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Inhibitory Effects of Ginsenoside-Rg2 on Catecholamine Secretion from the Perfused Rat Adrenal Medulla

조선대학교 대학원

의학과

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진세노사이드-Rg2 의 억제작용

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하 강 수

지도교수 : 김 상 훈

조선대학교 대학원 의학과

최근 임등(2014)은 흰쥐 적출 관류부신수질에서 panaxadiol계 사포닌 Ginsenoside-Rb2가 카테콜아민(CA) 분비를 억제한다고 발표하였으며, 더욱이, 총인삼사포닌은 흰쥐 (Hong등, 1999) 및 자연발증고혈압쥐(SHRs) (Jang등, 2011)의 적출관류부신에서 역시 카테콜아민 분비를 억제한다고 하였다 그러나 이전의 연구에서 panaxadiol계 사포닌 (Lim등, 1988)이 토끼 적출 관류부신에서, 그리고 최근에 고려인삼에서 분리한 Gintonin (Na등, 2016)이 흰쥐 관류 부신수질에서 카테콜아민 분비를 증가시킨다고 보고하였다. 따라서 본 연구의 목적은 고려홍삼에서 분리한 panaxatriol계 사포닌의 주요성분인 Ginsenoside-Rg2가 흰쥐에서 분리 적출한 부신의 관류모델에서 CA유리작용에 미치는 영향을 검색하고, 그 작용기전을 규명코자 본 연구를 수행하여 얻어진 결과는 다음과 같다.

Ginsenoside-Rg2 (3~30 μM)를 부신정맥 내로 90분간 관류시 아세틸콜린 (5.32 mM, ACh) 에 의한 CA 분비작용을 비교적 용량 및 시간 의존적으로 감소시켰다. 또한 Ginsenoside-Rg2 (10 μM)존재하에서 DMPP (선택성 니코틴수용체 작용제), McN-A-343 (100 μM, 선택성 무스카린 M_1수용체 작용제) 및 angiotesin II (Ang II, 100 ηM) 에 의한 CA 분비반응이 시간 의존적으로 억제되었다. 그러나, Ginsenoside-Rg2 자체는 기초

CA 유리효과에 영향을 미치지 못하였다. 또한, Ginsenoside-Rg2 (10 μM)존재 하에서, 직접 막탈분극제인 고칼륨 (56 mM), 선택성 전압의존적 나트륨통로 활성화제, veratridine (50 μM), 전압의존적 L형 dihyropyridine계 칼슘통로 활성화제, Bay-K-8644 (10 μM) 및 세포질 내 내형질세망막에서 Ca^{2+}-ATPase 억제제, cyclopiazonic acid (10 μM)에 의한 CA 분비반응이 역시 시간 의존적으로 감소하였다. 흥미롭게도, Ginsenoside-Rg2 (10 μM)와 L-NAME (30 μM, NO Synthase 억제제)를 90분간 동시 처치하는 동안 ACh, DMPP, McN-A-343, Ang II, high K^+, Bay-K-8644, veratridine 및 cyclopiazonic acid에 의한 CA 분비효과가 Ginsenoside-Rg2 (10 μM)단독처치 시의 억제효과와 비교하여 거의 상응하는 대조치의 수준까지 회복되었다. 또한 실제로 NO 유리량이 Ginsenoside-Rg2 (10 μM) 관류 후에 기초 유리량에 비교하여 뚜렷이 상승하였다. 또한, Ginsenoside-Rg2 (10 μM)과 fimasartan (15 μM, Ang II AT_1 수용체차단제)을 동시 처치하였을 때 ACh의 CA 분비에 대한 억제작용이 fimasartan (15 μM) 단독처치 시의 억제효과에 비교하여 현저히 증강되었다.

이와 같은 연구결과를 종합하여 보면, Ginsenoside-Rg2는 흰쥐 적출 부신수질의 관류모델에서 콜린수용체(니코틴 및 무스카린 수용체) 및 안지오텐신 AT_1 수용체 활성화에 의한 CA 분비작용에 대하여 뚜렷한 억제작용을 나타내었다. 이러한 Ginsenoside-Rg2의 억제작용은 흰쥐 부신에서 NO Synthase의 활성화에 의한 NO 생산상승으로 인하여 부신수질크롬친화세포 내로 나트륨 및 칼슘통로를 통한 이들의 유입억제와 세포 내 칼슘저장고로부터 칼슘유리의 억제작용에 기인되며, 이는 니코틴수용체 및 안지오텐신 AT_1 수용체 차단과 관련이 있는 것으로 생각된다. 또한 Ginsenoside-Rg2와 fimasartan병용투여 시 CA분비억제작용이 증강되는 점은 고혈압 및 협심증과 같은 심혈관계 질환 치료에 임상적으로 유용할 것으로 사료된다.

I. INTRODUCTION

Recently, it has been reported that gintonin, known as a novel G protein-coupled LPA receptor ligand isolated from Korean ginseng, greatly enhanced the secretion of catecholamines (CA) from the isolated perfused rat adrenal medulla (Na et al., 2016). This facilitatory effect of gintonin seems to be relevant to activation of lysophosphatidic acid (LPA) receptors and cholinergic receptors, which are relevant to the cytoplasmic Ca^{2+} increase by stimulation of the Ca^{2+} influx as well as by the inhibition of Ca^{2+} uptake into the cytoplasmic Ca^{2+} stores, without the increased nitric oxide (Na et al., 2016). Moreover, in previous studies, all of total ginseng saponin (TGS, Lim et al., 1987), panaxadiol (Lim et al., 1988) and panaxatriol (Lim et al., 1989) 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 (Lim et al., 2014). Also, TGS has been reported rather to inhibit the CA secretion from the isolated perfused rat adrenal glands (Hong et al., 1999) and spontaneously hypertensive rats (Jang et al, 2011). Several investigators also showed that ginsenoside components isolated from *Panax ginseng* reduce the ACh-induced CA secretion from bovine adrenal chromaffin cells (Kudo et al., 1992; Tachikawa et al., 1995; Kudo et al., 1998; Tachikawa et al., 2001).

In previous study, Tachikawa and his co-workers (1995) 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 (2000a) 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 (Kim et al., 2003). Han

and his co-workers (2005) 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 (Kim et al., 1994) and that Rg3 is the most potent ginsenoside that activates eNOS in rat aorta (Kim et al., 1999). Although some investigators have demonstrated that Rg3 induces eNOS activation in the vasculature of animal models (Kim et al., 1994; Kang et al., 1995), Hien and his co-workers (2010) have found that Rg3 activates eNOS via eNOS phosphorylation in ECV 304 human endothelial cells, and elevates eNOS expression.

Several studies have demonstrated that ginsenoside Rg1 can induce endothelial-dependent relaxation in the rat aorta (Kang et al., 1995) and cause the increased endogenous NO production in human umbilical vein endothelial cells (Leung et al., 2006), rat kidney (Han and Kim, 1996) and in porcine coronary arteries (Chai et al., 2005).

Despite of these many studies on various ginseng saponins, there are still a little known effects 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.

Ⅱ. MATERIALS AND METHODS

Experimental procedure

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 (Cheil 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.

