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# Suppressive Impact of Ginsenoside-Rg2 on Catecholamine Secretion from the Rat Adrenal Medulla

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**Abstract** – This study was designed to characterize the effect of ginsenoside-Rg2 (Rg2), one of panaxatriol saponins isolated from Korean ginseng root, on the release of catecholamines (CA) in the perfused model of the rat adrenal medulla, and also to establish its mechanism of action. Rg2 (3~30 μM), administered into an adrenal vein for 90 min, depressed acetylcholine (ACh)-induced CA secretion in a dose- and time-dependent manner. Rg2 also time-dependently inhibited the CA secretion induced by 3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride (McN-A-343), 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), and angiotensin II (Ang II). Also, during perfusion of Rg2, the CA secretion induced by high $K^+$, veratridine, cyclopiazonic acid, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoro-methyl-phenyl)-pyridine-5-carboxylate (Bay-K-8644) depressed, respectively. In the simultaneous presence of Rg2 and $N^{\omega}$-nitro-L-arginine methyl ester hydrochloride (L-NAME), the CA secretion induced by ACh, Ang II, Bay-K-8644 and veratridine was restored nearly to the extent of their corresponding control level, respectively, compared to those of inhibitory effects of Rg2-treatment alone. Virtually, NO release in adrenal medulla following perfusion of Rg2 was significantly enhanced in comparison to the corresponding spontaneous release. Also, in the coexistence of Rg2 and fimasartan, ACh-induced CA secretion was markedly diminished compared to the inhibitory effect of fimasartan-treated alone. Collectively, these results demonstrated that Rg2 suppressed the CA secretion induced by activation of cholinergic as well as angiotensinergic receptors from the perfused model of the rat adrenal gland. This Rg2-induced inhibitory effect seems to be exerted by reducing both influx of $Na^+$ and $Ca^{2+}$ through their ionic channels into the adrenomedullary cells as well as by suppressing $Ca^{2+}$ release from the cytoplasmic calcium store, at least through the elevated NO release by activation of NO synthase, which is associated to the blockade of neuronal cholinergic and $AT_1$-receptors. Based on these results, the ingestion of Rg2 may be helpful to alleviate or prevent the cardiovascular diseases, via reduction of CA release in adrenal medulla and consequent decreased CA level in circulation.

**Keywords** – Ginsenoside-Rg2, adrenal medulla, catecholamine secretion, NO release

## Introduction

Previously, we have reported that gintonin, known as a novel G protein-coupled lysophosphatidic acid (LPA) receptor ligand isolated from Korean ginseng, greatly enhanced the CA secretion from the isolated perfused rat adrenal medulla.[1] This facilitatory effect of gintonin seems to be relevant to activation of LPA receptors and cholinergic receptors, which are relevant to the cytoplasmic $Ca^{2+}$ increase by stimulation of the $Ca^{2+}$ influx as well as

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by the inhibition of $Ca^{2+}$ uptake into the cytoplasmic $Ca^{2+}$ stores, without the increased nitric oxide.[1] Moreover, all of total ginseng saponin (TGS),[2] panaxadiol[3] and panaxatriol[4] are found to cause the increased CA secretion from the isolated perfused rabbit adrenal glands in a $Ca^{2+}$-dependent fashion, which are mediated by the activation of cholinergic (both nicotinic and muscarinic) receptors and partly the direct action on the rabbit adrenomedullary chromaffin cells. However, it has been shown that ginsenoside-Rb2, one of panaxadiol type saponins, inhibits the CA secretion from the isolated perfused rat adrenal glands.[5] Also, TGS has been reported rather to inhibit the CA secretion from the isolated perfused rat adrenal glands[6] and spontaneously hypertensive rats[7] Several investigators also showed that ginsenoside components isolated from *Panax ginseng* reduce the ACh-induced CA secretion from bovine adrenal chromaffin cells.[8-11]

In previous study, Tachikawa and his co-workers[9] have found that most of the ginsenosides (1 - 100 μM) produced a tendency to suppress the ACh-induced CA secretion. They showed that the order of inhibitory potency (at the concentration of 10 μM) was as follows: Rg2 > Rf > Re > Rh1 > Rb2, Rg1 > Rb1 > Rc > Rb3, Rd, Ro, $Rs_1$. The inhibition of ginsenoside- Rg2 at 10 μM was 72%, but ginsenosides Rb3, Rd, Ro and $Rs_1$ did not show the inhibitory effect.

Jeon and his co-workers[12] have found that Korean Red Ginseng (KRG)-evoked releasing effect of NO in the conscious rats can partly contribute to the hypotensive effect. Rg3 also has been reported to relax the rat thoracic aorta as a consequence of NO production.[13] Han and his co-workers[14] have found evidence that NO levels in exhaled breath of human volunteers by KRG were increased along with reduced blood pressure and heart rate. In a series of studies, it has been found that ginsenosides reduce blood pressure via increased production of endothelial nitric oxide[15] and that Rg3 is the most potent ginsenoside that activates endothelial nitric oxide synthase (eNOS) in rat aorta.[16] Although some investigators have demonstrated that Rg3 induces eNOS activation in the vasculature of animal models.[15,17]

Despite of these many studies on various ginseng saponins, there are still a little-known effect of ginsenoside-Rg2, one of panaxatriol-type saponins, on adrenal CA secretion. Therefore, the present study was for the first time attempted to determine whether ginsenoside-Rg2 influences several secretagogues-induced CA secretory responses from the perfused model of the isolated rat adrenal medulla and to clarify its mechanism of action.

## Experimental

**Drugs and their sources** – The following drugs were used: ginsenoside-Rg2 (provided by the Society of Korean Ginseng, Seoul, Korea), fimasartan (donated from Boryung Pharmaceutical Company, Seoul, Korea), 3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride [McN-A-343] (RBI, U.S.A.), cyclopiazonic acid, norepinephrine bitartrate, acetylcholine chloride, veratridine hydrochloride, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), potassium chloride (KCl), Sodium bicarbonate, calcium chloride, $N^{\omega}$-nitro-L-arginine methyl ester hydrochloride (L-NAME), angiotensin II, sodium chloride, potassium phosphate, glucose, ascorbic acid, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoro-methyl-phenyl)-pyridine-5-carboxylate [Bay–K-8644], disodium EDTA, magnesium chloride (Sigma Chemical Co., U.S.A.). Drugs were dissolved in distilled water (stock) and added to the normal Krebs-bicarbonate solution, 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%). Concentrations of all drugs used in this study are depicted in terms of their molar base.

**Experimental procedure** – All operating procedures including the animal experiments were performed strictly in accord with the guidance for the care and employment of Laboratory Animals of the Committee of Experimental Animals, School of Medicine, Chosun University and approved (No. 2015-06, March, 2015).

Male mature Sprague-Dowley rats (DAMOOL SCIENCE, International Customer Service, Seoul, Korea), weighing 180 to 320 grams, were used in this study. The experimental animals were individually housed in separate cages, and food (Jeil Animal Chow, Korea) and tap water were allowed freely for about ten days to adapt to circumstances. On the day of experiment, the animal was anesthetized with intraperitoneal injection of thiopental sodium (50 mg/kg), and tied in supine position on fixing platform.

