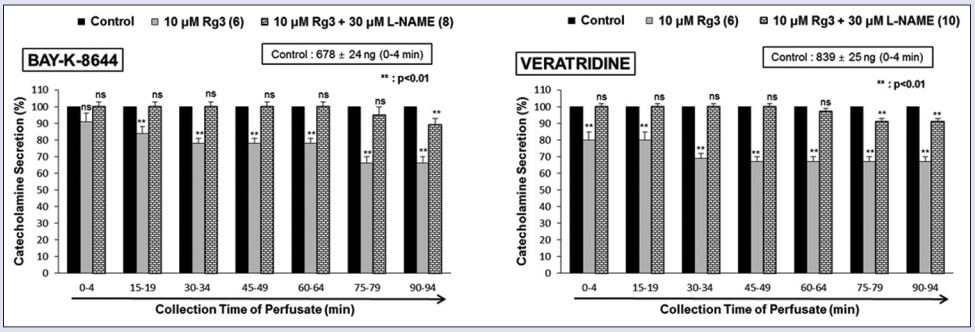


**Figure 6:** Effects of Rg3 plus L-NAME the secretion of CA produced by Bay-K-8644 (left) and veratridine (right) in the isolated rat adrenal medullae. The release of CA evoked by infusion of Bay-K-8644 (100 μM) and veratridine (50 μM) was produced at 15-min intervals during concurrent perfusion of Rg3 (10 μM) plus L-NAME (30 μM) for 1.5 h. Significant difference was statistically analyzed by comparing the value of the control with Rg3-treatment only group or treatment group with Rg3+L-NAME. Other legends are identical to those of Figure 1. $^{**}P < 0.01$. Rg3: Ginsenoside-Rg3; ns: Statistically nonsignificant; CA: Catecholamines; Ang II: Angiotensin II; L-NAME: $N^{\omega}$-nitro-l-arginine methyl ester; ACh: Acetylcholine

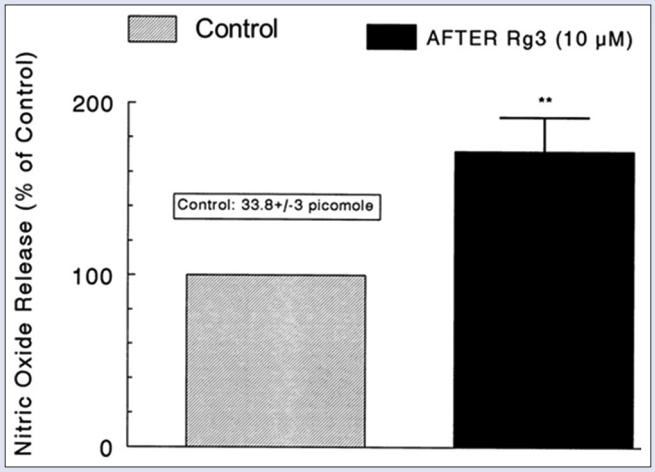


**Figure 7:** Influence of Rg3 on production of NO in the isolated rat adrenal medullae. The sample of perfusate sample was collected for 8 min following infusion of Rg3 (10 μM) with a rate of 0.31 mL/min. Ordinate: the quantities of liberated NO in the adrenal medulla (% of control). Abscissa: Perfusion-treatment (before and after infusion of Rg3). Significant difference was statistically analyzed by comparing the value of control with Rg3-treatment group. $^{**}P < 0.01$. NO: Nitric oxide; Rg3: Ginsenoside-Rg3

## Effect of ginsenoside-Rg3 on nitric oxide liberation in the perfused rat adrenal medullae

Figures 5 and 6 showed that the secretion of CA produced by ACh, Bay-K-8644, Ang II, and veratridine was restored nearly to the control level during simultaneous loading with Rg3 and L-NAME. Thus, we determined the amount of NO liberated in the perfused adrenal medulla following infusion of Rg3, since it has been reported that ginsenosides lower blood pressure through the increased release of endothelial NO,[15] and Rg3 is the most powerful ginsenoside, dynamizing eNOS in the isolated rat aorta.[16] Moreover, it has also been shown that Rg3 produces activation of eNOS in the vasculature of animal models.[15,17]

The spontaneous NO release in the rat adrenal medulla prior to loading with Rg3 in 10 adrenal medullae was 33.8 ± 3 picomoles. However, 8 min following peristaltic infusion of Rg3 (10 μM), it was enhanced significantly to 58 ± 7 picomoles, which revealed 172% of the spontaneous NO release [Figure 7].