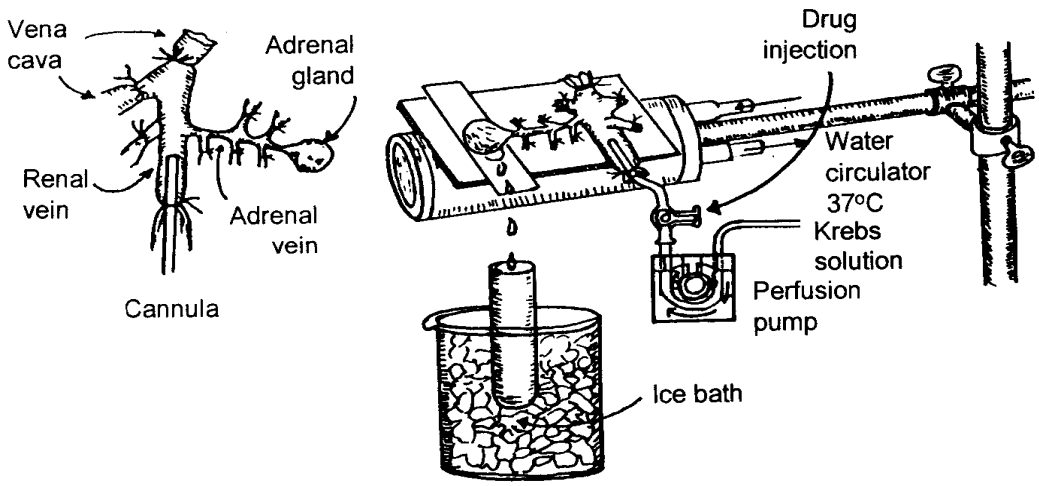

Fig. 1. Schematic drawing of the preparation used to study the CA secretion in the isolated perfused rat adrenal gland.

Isolation of adrenal glands: The adrenal gland was isolated by some modification of previous method (Wakade, 1981). 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℃ (Fig. 1).

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 (1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) 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 meulla 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 fimarsartan into rat adrenal medulla, as previously described (McVeigh et al., 2002). 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 fimarsartan 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* and ANOVA 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 (1987).

Drugs and their sources

The following drugs were used: ginsenoside-Rg2 (a gift from the Society of Korean Ginseng, Seoul, Korea), fimasartan (a gift 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^{ω}-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.

III. RESULTS

Effects of Ginsenoside-Rg2 on the CA secretion induced by ACh, DMPP, McN-A-343 and angiotensin II from the perfused rat adrenal medulla

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 (Tachikawa et al., 1995), 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 in the perfused rat adrenal medulla (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 CA release was significantly reduced in relatively concentration- and time-dependent manner. As shown in Fig. 2, 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. 3, DMPP-induced CA secretion in the presence of Rg2 (10 μM) for 90 min was greatly reduced to 72% of the control secretion.

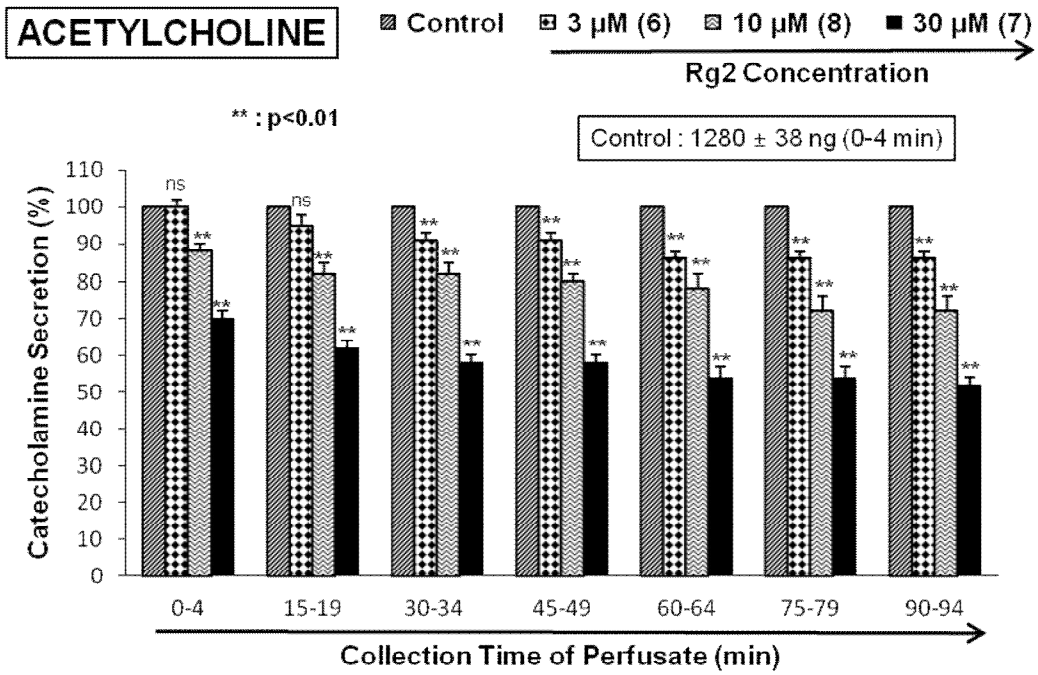

Fig. 2. Dose-dependent effects of ginsenoside-Rg2 (Rg2) on the secretory responses of catecholamines (CA) induced by acetylcholine (ACh) in the perfused rat adrenal medulla. The CA secretion by a single injection of ACh (5.32 mM) in a volume of 0.05 mL was induced at 15 min intervals during loading with 3, 10 and 30 μM of Rg2 for 90 min as indicated by the arrow marks, respectively. The numbers in parentheses express the number of rat adrenal medulla. Vertical bars on the columns indicate the standard error of the mean (S.E.M.). Ordinate: the amounts of CA released from the adrenal medulla (% of control). Abscissa: collection time of perfusate (min). Statistical difference was calculated by comparing the corresponding control with each concentration-treated group of Rg2. ACh-induced perfusate was collected for 4 minutes. **: $p<0.01$. ns: Not statistically significant.

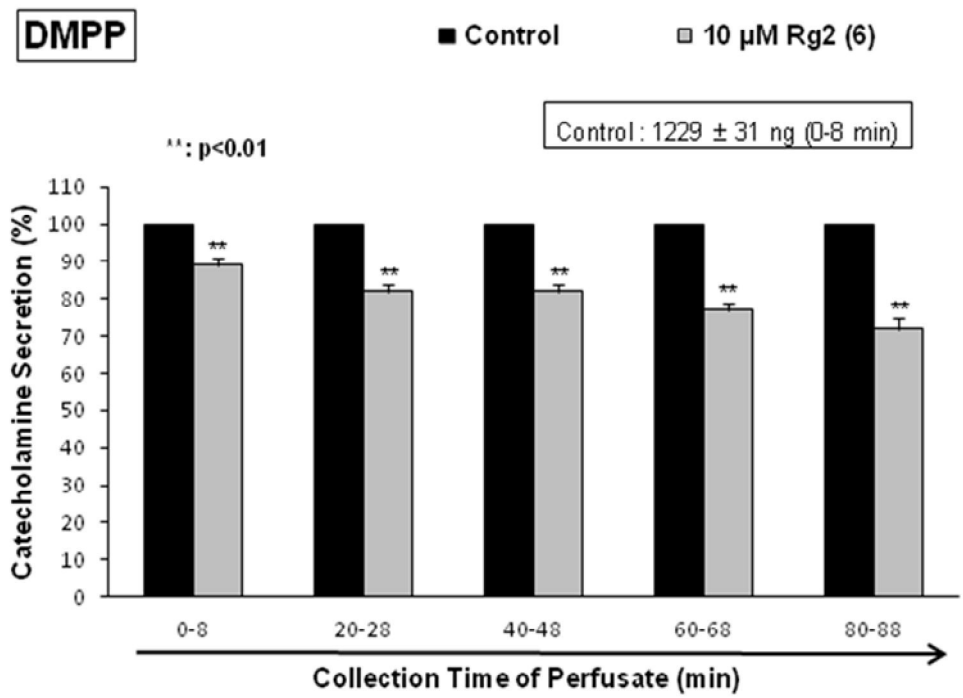

Fig. 3. Time-course effects of ginsenoside-Rg2 on DMPP-induced CA secretion in the perfused rat adrenal medulla. The CA secretion by perfusion of DMPP (100 μM) for 2 min was induced at 20 min interval during loading with 10 μM Rg2 for 90 min. DMPP-induced perfusate was collected for 8 minutes. Other legends are the same as in Fig. 2. **: p<0.01.