**Isolation of adrenal glands** – The adrenal gland was isolated by some modification of previous method.[18] The abdomen was opened by a midline incision, and the left adrenal gland and surrounding area were exposed by the placement of three-hook retractors. The stomach, intestine and portion of the liver were not excised out, but pushed over to the right side and covered by saline-soaked gauge pads, and urine in bladder was drained in order to secure enough working space for tying blood vessels and cannulations. A cannula, employed for perfusion of the adrenal gland, was placed into the distal end of the renal

vein following all branches of adrenal vein (if any), vena cava and aorta were ligated. Prior to ligating vessels and cannulations, heparin (400 IU/mL) was given into vena cava to avoid blood coagulation. The adrenal cortex was cut down to make a small slit into just opposite side of adrenal vein. The gland was started to perfuse, checking up there is no leakage, and the perfusion fluid flowed out only from the slit made in adrenal cortex. Then the adrenal gland, including ligated blood vessels and the cannula, was cautiously excised from the rat and placed on a platform of a leucite chamber. The chamber was incessantly circulated with water heated at 37 ± 1 ºC.

**Perfusion of adrenal gland** – The perfusion of the isolated adrenal glands was performed by means of peristaltic pump (Isco, St. Lincoln, NE, U.S.A.) at a rate of 0.31 mL/min. The perfusion was made with Krebs-bicarbonate solution containing the following composition (mM): NaCl, 118.4; $NaHCO_3$, 25; $KH_2PO_4$, 1.2; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; glucose, 11.7. The perfusion solution was continuously bubbled with 95% $O_2$ + 5% $CO_2$ and the final pH of the Krebs-bicarbonate solution was continued at 7.4 ~ 7.5. Disodium EDTA (10 μg/mL) and ascorbic acid (100 μg/mL) to block oxidation of catecholamines were added into the perfusion solution.

**Drug administration** – DMPP (100 μM) and angiotensin II (100 nM) for 2 minutes and/or a single injection of ACh (5.32 mM) and KCl (56 mM) in a volume of 0.05 mL were administered into perfusion stream via a three-way stopcock, respectively. McN-A-343 (100 μM), veratridine (50 μM), Bay-K-8644 (10 μM) and cyclopiazonic acid (10 μM) were also given by perfusion for 4 min, respectively.

In the preliminary studies, it was shown that upon injection or perfusion of these drugs, secretory responses to ACh, KCl, McN-A-343, angiotensin II, veratridine Bay-K-8644 and cyclopiazonic acid turned back to pre-injection level in about 4 min, but the responses to DMPP in 8 min.

**Collection of perfusate** – Generally, before stimulation with various secretagogues, the collection of perfusate was performed for 4 min to assay the spontaneous CA secretion (background sample). Immediately following the collection of the background sample, the perfusates were collected continuously in another tube as soon as the perfusion solution containing the stimulatory secretagogue reached the adrenal medulla. Stimulated sample's perfusate was collected for 4 or 8 min. The amounts released in the background sample have been subtracted from that released from the stimulated sample to get the net CA secretion, which is shown in all of the figures.

Prior to study the effect of Rg2 on the spontaneous and induced CA release, the perfusion of adrenal gland was performed with normal Krebs solution for 90 min, and then the collection of perfusate was made for a certain period (background sample). Then the solution was displaced by the one containing the facilitatory secretagogue or along with Rg2, and the collection of perfusates was performed for the same period as that for the background sample. The perfusate of the adrenal gland was collected in chilled tubes.

**Measurement of catecholamines** – The content of CA (all of epinephrine, norepinephrine and dopamine) in perfusate was measured directly by the fluorometric method of Anton and Sayre[19] without the intermediate purification alumina for the reasons described earlier[18] using fluorospectrophotometer (Kontron Co., Milano, Italy).

0.2 mL volume of the perfusate was employed for the assay reaction. The CA content in the perfusate of stimulated medulla by secretagogues employed in the present work was fully enough to secure readings several folds greater than the reading of unstimulated samples (control). The sample blanks were also lowest for perfusates of stimulated and non-stimulated samples. The CA concentration in the perfusate was shown in terms of norepinephrine (base) equivalents.

**Measurement of NO release** – The NO-selective microelectrode (ami700, Innovative Instruments Inc) and an amplifier (inNo meter, Innovative Instruments Inc) were employed for measurement of NO released from the perfused adrenal medulla. NO production released from adrenal medulla was quantified as the integrated signal detected by the microelectrode after perfusion of fimasartan into rat adrenal medulla, as previously described.[20] The value of electrode was calibrated by establishing standardized concentrations of NO in 0.5% (wt/vol) KI in 0.1 Mol/L $H_2SO_4$ from $NaNO_2$ standards. NO release was estimated as the current detected at the electrode following perfusion of fimasartan into adrenal medulla. The net NO release was calculated as picomoles.

**Statistical analysis** – The difference between the control group and the drug-treated group was statistically analyzed by the Student's *t* tests. A P-value of less than 0.05 was regarded statistically to elicit significant changes unless specifically described in the text. Values expressed in the text refer to means and the standard errors of the mean (S.E.M.). The experimental data were statistically assayed by computer program described by Tallarida and Murray. [21]

## Results and Discussion

Following the perfusion of oxygenated Krebs-bicarbonate

solution for 60 min, the spontaneous CA secretion from the isolated perfused rat adrenal glands was 21 ± 2 ng for 2 min (n = 10). Since it has been reported that most of the ginsenosides (1 - 100 μM) elicited tendency to reduce the ACh-induced CA secretory response,[9] it was tried initially to examine the effects of Rg2 itself on CA secretion from the perfused model of the rat adrenal glands. However, in the present study, Rg2 itself failed to affect spontaneous CA release (data not shown). Therefore, it was decided to examine effects of Rg2 on the CA secretory responses induced by stimulation of cholinergic receptors as well as angiotensin II receptors. Secretagogues were administered at 15 to 20 min-intervals. Rg2 was perfused for 90 minutes following the corroboration of the control secretion.