## Combined effects of ginsenoside-Rg3 and fimasartan on acetylcholine-produced secretion of catecholamines in the perfused adrenal medulla of the rat

In the present work, Rg3 as well as fimasartan (an Ang II type 1 [$AT_1$] receptor-selective antagonist[33]) reduced the release of CA by activation of cholinergic and Ang II $AT_1$ receptors in the perfused adrenal medulla of the rat. Thus, so as to distinguish the combined effects of fimasartan and Rg3, inhibitory action of Rg3 plus fimasartan on ACh-produced release of CA was examined.

During the simultaneous perfusion of Rg3 (10 μM) and fimasartan (15 μM) for 1.5 h, ACh (5.32 mM)-produced release of CA was more potently depressed to ~ 50% of control level (1214 ± 27 ng/0–4 min), than in the fimasartan-treatment alone (73% of the control) from 7 adrenal medullae, as depicted in Figure 8. In addition, there was statistically significant difference in inhibition on ACh-produced release of CA between fimasartan versus fimasartan plus Rg3.

## DISCUSSION

Our study showed the first evidence that Rg3 greatly suppresses the release of CA produced by activation of cholinergic (nicotinic and muscarinic) and angiotensinergic $AT_1$ receptors in the perfused rat adrenal medullae. Rg3-produced inhibition appears to be revealed by depressing inflow of $Na^+$ and $Ca^{2+}$ ions into adrenomedullary cells and by diminishing $Ca^{2+}$ release in intracellular $Ca^{2+}$ storage at least via the rise of NO production by activating NO synthase, which is in relation to the blocking action of angiotensinergic $AT_1$ and AChergic nicotinic receptors.

In our study, during concurrent perfusion of Rg3 and L-NAME (an inhibitor of NO synthase), release of CA produced by ACh, Ang II, Bay-K-8644, and veratridine were restored nearly to the level of each control, in contrast to that of Rg3-treatment alone. These findings are well accordance with the previous data that, in the previous studies, ginsenoside-Rb2 reduces the release of CA in the perfused adrenal medulla of the rat via the elevated production of NO by dynamization of neuronal NO synthase (nNOS),[1] ginsenosides lower blood pressure through the elevated production of endothelial NO,[15] and that Rg3 is the most powerful ginsenoside dynamizing eNOS in the isolated rat aorta.[16] Some studies have verified that Rg3 revealed the dynamization of eNOS in vasculature of animal models.[15,17] Furthermore, Rg3 has been found to dynamize eNOS through phosphorylation of NOS in ECV 304 human

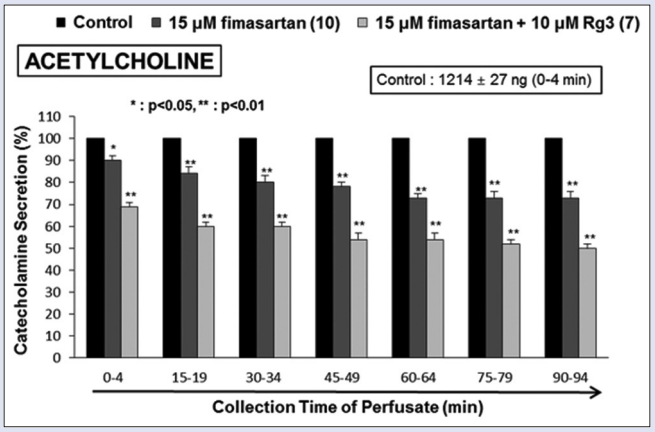


**Figure 8:** Comparative time-dependent effect of fimasartan and Rg3 plus fimasartan on ACh-produced secretion of CA in the isolated rat adrenal medullae. The release of CA evoked by an injection of ACh (5.32 mM) with a volume of 50 μL was produced at 15-min intervals during perfusion of fimasartan (15 μM) or fimasartan (15 μM) plus Rg3 (10 μM) for 1.5 h, as designated by the bolt mark. The collection of perfusates produced by ACh was performed for 4 min. Other legends are identical to those of Figure 1. Significant difference was statistically analyzed by comparing the value of control group versus fimasartan (15 μM)-treatment group or fimasartan (15 μM) + Rg3 (10 μM)-treatment group. *$P < 0.05$ or **$P < 0.01$. Rg3: Ginsenoside-Rg3; ACh: Acetylcholine; CA: Catecholamines