McN-A-343 (100 μM), a selective muscarinic M_1-receptor agonist (Hammer and Giachetti, 1982), 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. 4.

Since it has been found that Ang II increases epinephrine release from the adrenal medulla via the AT_1 receptors (Hano et al., 1994), 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. 5).

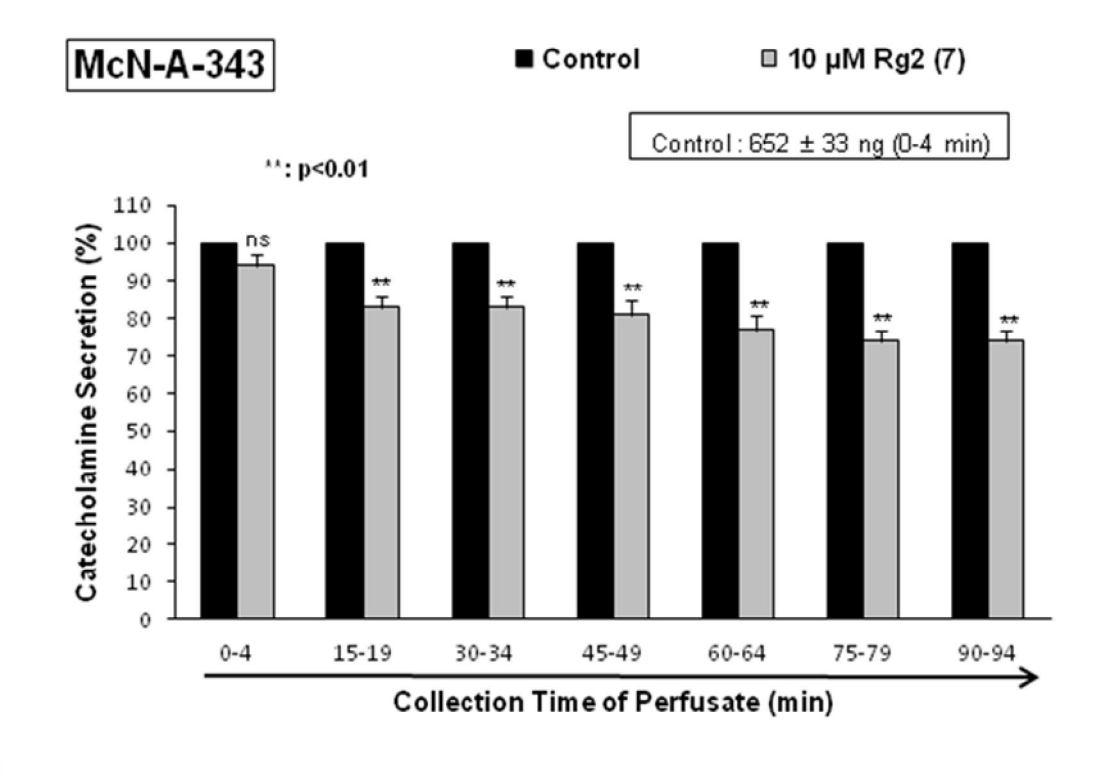

Fig. 4. Time-course effects of ginsenoside-Rg2 on McN-A-343-induced CA release in the perfused rat adrenal gland. The CA secretion by perfusion of McN-A-343 (100 μM) for 4 min was induced at 15 min interval during loading with 10 μM of Rg2 for 90 min. McN-A-343-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: p<0.01. ns: Not statistically significant.

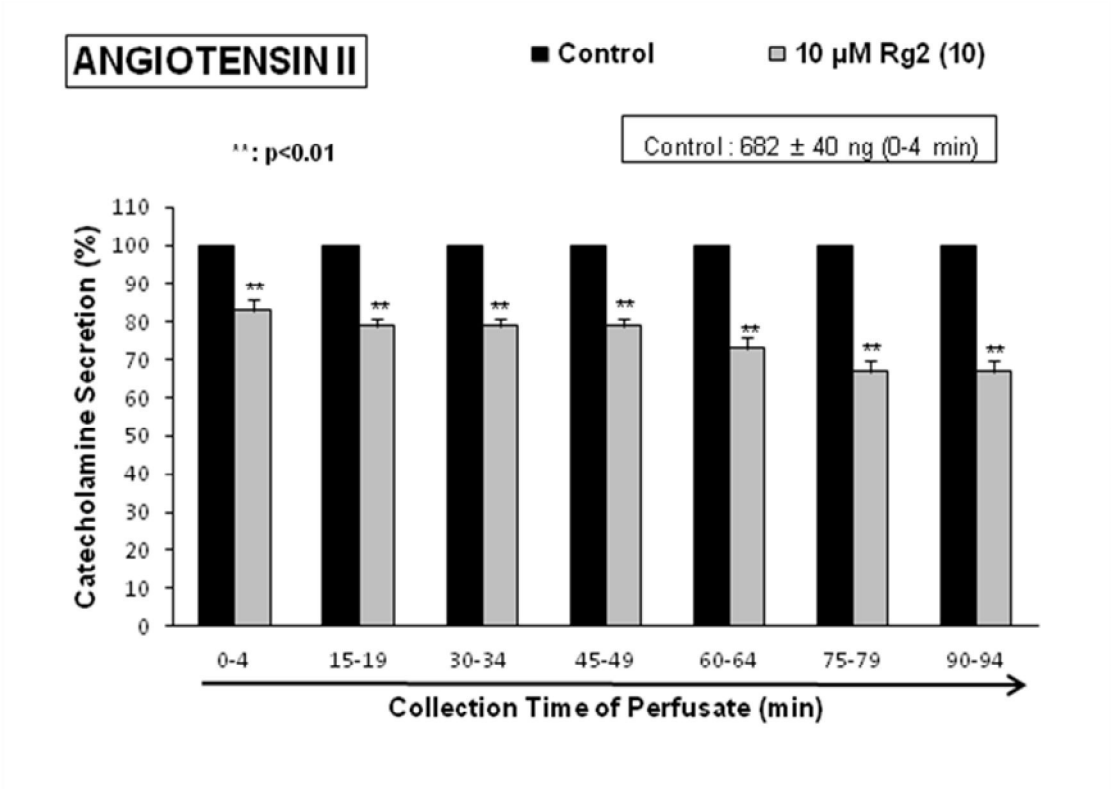

Fig. 5. Time-course effects of ginsenoside-Rg2 on angiotensin II-induced CA secretion in the perfused rat adrenal medulla. Angiotensin II (100 nM) was perfused into an adrenal vein for 1 min at 15 min intervals during loading with Rg2 (10 μM) for 90 min. Angiotensin II-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: p<0.01.

Effects of Ginsenoside-Rg2 on the CA secretion induced by high K^+, Bay-K-8644, cyclopiazonic acid and veratridine from the perfused rat adrenal medulla

Also, high KCl, a 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. 6.

Since Bay-K-8644 is known to be a calcium channel activator, which enhances basal Ca^{2+} uptake (Garcia et al., 1984) and CA release (Lim et al., 1992), it was of interest to determine the effects of Rg2 on Bay-K-8644-induced CA secretion from the perfused rat adrenal glands. 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 medullas, as shown in Fig. 7.

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 (Goeger and Riley, 1989; Seidler et al., 1989). The inhibitory action of Rg2 on cyclopiazonic acid-induced CA secretion was obtained as shown in Fig. 8. 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 (Wada et al., 1985a). As shown in Fig. 9, 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.