When ACh (5.32 mM) in a volume of 0.05 mL was given into the perfusion stream, the amount of CA release was 1280 ± 38 ng for 4 min. However, in the presence of Rg2 in the range of 3 ~ 30 μM for 90 min, ACh-induced

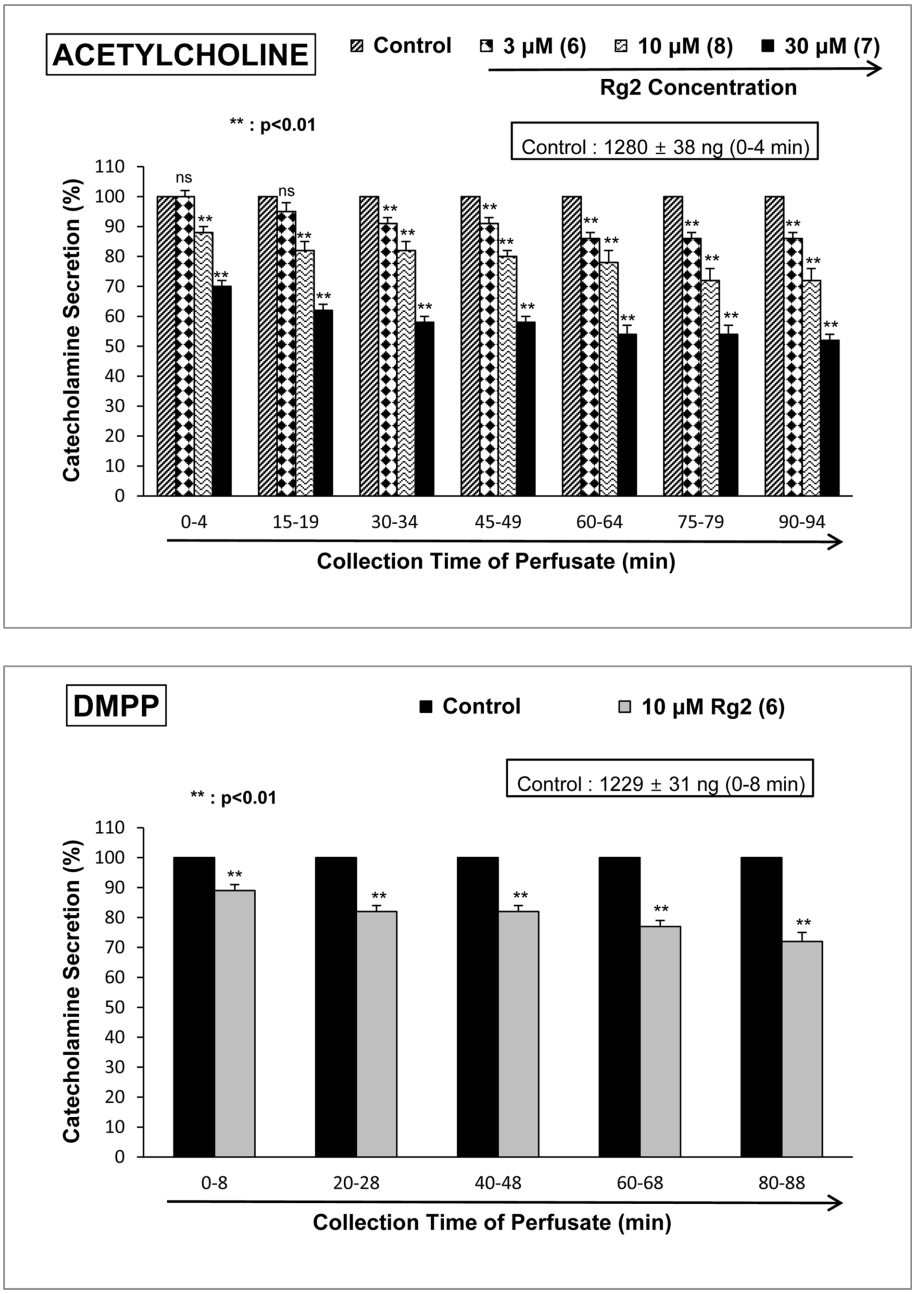


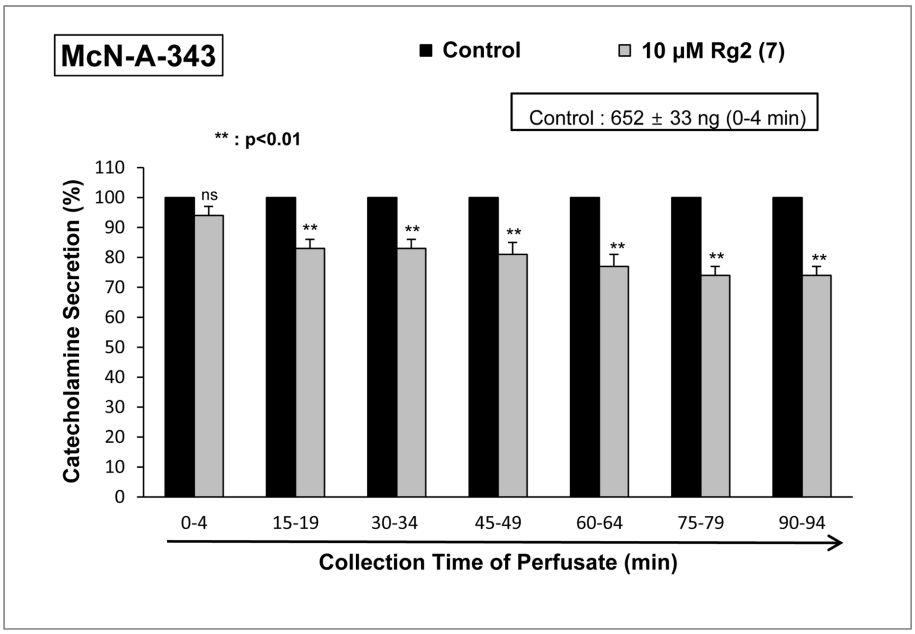

**Fig. 1.** Inhibitory responses of ginsenoside-Rg2 (Rg2) on release of catecholamines (CA) produced by acetylcholine (upper) and DMPP (lower).

CA release was significantly reduced in relatively concentration- and time-dependent manner. As shown in Fig. 1 (upper), under the existence of Rg2, the CA secretory responses were depressed maximally to 52% of the corresponding control secretion (100%).

DMPP (100 μM), a selective agonist of neuronal nicotinic receptor in autonomic sympathetic ganglia, induced a sharp and rapid increase in CA secretion (1229 ± 31 ng for 0 - 8 min). However, as shown in Fig. 1 (lower), DMPP-induced CA secretion in the presence of Rg2 (10 μM) for 90 min was greatly reduced to 72% of the control secretion.

McN-A-343 (100 μM), a selective muscarinic $M_1$-receptor agonist,[22] when perfused into an adrenal gland for 4 min, also increased the CA secretion (652 ± 33 ng for 0 - 4 min). However, under the existence of Rg2 (10 μM), McN-A-343-induced CA secretion was markedly reduced to 74% of the corresponding control secretion as shown in Fig. 2 (upper).