endothelial cells and to elevate the expression of eNOS.[18] Furthermore, in our study, after intravenous perfusion of Rg3, NO production substantially increased as depicted in Figure 7. In light of these results, it seems that Rg3 reduces several secretagogues-produced CA-releasing effect through elevation of NO production in rat adrenomedullary cells, because during concurrent loading of Rg3 and L-NAME, the secretion of CA produced by ACh, Bay-K-8644, Ang II, and veratridine were restored nearly to each control level in comparison with that of Rg3-treatment alone, and also actually, Rg3 significantly elevated NO liberation.

In supporting our results, it was previously found that L-NAME (an NOS inhibitor) increased $K^+$-produced secretion of CA in the cultured bovine adrenochromaffin cells,[34] and sodium nitroprusside (SNP) suppressed ACh-produced release of CA in cultured bovine adrenochromaffin cells.[35] These findings have demonstrated that NO can play a pivotal role in regulation of the release of CA in the adrenal medulla. Furthermore, when the endothelial cells are existed in cultured bovine chromaffin cells, it has been shown that $K^+$-produced or nicotinic receptor agonist DMPP-produced secretion of CA are reduced,[34] implying that eNOS as well as nNOS may play important roles in regulating secretion of CA. In light of these previous findings, our results strongly suggest that Rg3 may at least activate nNOS in adrenal medulla, reducing the CA release due to enhanced NO production, in addition to its direct action on the CA release. In support of our findings, in the midst of ginsenosides pertaining to protopanaxatriol- and protopanaxadiol-type saponin groups, Rg3 is known to be the most powerful vasorelaxant.[13,16] It has been previously found that Rg3 inhibits not only calcium-produced vasoconstriction[16] as well as phenylephrine-produced vasoconstriction as a consequence of increased NO production.[13]

On the other hand, it has been shown that L-NAME attenuates ACh-produced secretion of CA in cultured bovine adrenochromaffin cells[35] and also that SNP (an NO donor) potentiates nicotine-produced secretion of CA in the cultured bovine adrenochromaffin cells.[36] These results imply that NO can rather enhance cholinergic agonist-produced release of CA. Moreover, a few cases of *in vivo* studies showed that NO failed to play a role in the modulation of adrenal CA release.[37,38]

Upon neurogenic activation of the adrenal medulla, ACh, the physiological presynaptic transmitter, is liberated from splanchnic nerve terminals and stimulates ACh receptors on the adrenochromaffin cell membrane.[39] This stimulation generates a series of neurogenic reactions as stimulus-secretion coupling, resulting in exocytotic release of CA and other constituents from releasing pool of synaptic vesicles into synaptic cleft. In general, these two mechanisms are included in liberation of adrenomedullary hormones. In view of this fact, our findings indicate that Rg3 can reduce the secretion of CA produced by activation of AChergic (nicotinic and muscarinic) and angiotensinergic $AT_1$ receptors in the adrenal medulla. Ginsenosides isolated from *Panax ginseng* are known to lower the blood pressure in both hypertensive patients and experimental animals.[15,40-42] These previous findings support that Rg3-produced inhibition on release of CA produced by activation of acetylcholinergic as well as angiotensinergic $AT_1$ receptors can attribute at least to its hypotensive action. ACh is liberated by triggering depolarization of splanchnic nerve endings which in turn stimulates AChergic nicotinic receptors, evokes release of CA, and produces induction of dopamine β-hydroxylase by $Ca^{2+}$-sensitive secretory procedure.[43,44] On the basis of these results, our findings demonstrate that Rg3 can reduce the secretion of secretion produced by cholinergic activation from the splanchnic nerve terminal, antagonizing nicotinic receptors. The exocytotic secretion of CA in adrenochromaffin cells seems to be originally analogous to those happening in adrenergic axons.[45,46] ACh-produced secretion of CA has known to be evoked via stimulation of AChergic nicotinic and muscarinic receptors in the adrenal gland of the guinea pig[47] as well as in perfused adrenal medulla of the rat.[48] In supporting of this finding, it is known that ginseng saponins, ginsenoside-Rg3, a panaxadiol,[7] as well as ginsenoside-Rg2, a panaxatriol[5] block AChergic nicotinic receptors or receptor-gated $Na^+$ channels (but not voltage-sensitive $Na^+$ and $Ca^{2+}$ channels), reduce $Na^+$ inflow through the channels and in consequence inhibit both $Ca^{2+}$ inflow and release of CA in cultured bovine adrenochromaffin cells.