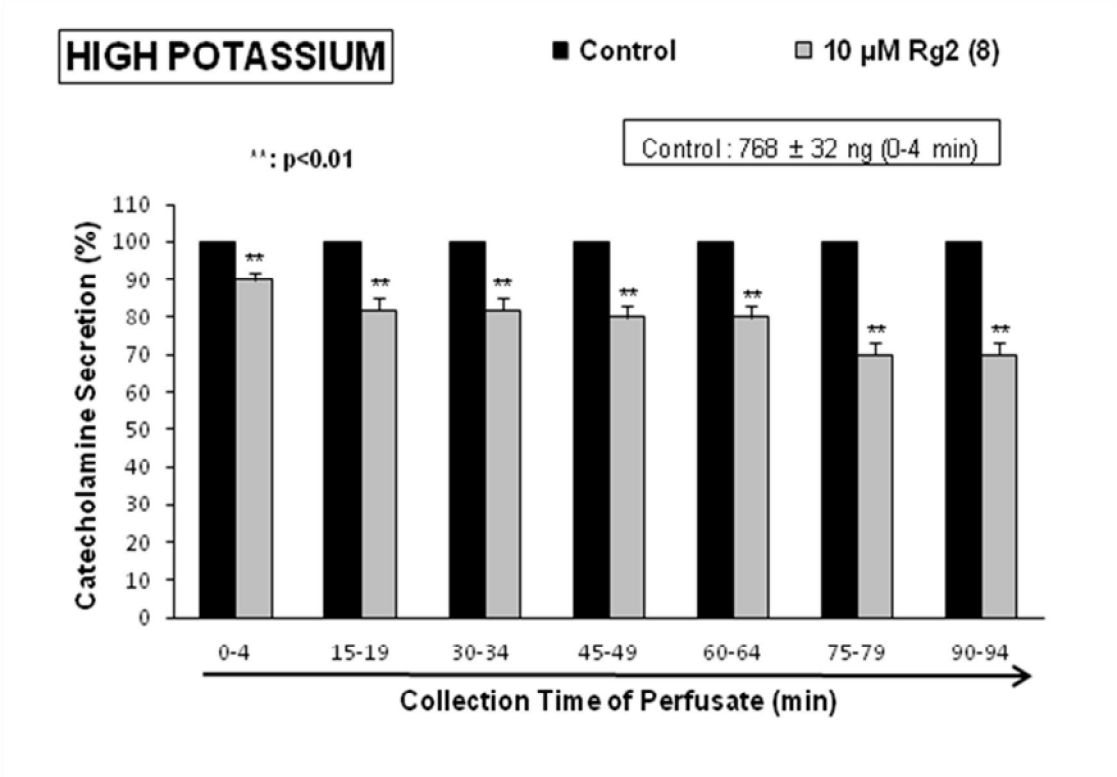

Fig. 6. Time-course effects of ginsenoside-Rg2 on the high K^+-induced CA secretion in the perfused rat adrenal medulla. The CA secretion by a single injection of K^+ (56 mM) in a volume of 0.05 mL was induced at 15 min intervals during loading with 10 μM Rg2 for 90 min as indicated by the arrow marks. High K^+-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$.

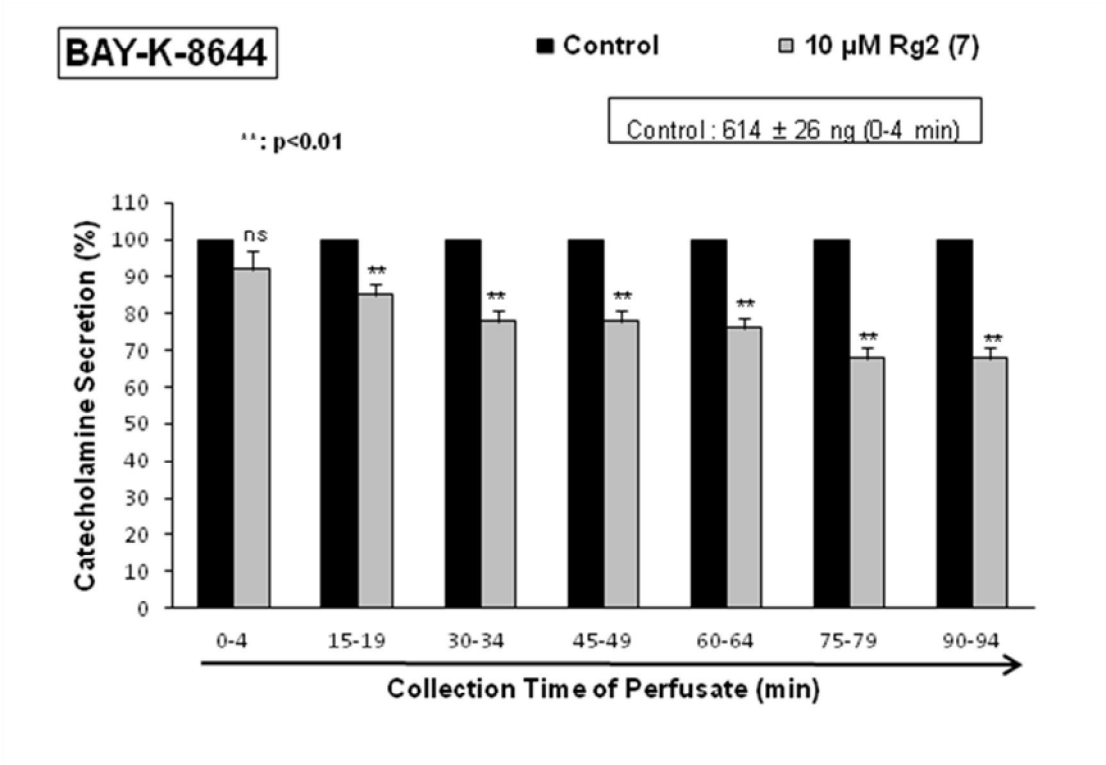

Fig. 7. Time-course effects of ginsenoside-Rg2 on Bay-K-8644-induced CA secretion in the perfused rat adrenal medulla. Bay-K-8644 (10 μM) was given into an adrenal vein for 4 min at 15 min intervals during loading with Rg2 (10 μM) for 90 min. Bay-K-8644-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