Since it has been found that Ang II increases



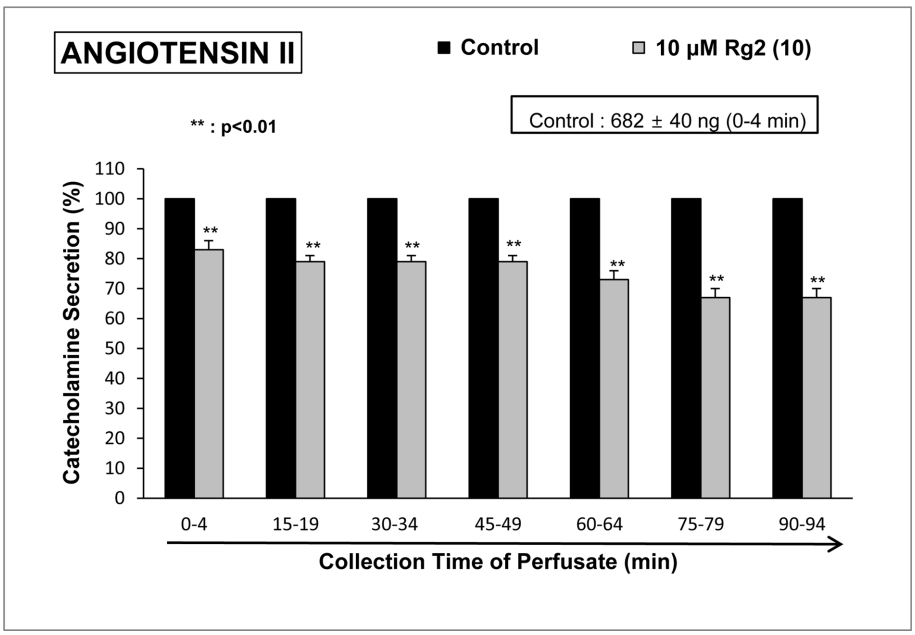




**Fig. 2.** Time-dependent effects of ginsenoside-Rg2 (Rg2) on the secretion of CA produced by McN-A-343 (upper) and Ang II (lower).

epinephrine release from the adrenal medulla via the $AT_1$ receptors,[23] it was attempted to examine the effect of Rg2 on Ang II-induced CA secretion. Ang II (100 nM) greatly elevated the CA secretion (682 ± 40 ng for 0 - 4 min), whereas in the presence of Rg2 (10 μM), Ang II-induced CA secretion was greatly reduced to 67% of the corresponding control secretion (Fig. 2-lower).

Also, high KCl, a direct membrane-depolarizing agent, markedly enhanced the CA secretion (768 ± 32 ng for 0 - 4 min). High $K^+$ (56 mM)-induced CA secretion in the presence of Rg2 (10 μM) for 90 min was maximally reduced to 70% of the control in 75 ~ 94 min periods, as shown in Fig. 3 (upper).

Since Bay-K-8644 is known to be a calcium channel activator, which enhances basal $Ca^{2+}$ uptake[24] and CA release,[25] it was of interest to determine the effects of Rg2 on Bay-K-8644-induced CA secretion. Bay-K-8644 (10 μM)-induced CA release in the presence of Rg2 (10 μM) was decreased to 68% of the control except for the early 15 min period in comparison to the corresponding control secretion (614 ± 26 ng for 0 - 4 min) from 7 rat adrenal medullae, as shown in Fig. 3 (lower).

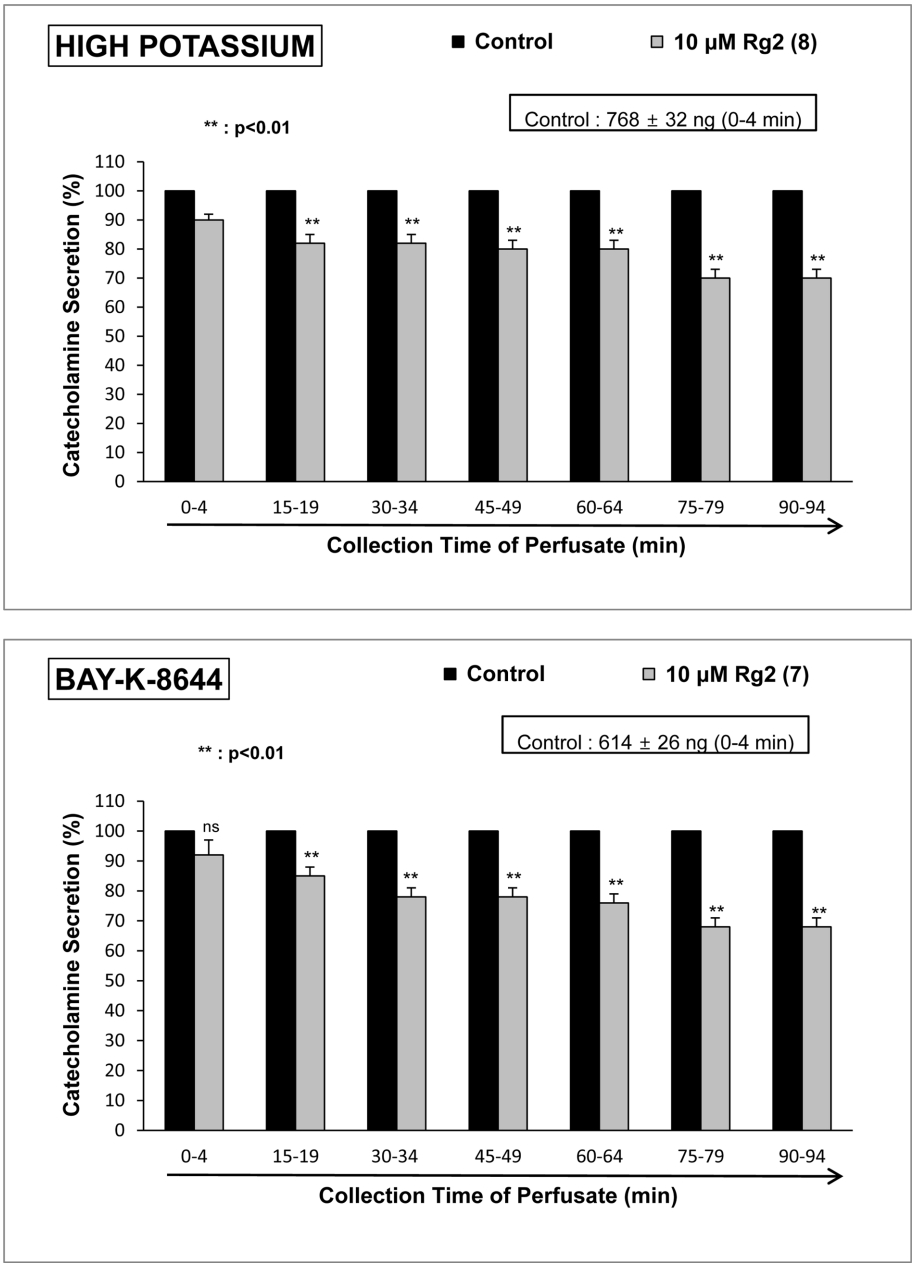



**Fig. 3.** Time-dependent effects of Rg2 on the secretion of CA produced by high K+ (upper) and Bay-K-8644 (lower).

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of $Ca^{2+}$-ATPase in skeletal muscle sarcoplasmic reticulum.[26,27] The inhibitory action of Rg2 on cyclopiazonic acid-induced CA secretion was obtained as shown in Fig. 4 (upper). In 6 rat adrenal glands, under the existence of Rg2 (10 μM) for 90 min, cyclopiazonic acid ($10^{-5}$ M)-induced CA secretion was also suppressed to 71% of the control release (619 ± 24 ng for 0 - 4 min), although it was not influenced only for the first period (0 - 4 min).