Our study found that Rg3 diminished the secretion of CA produced by Ang II, ACh, McN-A-343, and DMPP. This finding implies that Rg3 can exert the same effect as in adrenal medulla of the rat with normal blood pressure[2] and in cultured bovine adrenochromaffin cells.[5,7] Previously, $Rg_3$ has been found to reduce both ACh-produced $Na^+$ and $Ca^{2+}$ inflow in a dose-dependent fashion similarly to the ACh-produced release of CA in cultured bovine adrenochromaffin cells.[7] However, it caused no or merely a little effect on release of CA and $Ca^{2+}$ inflow produced by veratridine (an activator of the voltage-sensitive $Ca^{2+}$ or $Na^+$ channels) or high $K^+$.[4,5] These findings toughly imply that Rg3 acts on AChergic nicotinic receptor-gated cation channels but not on voltage-dependent $Na^+$ or $Ca^{2+}$ channels. Moreover, Rg3-produced inhibitory effect on ACh-produced release of CA was not surmounted by raising the extracellular ACh and $Ca^{2+}$ levels[7] showing that Rg3-induced inhibition is different from that of the competitive AChergic nicotinic receptor antagonists, like trimethaphan,[49,50] and that of the L-type VDCC blockers, which are surmountable with extracellular $Ca^{2+}$ levels, like diltiazem.[51] Actually, antagonistic action of $Rg_3$ was nonsurmountable with nicotine.[7] This finding appears to be much distinct from that of our study that Rg3 greatly diminished the secretion of CA produced by AChergic nicotinic agonist as well as by activator of VDCC or voltage-dependent $Na^+$ channels from the perfused adrenal medulla of the rat. The inconsistency seems to be contributed to experimentally utilization of different components and different methodology between previous and our studies.

In our study, Rg3 time-dependently suppressed the secretion of CA produced by Bay-K-8644, which triggers L-type VDCC,[28,52] as well as by

high $K^+$ (a direct membrane depolarizer). The present finding indicates that Rg3 can suppress inflow of $Ca^{2+}$ via VDCC into adrenomedullary chromaffin cells. In the preceding studies, nicotinic (but not muscarinic) stimulation produces the $Ca^{2+}$-sensitive release of CA in bovine adrenomedullary cells.[53,54] Stimulation of nicotinic AChergic receptors has usually been known to facilitate secretion of CA by raising $Ca^{2+}$ inflow via receptor-operated and/or VDCC in both perfused adrenal gland of the rat[48,55] and cultured bovine adrenochromaffin cells.[55-58] Previously, it is well-known that the adrenochromaffin cells possess (i) AChergic nicotinic receptor-gated channels of ion, accountable to carbachol-produced $Na^+$ inflow; (ii) voltage-sensitive channels of $Na^+$, accountable to veratridine-produced $Na^+$ inflow; and (iii) VDCC, implying that the inflow of $Na^+$ produced by carbachol as well as veratridine causes to dynamize VDCC by changing membrane potentials, while high $K^+$ dynamizes directly VDCC without elevation of $Na^+$ inflow.[59] In our experiments, during loading of Rg3, the secretion of CA produced by high $K^+$ or Bay-k-8644 was vastly reduced. The inhibitory activity of Rg3 appears to be revealed by reducing inflow of $Ca^{2+}$ via VDCC to adrenomedullary cells. Moreover, a little increase in concentration of the extracellular $K^+$ rises the frequency of basal action potentials along with increased release of CA,[60] implying that inflow of $Ca^{2+}$ that happens while generating action potentials is directly connected to secretion rate. In our study, Rg3 reduced the CA release produced by high $K^+$ and Bay-K-8644. This data imply that Rg3 can suppress the VDCC. In the cultured bovine adrenochromaffin cells, activation of AChergic nicotinic (but not muscarinic) receptors is found to produce the release of CA through elevation of $Ca^{2+}$ inflow principally through VDCC.[61,62] Thus, this Rg3-produced inhibitory action on release of CA produced by DMPP, ACh, veratridine, and Bay-K-8644 appears to be revealed by inhibiting inflow of $Ca^{2+}$ via VDCC by stimulation of AChergic nicotinic receptor-gated channels of ion, accountable to carbachol-produced inflow of $Na^+$ as well as of voltage-sensitive channels of $Na^+$, accountable to veratridine-produced inflow of $Na^+$.