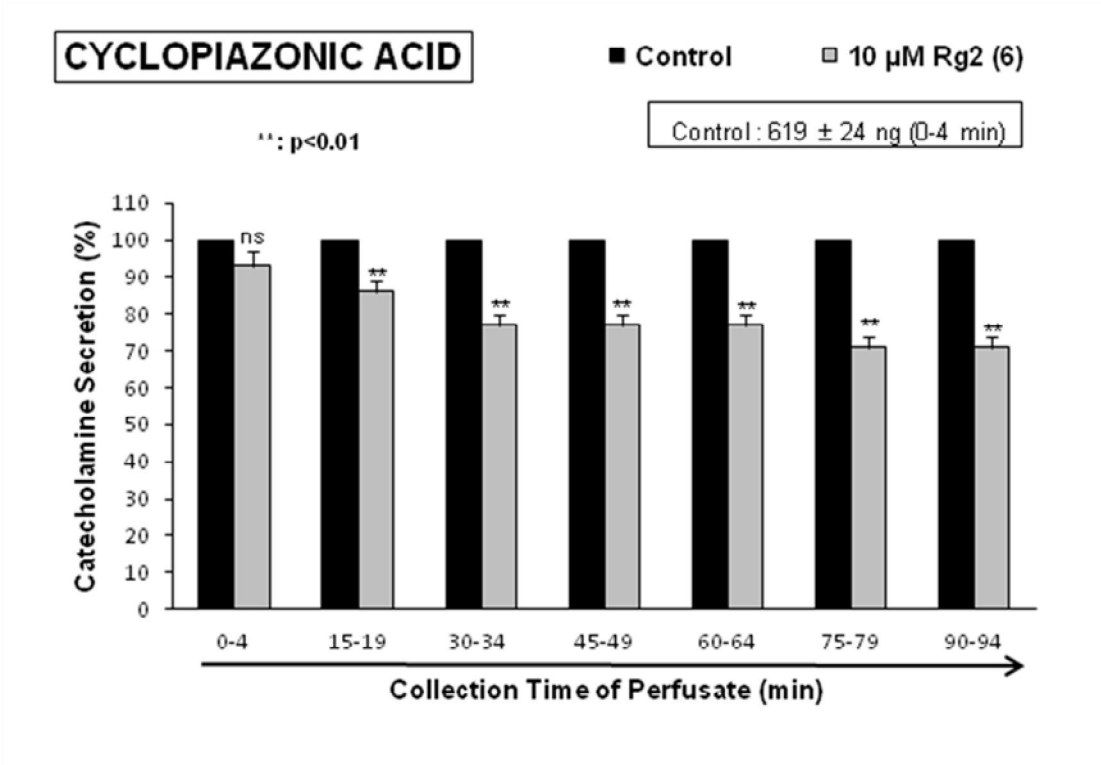

Fig. 8. Time-course effects of ginsenoside-Rg2 on cyclopiazonic acid-induced CA secretion in the perfused rat adrenal medulla. Cyclopiazonic acid (10 μM) was given into an adrenal vein for 4 min at 15 min intervals during loading with Rg2 (10 μM) for 90 min. Cyclopiazonic acid-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

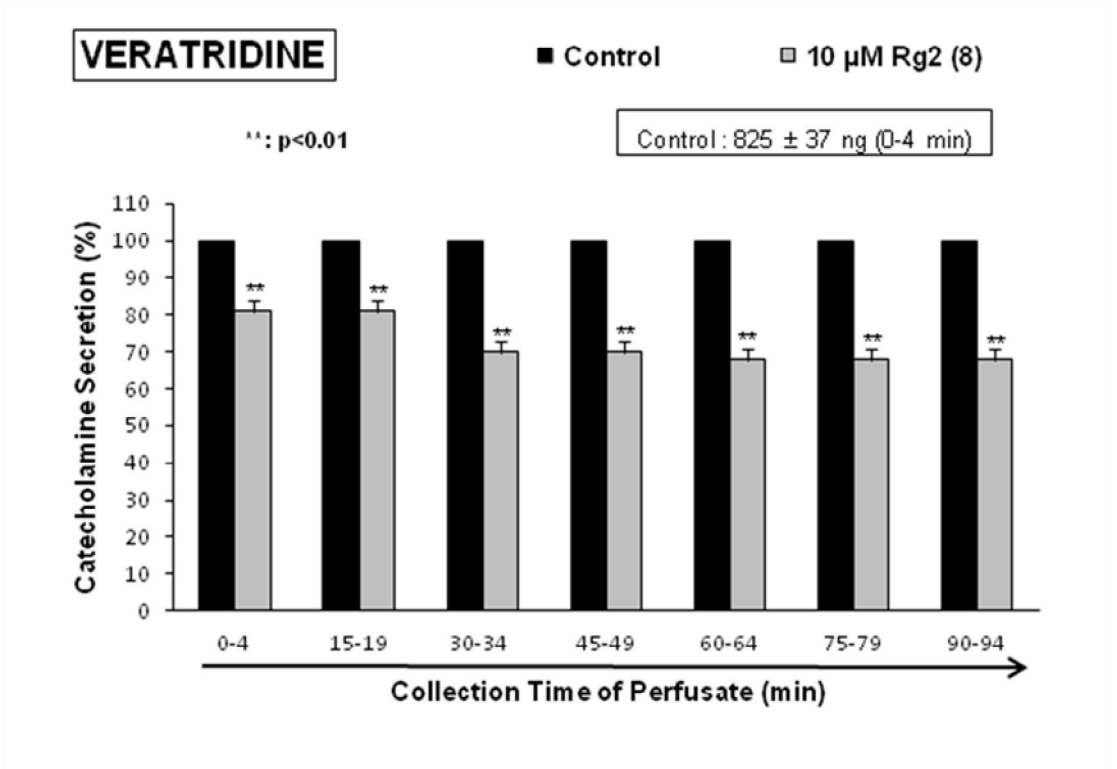

Fig. 9. Time-course effects of ginsenoside-Rg2 on veratridine-induced CA secretion in the perfused rat adrenal medulla. Veratridine (50 μM) was given into an adrenal vein for 4 min at 15 min intervals during loading with Rg2 (10 μM) for 90 min. Veratridine-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$.

Influence of Ginsenoside-Rg2 plus L-NAME on CA secretion induced by ACh, DMPP, McN-A-343, Angiotensin II, High K^+, Bay-K-8644, Cyclopiazonic acid and Veratridine from the perfused rat adrenal medulla

It has also been found that, in this study, Rg2 markedly reduced the CA secretory response induced by stimulation of cholinergic receptors as well as angiotensin II receptors in the perfused rat adrenal medulla. 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 on Rg2-induced inhibitory responses of CA secretion induced by ACh, DMPP, McN-A-343, Ang II, high K^+, Bay-K-8644, cyclopiazonic acid 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. 10.

Also, the simultaneous perfusion of L-NAME (30 μM) and Rg2 (10 μM) for 90 min made the recovery of the DMPP- and McN-A-343-induced CA secretory responses mostly to their control levels (100~92%) compared to that of the Rg2-treatment alone (Fig. 11 and 12).

Moreover, in the coexistence of L-NAME (30 μM) and Rg2 (10 μM) in 7 rat adrenal medullas, 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. 13.

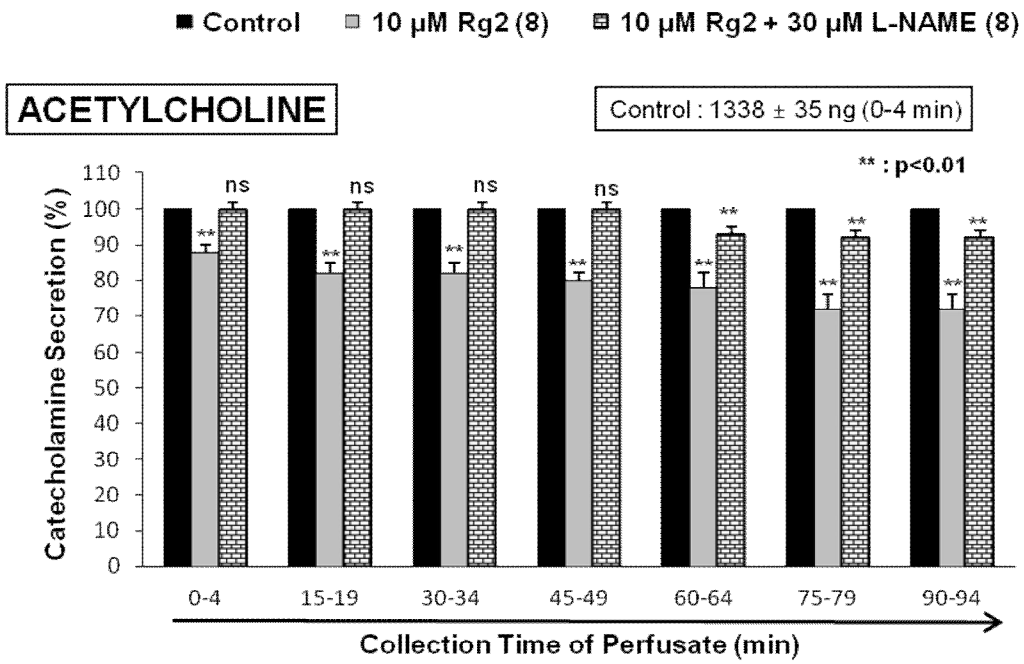

Fig. 10. Effects of ginsenoside-Rg2 plus L-NAME on acetylcholine-induced CA secretion in the perfused rat adrenal medulla. The CA secretion by a single injection of ACh (5.32 mM) in a volume of 0.05 mL was induced at 15 min intervals during simultaneous loading with Rg2 (10 μM) plus L-NAME (30μM) for 90 min. Statistical difference was calculated by comparing the corresponding control (CONTROL) with only Rb2-treated group or group treated with Rg2+L-NAME. Acetylcholine-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