It has been found that veratridine-induced $Na^+$ influx mediated through voltage-dependent $Na^+$ channels increased $Ca^{2+}$ influx via activation of voltage-dependent $Ca^{2+}$ channels and produced the exocytotic CA secretion in cultured bovine adrenal medullary cells.[28] As shown in Fig. 4 (lower), veratridine (50 μM) sharply increased the CA release (825 ± 37 ng for 0 - 4 min). In 8 rat adrenal medulla, Rg2 (10 μM) also attenuated veratridine-induced CA secretion to 68% of the corresponding control release.

It has also been found that, in this study, Rg2 markedly

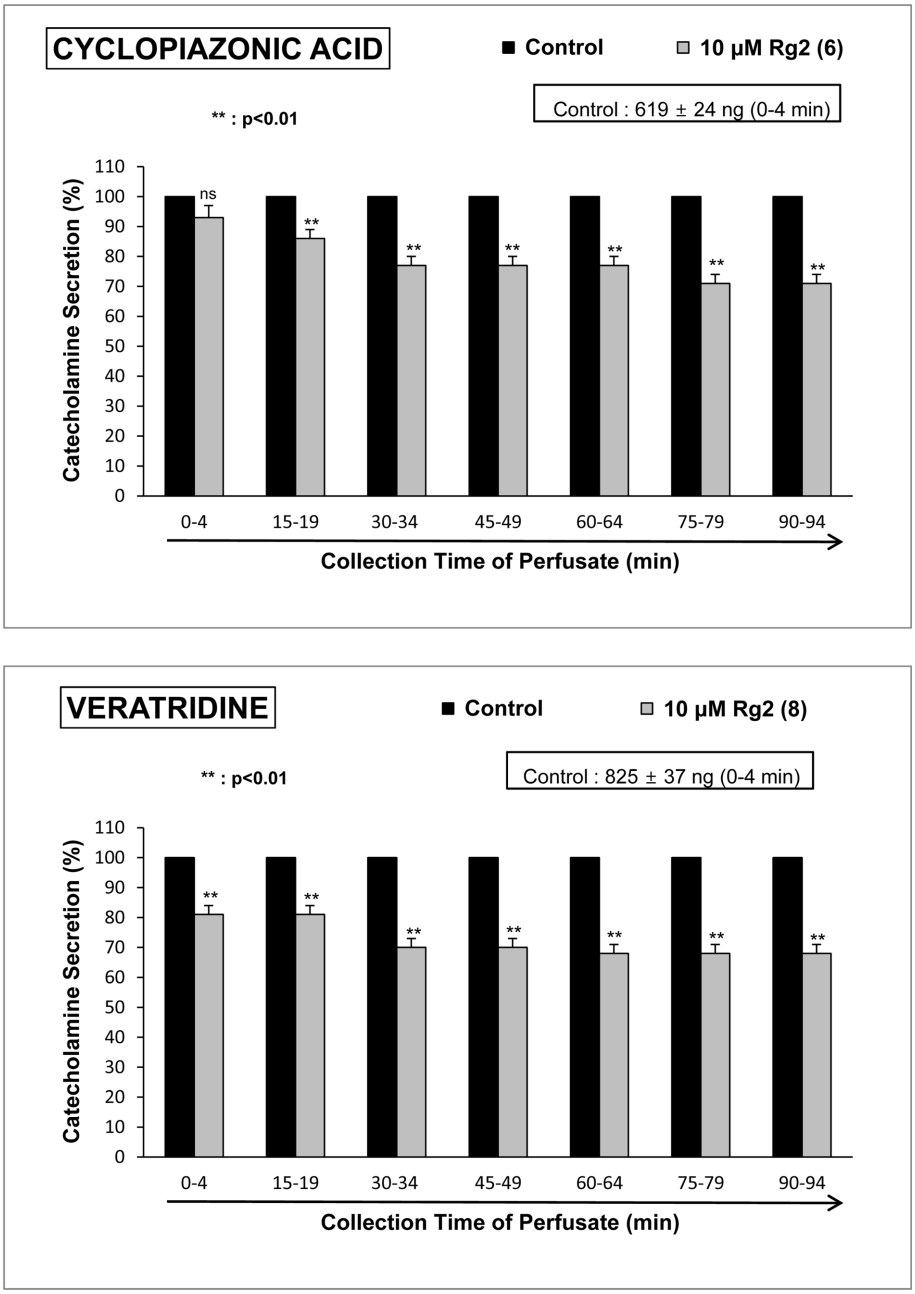



**Fig. 4.** Time-dependent effects of Rg2 on the secretion of CA produced by cyclopiazonic acid (upper) and veratridine (lower).

reduced the CA secretory response induced by stimulation of cholinergic receptors as well as angiotensin II receptors. Therefore, in order to study the relationship between NO and Rg2-induced inhibitory action on the CA secretion from the rat adrenal medullas, the influence of L-NAME (a NO synthase inhibitor) on Rg2-induced inhibitory responses of CA secretion induced by ACh, Ang II, Bay-K-8644, and veratridine was examined.

In the present study, during the coexistence of L-NAME (30 μM) and Rg2 (10 μM) for 90 min, in 8 rat adrenal medullas, ACh (5.32 mM)-induced CA release was mostly recovered to 100 ~ 92% of the corresponding control level (1338 ± 35 ng for 0 - 4 min) compared to that of Rg2 (10 μM)-treated alone, as shown in Fig. 5 (upper).

Moreover, in the coexistence of L-NAME (30 μM) and Rg2 (10 μM) in 7 rat adrenal medullae, the Ang II (100 nM)-induced CA secretory response was restored to 100 ~ 89% of the corresponding control release (645 ± 31 ng for 0 - 4 min), compared to the inhibitory effect of Rg2-treatment alone on Ang II-induced CA secretion, as shown in Fig. 5 (lower).