In our experiments, it has been shown that Rg3 diminishes cyclopiazonic acid-produced secretion of CA. Cyclopiazonic acid is known to be an exceptionally selective inhibitor of $Ca^{2+}$-ATPase in sarcoplasmic reticulum of skeletal muscle[30,31] and a pharmacologically precious tool for exploring intracellular movement of $Ca^{2+}$ and currents of ion modulated by intracellular $Ca^{2+}$.[63] Pursuantly, it seems that Rg3-produced inhibitory action on cyclopiazonic acid-produced release of CA may be in relation to the intracellular $Ca^{2+}$ movement from the store of cytoplasmic $Ca^{2+}$. This effect is in accordance with the results obtained from skinned longitudinal smooth muscle fibers in the guinea-pig ileum, where uptake of $Ca^{2+}$ was prevented by cyclopiazonic acid.[64] Cyclopiazonic acid is known readily to traverses into cytoplasm via plasma membrane and inhibits $Ca^{2+}$-ATPase activity in sarcoplasmic/endoplasmic reticulum, consequently causing a raised release of $Ca^{2+}$ from their sites of storage.[63] Furthermore, in cultured bovine adrenochromaffin cells, AChergic muscarinic activation is suggested to facilitate metabolism of phosphoinositide (PI), producing the consequent production of inositol 1,4,5-trisphosphate, which evokes $Ca^{2+}$ movement from internal store.[65,66] Consequently, in our study, Rg3-produced inhibition to the secretion of CA induced by cyclopiazonic acid or McN-A-343 seems to be associated to the suppression of intracellular $Ca^{2+}$ movement from the storage of cytoplasmic $Ca^{2+}$. However, it is still unclarified whether Rg3-produced inhibition to release of $Ca^{2+}$ release from internal store is contributed to the indirect action on the PI metabolism or the direct action. To verify this characteristic activity of Rg3, more research should be done in the future.

In our study, when both fimasartan (an antagonist of angiotensinergic $AT_1$ receptors) and Rg3 were simultaneously given, the inhibitory action on ACh-produced CA secretion was significantly potentiated. In support of this idea, the combination of losartan (an $AT_1$ antagonist)-enalapril (an inhibitor of Ang II-converting enzyme) is found to be more powerful effectiveness in reduction of blood pressure as well as in elevation of active plasma renin than twofold of enalapril dose in male volunteers with normal blood pressure.[67] Similarly, in our findings, it is thought that the clinically concurrent administration of Rg3 and fimasartan can be beneficial to the alleviation or treatment of angina pectoris and hypertension.

In summary, our study shows the first verification that Rg3 can suppress the secretion of CA by activation of AChergic (nicotinic as well as muscarinic) receptors and angiotensinergic $AT_1$ receptors in the perfused rat adrenal medullae. This Rg3-produced inhibitory action on the secretion of CA appears to be revealed not merely by repressing influx of $Na^+$ and $Ca^{2+}$ to adrenochromaffin cells but also by depressing $Ca^{2+}$ release from intracellular $Ca^{2+}$ storage at least through raised production of NO by the dynamization of neuronal NO synthase. Grounded on these findings, the chronic intake of Rg3 or concurrent administration of Rg3 and $AT_1$ antagonist (fimasartan) can be beneficial to improve the cardiovascular diseases by diminishing the secretion of CA from adrenochromaffin cells, and consequently reducing the level of CA in the circulating blood.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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