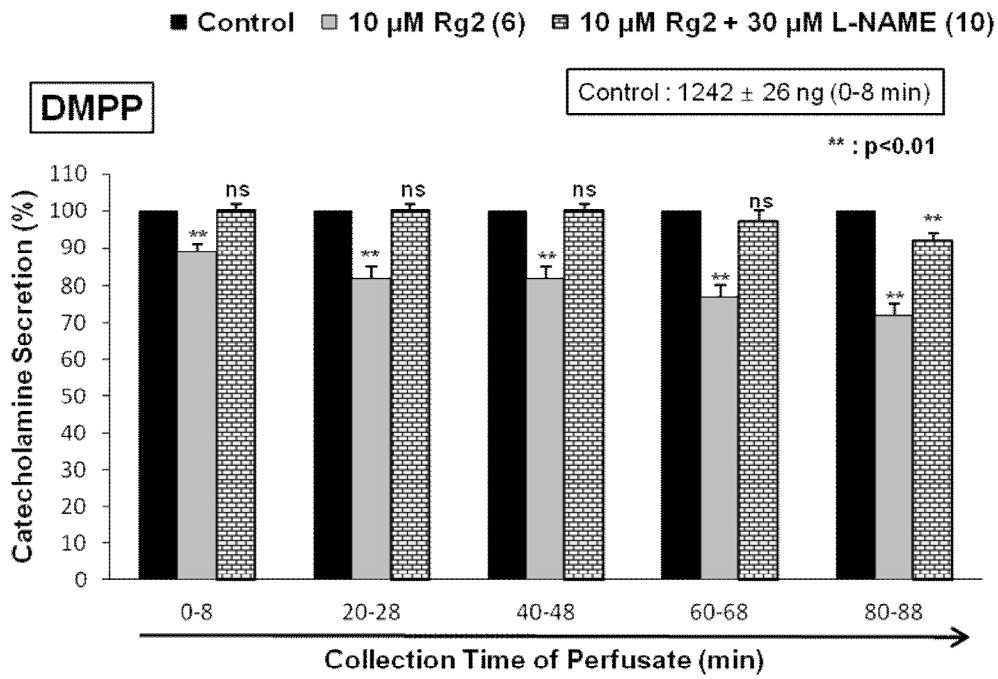

Fig. 11. Effects of ginsenoside-Rg2 plus L-NAME on DMPP-induced CA secretion in the perfused rat adrenal medulla. The CA secretion by perfusion of DMPP (100 μM) for 2 min was induced at 20 min intervals during simultaneous loading with Rg2 (10 μM) plus L-NAME (30μM) for 90 min. DMPP-induced perfusate was collected for 8 minutes. Other legends are the same as in Fig. 2. **: p<0.01. ns: Not statistically significant.

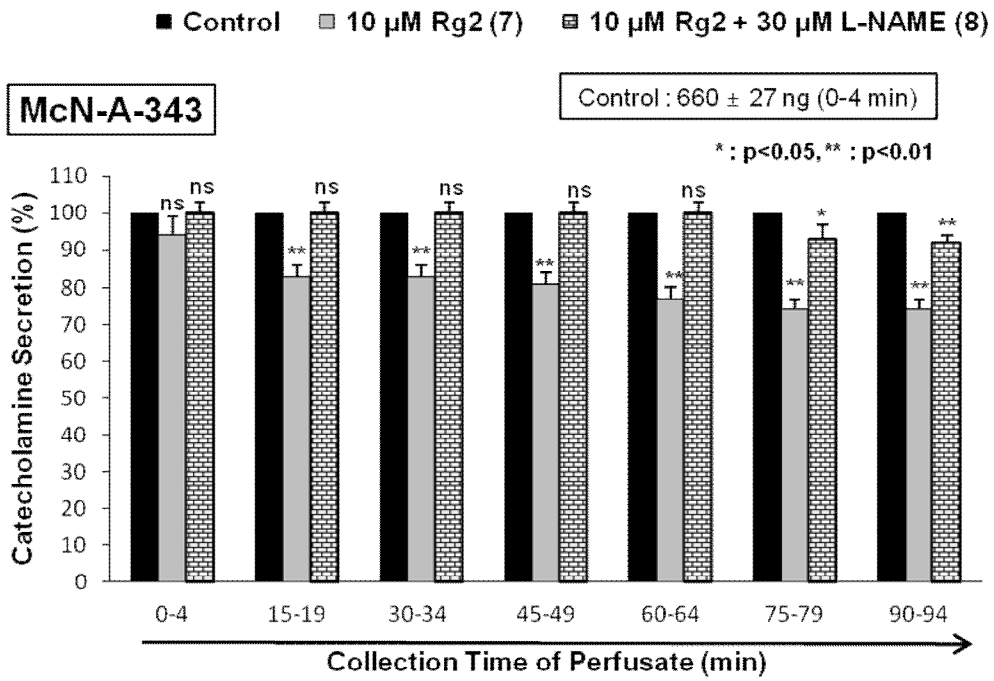

Fig. 12. Effects of ginsenoside-Rg2 plus L-NAME on McN-A-343-induced CA secretion in the perfused rat adrenal medulla. The CA secretion by perfusion of McN-A-343 (100 μM) for 4 min was induced at 15 min intervals during simultaneous loading with Rg2 (10 μM) plus L-NAME (30μM) for 90 min. McN-A-343-induced perfusate was collected for 8 minutes. Other legends are the same as in Fig. 2. *: $p<0.05$, **: $p<0.01$. ns: Not statistically significant.

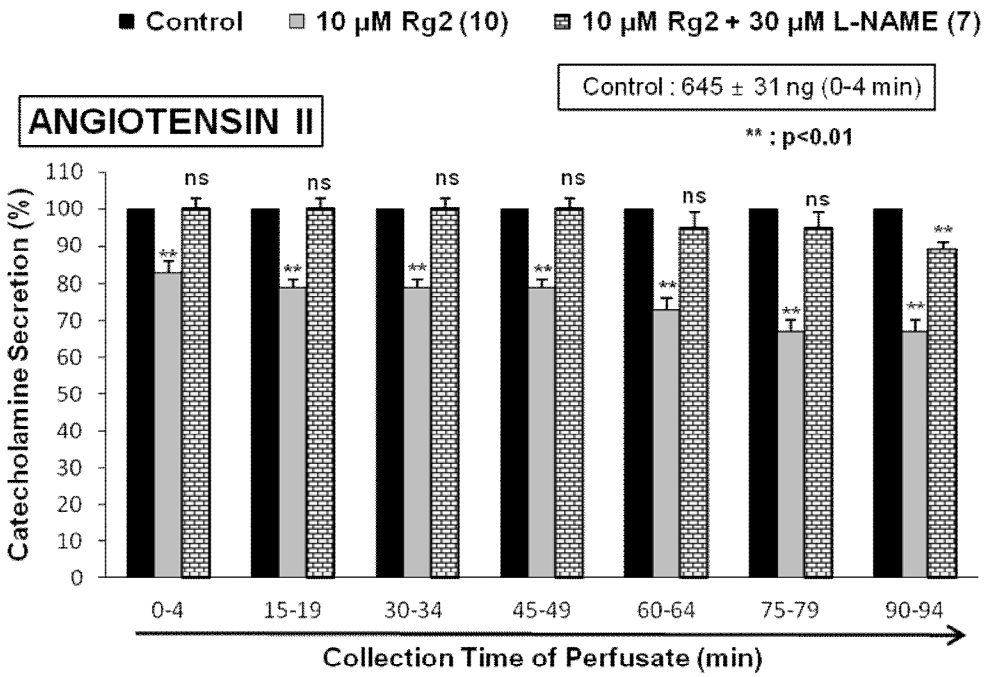

Fig. 13. Effects of ginsenoside-Rg2 plus L-NAME on angiotensin II-induced CA secretion in the perfused rat adrenal medulla. The CA secretion by perfusion of angiotensin II (100 nM) for 1 min was induced at 15 min intervals during simultaneous loading with Rg2 (10 μM) plus L-NAME (30μM) for 90 min. Angiotensin II-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