The simultaneous perfusion of Rg2 (10 μM) and L-

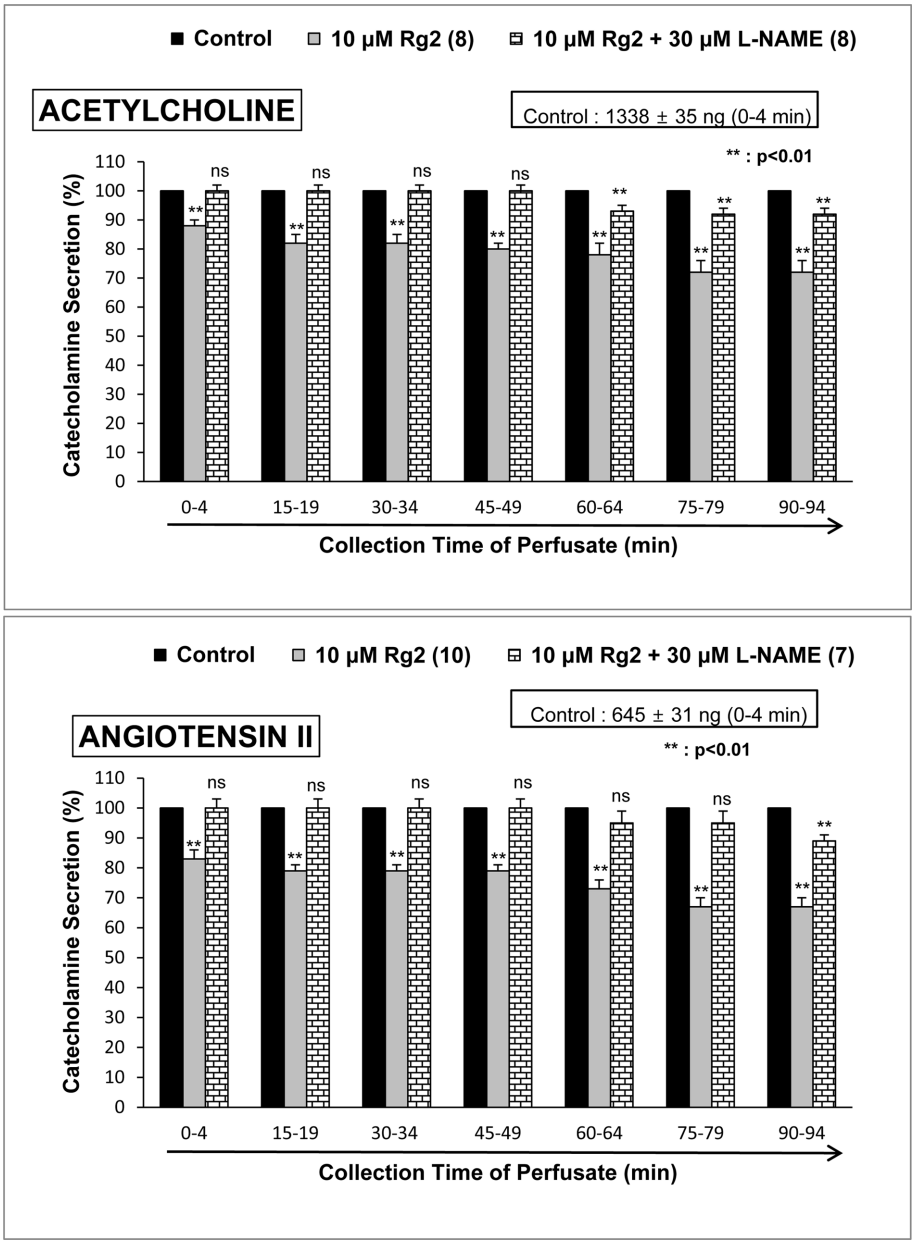



**Fig. 5.** Effects of Rg2 plus L-NAME on the secretion of CA produced by acetylcholine (upper) and angotensin II (lower).

NAME (30 μM) for 90 min restore the CA secretion induced by Bay-K-8644 (10 μM) almost to 100 ~ 88% (Bay-K-8644) of its corresponding control secretory response (627 ± 27 ng/0 - 4 min) in comparison to the inhibitory effect of Rg2-treatment alone, as shown in Fig. 6 (upper).

Under the coexistence of Rg2 and L-NAME, there was also a nearly full recovery (100 ~ 92%) of the control secretion (829 ± 31 ng for 0 - 4 min) in veratridine (100 μM)-induced CA release compared to that of the inhibitory effect of Rg2-treatment alone (Fig. 6-lower).

As shown in Fig. 5 and 6, the CA secretory responses induced by ACh, Ang II, Bay-K-8644 and veratridine were significantly restored to the control level under the coexistence of ginsenoside Rg2 and L-NAME. Thus, it was attempted directly to measure the level of NO released from rat adrenal medulla following the perfusion of Rg2. Moreover, it has been found that ginsenosides reduce blood pressure via increases in production of endothelial nitric oxide[15] and that ginsenoside Rg3 is the

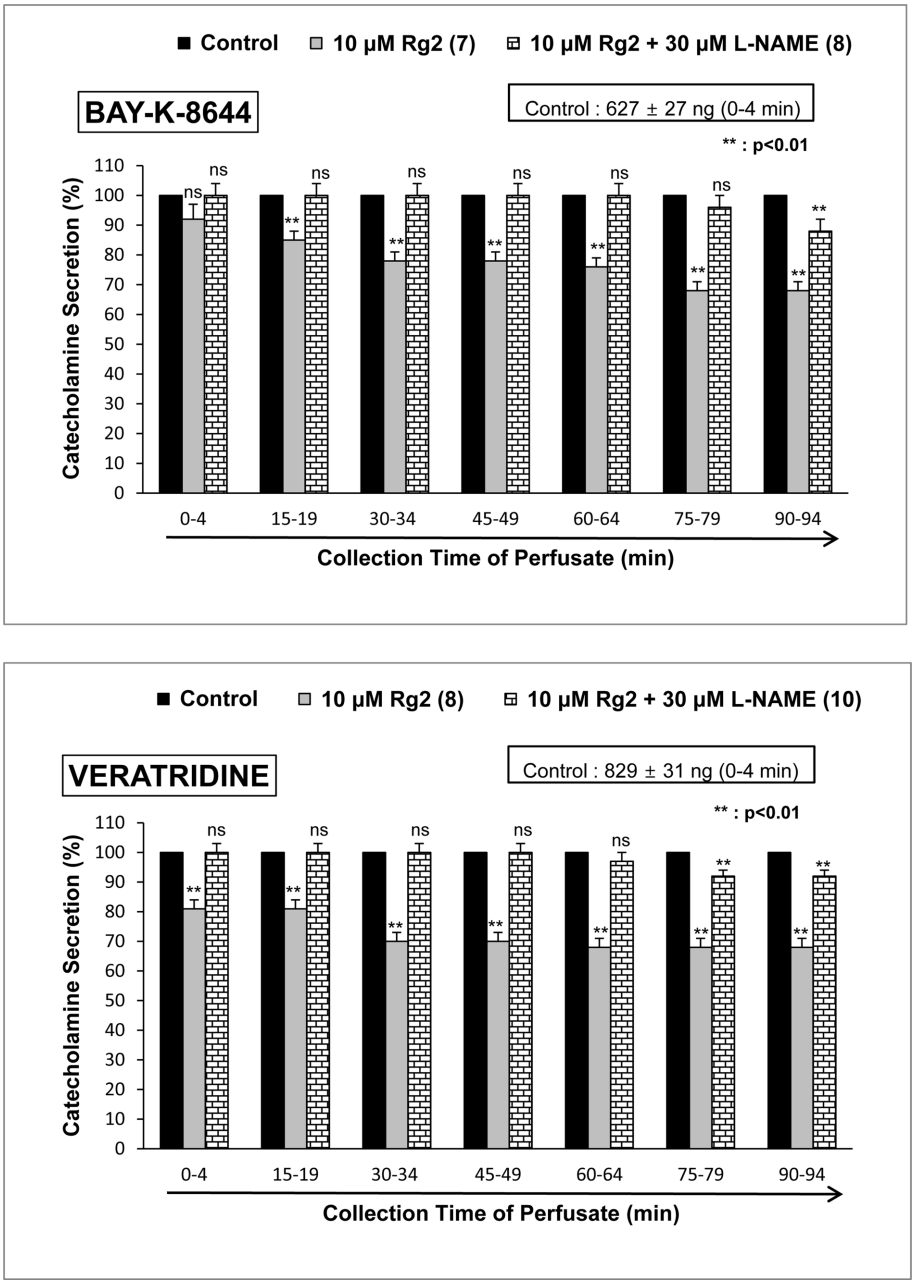



**Fig. 6.** Effects of Rg2 plus L-NAME the secretion of CA produced by Bay-K-8644 (upper) and veratridine (lower).

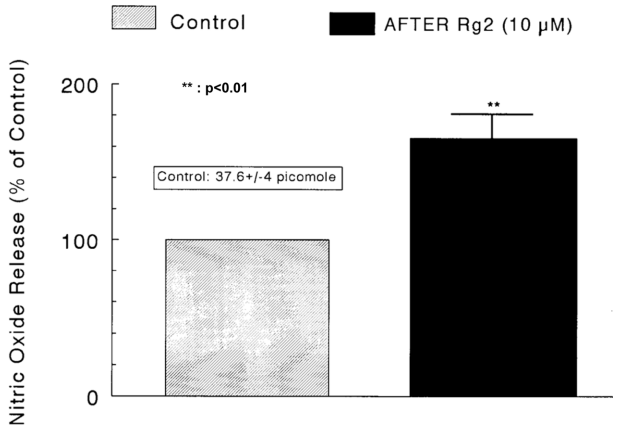



**Fig. 7.** Influence of Rg2 on production of nitric oxide (NO) in the isolated rat adrenal medullae.

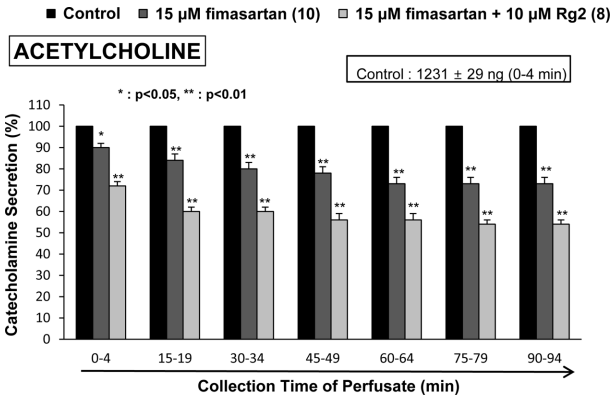



**Fig. 8.** Comparative time-dependent effect of fimasartan and Rg2 plus fimasartan on ACh-produced CA secretion.

most potent ginsenoside, activating eNOS in rat aorta.[16] Although some investigators have demonstrated that Rg3 induces eNOS activation in the vasculature of animal models.[15,17]

In 8 adrenal glands, the basal release of NO in the adrenal medulla prior to perfusion of Rg2 was 37.6 ± 4 picomoles. However, 8 min after loading with Rg2 (10 μM) it was greatly elevated to 62.6 ± 6 picomoles, which was 165% of the basal release, as shown in Fig. 7.

In the present study, ginsenoside-Rg2 or fimasartan (an angiotensin II type 1 ($AT_1$) receptor-selective antagonist[29] caused inhibitory effects on the CA release by stimulation of cholinergic receptors as well as Ang II $AT_1$ receptors. Therefore, in order to characterize the combined effects of ginsenoside-Rg2 and fimasartan on ACh-induced CA release, it was tried to examine inhibitory effects of ginsenoside-Rg2 plus fimasartan on ACh-induced CA secretion.

In the simultaneous presence of ginsenoside-Rg2 (10 μM) and fimasartan (15 μM) for 90 min, ACh (5.32 mM)-induced CA release more strongly was suppressed to ~54% of the corresponding control release (1231 ± 29 ng for 0 - 4 min), compared to the inhibitory effect induced by fimasartan-treatment alone as shown in Fig. 8. Also, there was statistically difference in inhibitory effect between fimasartan versus ginsenoside-Rg2 plus fimasartan on ACh-induced CA secretion, as shown in Fig. 8.

The present experimental results are the first report showing that Rg2 significantly reduces the CA secretory responses induced by activation of cholinergic nicotinic receptors as well as $AT_1$ receptors from the perfused model of the rat adrenal gland. It seems that this Rg2-induced inhibitory effect is mediated by suppressing influx of both $Na^+$ and $Ca^{2+}$ ions through their channels into the adrenal chromaffin cells and also by inhibiting $Ca^{2+}$ release from the cytoplasmic $Ca^{2+}$ pool at least via elevated NO release through activation of neuronal NO synthase, which is associated to the blockade of $AT_1$ receptors and neuronal nicotinic receptors.

In the present work, under the coexistence of Rg2 and L-NAME, the CA secretory responses induced by ACh, Ang II, Bay-K-8644 and veratridine were recovered nearly to the extent of the corresponding control level compared to those of Rg2-treatment alone. This result is well consistent with the reports that, in a series of studies, ginsenoside-Rb2 depresses the CA secretion through increase in nitric oxide production due to activation of nNOS,[5] ginsenosides reduce blood pressure via increased production of endothelial nitric oxide,[15] and that ginsenoside Rg3 is the most potent ginsenoside activating eNOS in rat aorta.[16] Moreover, several investigators have demonstrated that Rg3 induces eNOS activation in the vasculature of animal models,[15,17] Also, in this study, after loading of Rg2 into adrenal medulla, NO production was greatly elevated as shown in Fig. 7. Taking account of these findings, in the present work, it appears that Rg2 suppresses the CA secretory response induced by several secretagogues through elevated NO production in adrenomedullary chromaffin cells.

In the support of this finding, it has been reported that the NOS inhibitor, L-NAME enhances $K^+$-stimulated CA secretion in cultured bovine chromaffin cells[30] and also that sodium nitroprusside (SNP) inhibits ACh-induced CA secretion in bovine chromaffin cells.[31] Results of these studies indicate that NO may play an inhibitory role in the regulation of the CA secretion. Moreover, the presence of endothelial cells has been reported to reduce the $K^+$-induced or the nicotinic receptor agonist DMPP-induced CA secretion in cultured bovine chromaffin

cells,[30] suggesting that not only neuronal nitric oxide synthase (nNOS) but also eNOS may play roles in modulating adrenal CA secretion. In light of previous results reported so far, the present findings strongly indicate that Rg2 can at least activate nNOS in the adrenal chromaffin cells, producing inhibition of the CA release through enhancement of NO, in addition to the direct inhibitory action on the CA release. In supporting of this finding, previously, it has been found that Rg3 inhibits calcium-induced vascular contraction[16] as well as phenylephrine-induced vasocontraction as a consequence of NO production.[13]

In contrast, it has been reported that L-NAME inhibits ACh-induced CA secretion in bovine chromaffin cells,[35] and also that the NO donor SNP enhances nicotine-induced CA secretion in cultured bovine chromaffin cells.[32] These findings indicate that NO may enhance cholinergic agonist-induced CA secretion. On the other hand, a few in vivo studies have suggested that NO does not play a role in regulation of adrenal CA secretion.[33,34]