Under the coexistence of Rg2 (10 μM) and L-NAME (30 μM) for 90 min, high K^+ (56 mM)-induced CA release was also recovered to 100~88% of the corresponding control release (736±31 ng for 0-4 min), in which there was a significant difference in comparison to the inhibitory effect of Rg2-treatment alone on high K^+ (56 mM)-induced CA release (Fig. 14).

The simultaneous perfusion of Rg2 (10 μM) and L-NAME (30 μM) for 90 min restore the CA secretion induced by Bay-K-8644 (10 μM) and cyclopiazonic acid (10 μM) almost to 100~88% (Bay-K-8644) and 100~89% (cyclopiazonic acid) of their corresponding control secretory responses (627±27 ng/0-4 min for Bay-K-8644; 599±23 ng/0-4 min for cyclopiazonic acid), respectively, in comparison to the inhibitory effect of Rg2-treatment alone, as shown in Fig. 15 and 16.

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. 17).

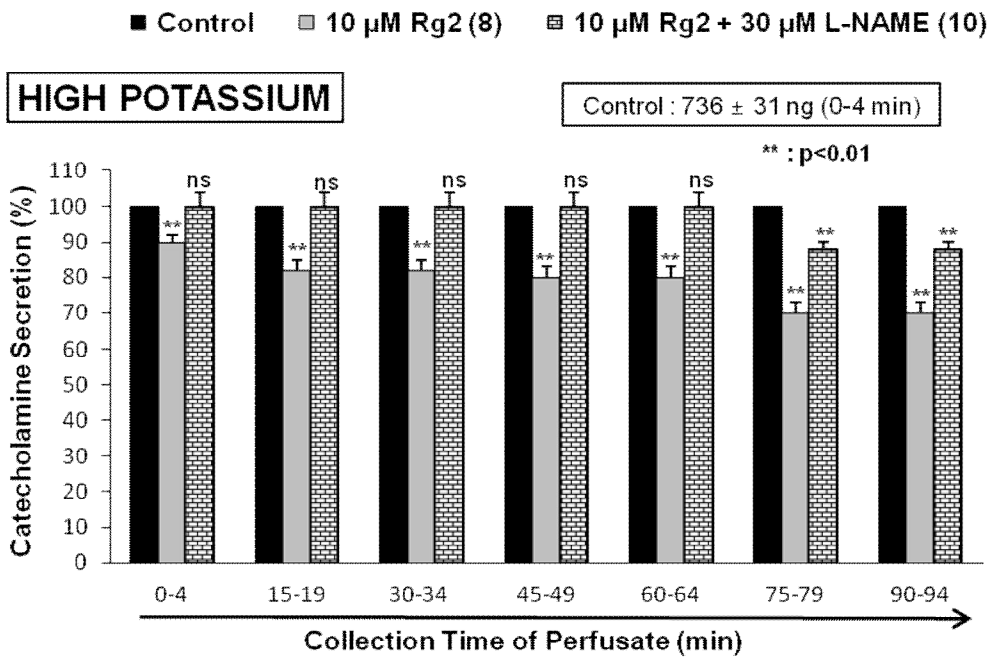

Fig. 14. Influence of ginsenoside-Rg2 plus L-NAME on high potassium-induced CA secretion in the perfused rat adrenal medulla. The CA secretion by a single injection of high potassium (56 mM) in a volume of 0.05 mL was induced at 15 intervals during simultaneous loading with Rg2 (10 μM) plus L-NAME (30μM) for 90 min. Statistical difference was obtained by comparing the corresponding control with group of Rg2-treated alone or group treated with Rg2+L-NAME. High potassium -induced perfusates were collected for 4 minutes. Other legends are the same as in Fig. 2., **: P < 0.01. ns: Not statistically significant.

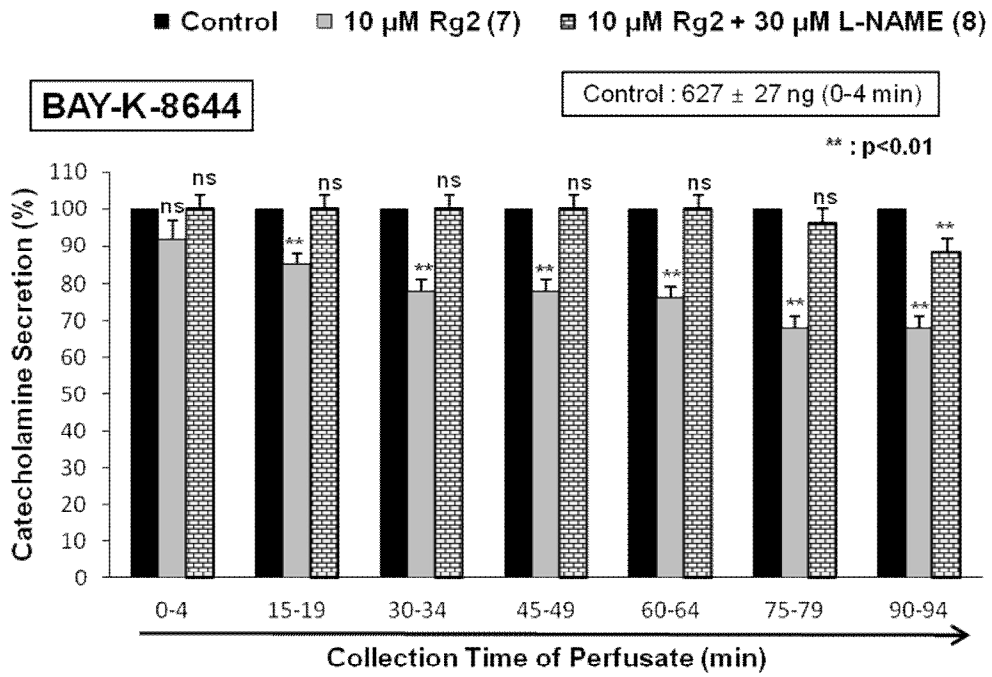

Fig. 15. Effects of ginsenoside-Rg2 plus L-NAME on Bay-K-8644-induced CA secretion in the perfused rat adrenal medulla. The CA secretion by perfusion of Bay-K-8644 (10 μM) for 4 min was induced at 15 min intervals during simultaneous loading with Rg2 (10 μM) plus L-NAME (30μM) for 90 min. Bay-K-8644-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