In the present study, Rg2 also time-dependently suppressed the CA secretory response induced by Bay-K-8644, which is known to activate L-type voltage-dependent $Ca^{2+}$ channels,[24,36] as well as by high $K^+$. This finding indicates that Rg2 may reduce $Ca^{2+}$ influx through voltage-sensitive $Ca^{2+}$ channels to the rat adrenal medullary cells. In support of this idea, in cultured bovine adrenal medullary cells, nicotinic (but not muscarinic) receptors mediate the $Ca^{2+}$-dependent CA secretion.[37,38] The stimulation of nicotinic receptors is known to be facilitate the CA secretion by increasing $Ca^{2+}$ entry through receptor-linked and/or voltage-dependent $Ca^{2+}$ channels in both perfused rat adrenal glands[35,39] and isolated bovine adrenal chromaffin cells.[39-42] It has been reported that the adrenomedullary chromaffin cells have (i) nicotinic receptor-operated ionic channels, responsible for carbachol-induced $Na^+$ influx, (ii) voltage-dependent $Na^+$ channels, responsible for veratridine-induced $Na^+$ influx and (iii) voltage-dependent $Ca^{2+}$ channels (VDCC), suggesting that the influx of $Na^+$ caused either by carbachol or by veratridine leads to activate voltage-dependent $Ca^{2+}$ channels by altering membrane potentials, whereas high $K^+$ directly activates voltage-dependent $Ca^{2+}$ channels without increasing $Na^+$ influx.[43] In the present study, the fact that the CA secretory responses induced by high $K^+$ as well as by Bay-k-8644 were greatly inhibited in the presence of Rg2 indicates that Rg2-induecd inhibitory effect is exerted by the direct inhibition of $Ca^{2+}$ influx through VDCC into the adrenal chromaffin cells. Furthermore, slight elevation in the extracellular $K^+$ concentration increases both the frequency of spontaneous action potentials and the CA secretion,[44] suggesting that the influx of $Ca^{2+}$ that occurs during action potentials is directly linked to the rate of secretion. These findings that Rg2 inhibited the CA secretion induced by Bay-K-8644 as well as by high $K^+$ suggest that Rg2 can inhibit directly the VDCC. In the bovine chromaffin cells, stimulation of nicotinic, but not muscarinic ACh receptors is known to cause CA secretion by increasing $Ca^{2+}$ influx largely through VDCC.[45] Therefore, it seems that these inhibitory effects of Rg2 on the CA secretion induced by ACh, DMPP, veratridine and Bay-K-8644 may be exerted by suppressing $Ca^{2+}$ influx through voltage-sensitive $Ca^{2+}$ channels by stimulation of nicotinic receptor-operative ionic channels, responsible for carbachol-evoked $Na^+$ influx, as well as of voltage-sensitive $Na^+$ channels, responsible for veratridine-evoked $Na^+$ influx.

In the present study, it seems that Rg2-induced inhibitory effect on cyclopiazonic acid-induced CA secretion may also be relevant to the movement of intracellular $Ca^{2+}$ from the cytoplasmic $Ca^{2+}$ pool. This is consistent with the findings obtained in skinned smooth muscle fibers of the longitudinal layer of the guinea-pig ileum, where $Ca^{2+}$-uptake was also inhibited by cyclopiazonic acid.[46] Moreover, in bovine adrenal chromaffin cells, stimulation of muscarinic ACh receptors is also proposed to cause activation of phosphoinositide (PI) metabolism, resulting in the formation of inositol 1,4,5-trisphosphate, which induces the mobilization of $Ca^{2+}$ from the intracellular pools.[47,48] Therefore, in the present work, it can be conjectured that Rg2-induced inhibitory effect on the CA secretion of McN-A-343 may be connected to the movement of intracellular $Ca^{2+}$ from the cytoplasmic $Ca^{2+}$ pool. This indicates that this Rg2 has an inhibitory action on the $Ca^{2+}$ release from the intracellular store induced by activation of muscarinic ACh receptors, which is slightly responsible for the CA release. The present results suggest that Rg2-induced inhibition of the CA secretion induced by cyclopiazonic acid and McN-A-343 may be mediated by the reduction of $Ca^{2+}$ release induced by activation of muscarinic ACh receptors from the intracellular pools. However, in the present study, it is uncertain whether Rg2-induced inhibitory effect on $Ca^{2+}$ mobilization from intracellular store is attributed to the indirect effects on the PI response or its direct effect. In future studies, it is necessary to establish the clear nature of these results.

In the present work, when both Rg2 and fimasartan were perfused in combination, their inhibitory effect on ACh-induced CA release was enhanced. In support of this

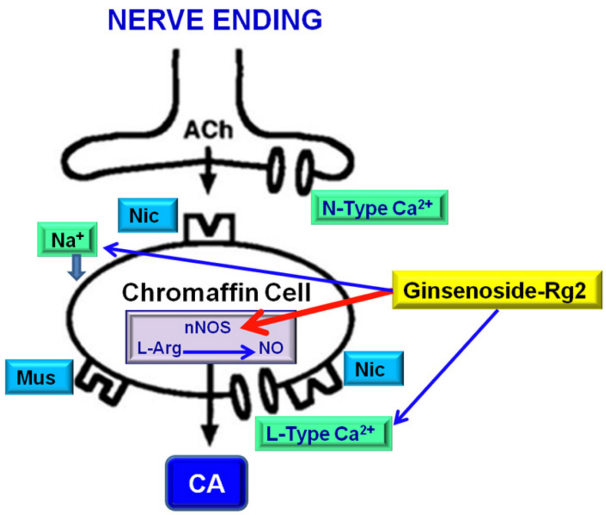



**Fig. 9.** Potential action site of Rg2 at splanchnic AChergic nerve-adrenomedullary synapse of the rat.

idea, losartan (an $AT_1$ antagonist)-enalapril (an angiotensin-converting enzyme inhibitor) combination are more effective in decreasing blood pressure and increasing plasma active renin than doubling of the enalapril dose in normotensive male volunteers.[49] Similarly, based on the present findings, the clinically combined use of both Rg2 and fimasartan may dedicate significantly to the alleviation of cardiovascular diseases such as angina pectoris, heart failure and hypertension.

In conclusion, as shown in Figure 9, the results of the present study have demonstrated that Rg2 reduces the CA release by activation of cholinergic nicotinic receptors as well as $AT_1$ receptors in the perfused model of the isolated rat adrenal glands. It seems that this Rg2-induced inhibitory effect is mediated by blocking influx of $Na^+$ and $Ca^{2+}$ through their ionic channels into the adrenal chromaffin cells as well as by reducing the $Ca^{2+}$ release from the cytoplasmic $Ca^{2+}$ pool partly via the elevated NO release due to the activation of NO synthase. Based on these results, the ingestion of Rg2 can be helpful to alleviate or prevent the cardiovascular diseases, via reduction of CA release in adrenal medullary cells and consequent decreased CA level in the circulation. The combined use of both Rg2 and fimasartan may contribute clinically to the alleviation of cardiovascular diseases.

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## Conflicts of interest

All contributing authors declare no conflicts of interest.

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