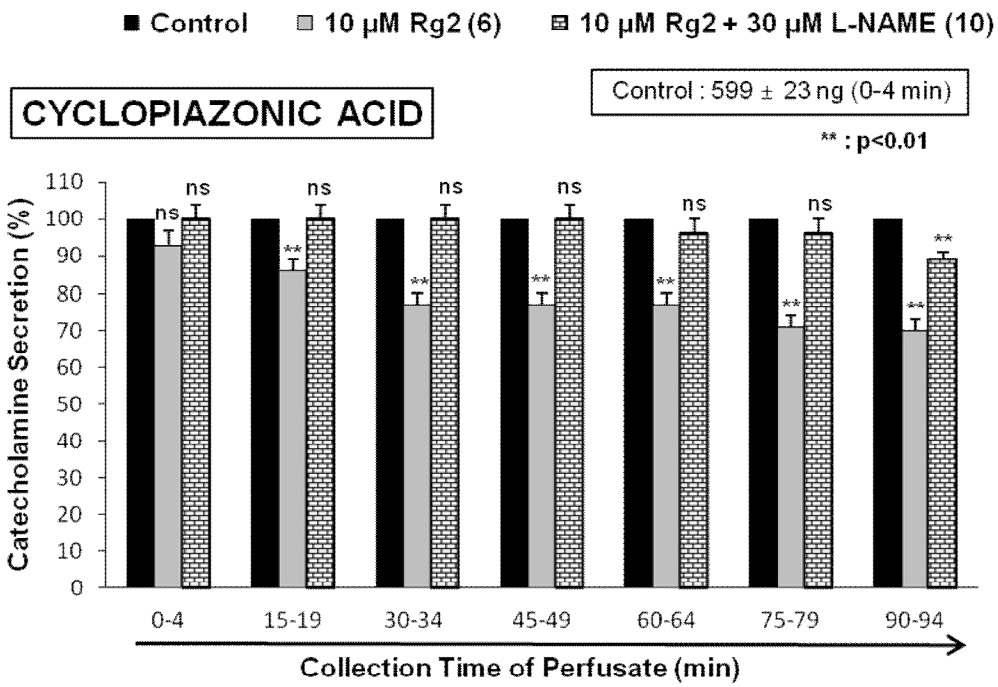

Fig. 16. Effects of ginsenoside-Rg2 plus L-NAME on cyclopiazonic acid-induced CA secretion in the perfused rat adrenal medulla. The CA secretion by perfusion of cyclopiazonic acid (10 μM) for 4 min was induced at 15 min intervals during simultaneous loading with Rg2 (10 μM) plus L-NAME (30μM) for 90 min. Cyclopiazonic acid -induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

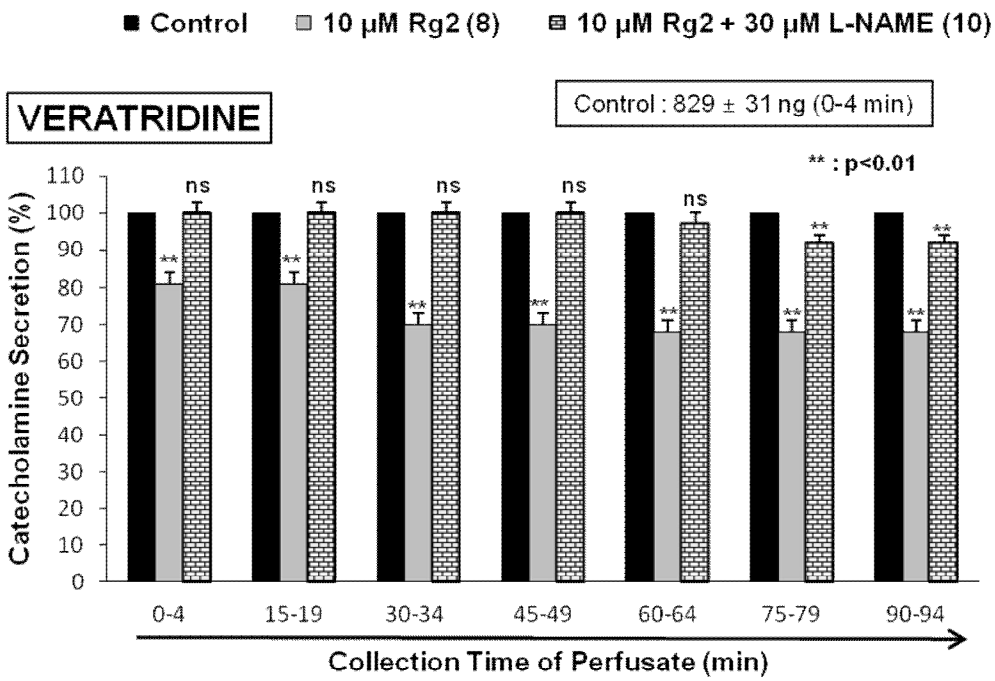

Fig. 17. Effects of ginsenoside-Rg2 plus L-NAME on veratridine-induced CA secretion in the perfused rat adrenal medulla. The CA secretion by perfusion of veratridine (100 μM) for 4 min was induced at 15 min intervals during simultaneous loading with Rg2 (10 μM) plus L-NAME (30μM) for 90 min. Veratridine-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

Influence of Ginsenoside-Rg2 on the level of nitric oxide in the perfused rat adrenal medulla

As shown in Fig. 10~17, the CA secretory responses induced by ACh, DMPP, McN-A-343, Ang II, high K^+, Bay-K-8644, cyclopiazonic acid 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 (Kim et al., 1994) and that ginsenoside Rg3 is the most potent ginsenoside, activating eNOS in rat aorta (Kim et al., 1999). Although some investigators have demonstrated that Rg3 induces eNOS activation in the vasculature of animal models (Kim et al., 1994; Kang et al., 1995).

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. 18.

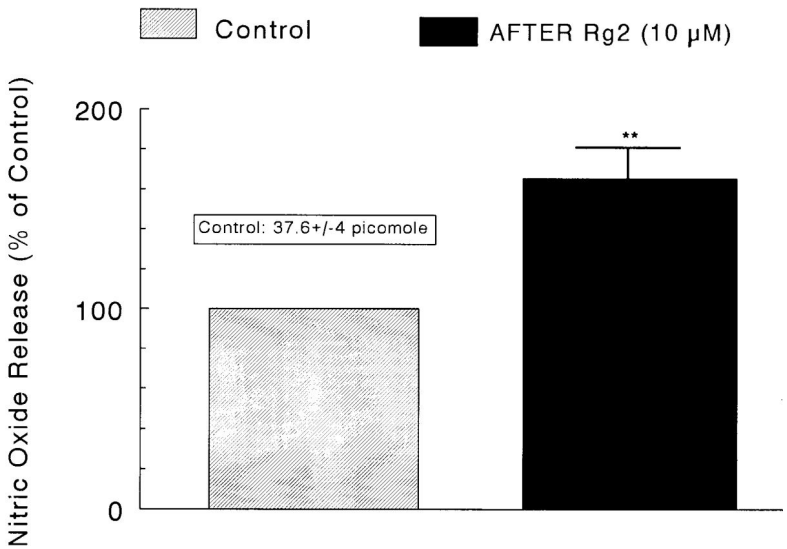

Fig. 18. Effects of ginsenoside-Rg2 on nitric oxide (NO) release in the perfused rat adrenal medulla. Perfusate sample was collected for 8 min after the perfusion of Rg2 (10 μM) at a rate of 0.31 mL/min. Ordinate: the amounts of NO release in the adrenal medulla (% of control). Abscissa: Treatment (before and after Rb2). Statistical difference was obtained by comparing the control group with Rg2-treated group. **: $p<0.01$.

Combined Effects of Ginsenoside-Rg2 and fimasartan on ACh-induced CA release in the perfused rat adrenal medulla

In the present study, ginsenoside-Rg2 or fimasartan (an angiotensin II type 1 (AT_1) receptor-selective antagonist [Kim et al., 2012]) caused inhibitory effects on the CA release by stimulation of cholinergic receptors as well as Ang II AT_1 receptors in the perfused rat adrenal medulla. 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. 19. Also, there was statistically difference in inhibitory effect between fimasartan versus ginsenoside-Rg2 plus fimasartan on ACh-induced CA secretion, as shown in Fig. 19.

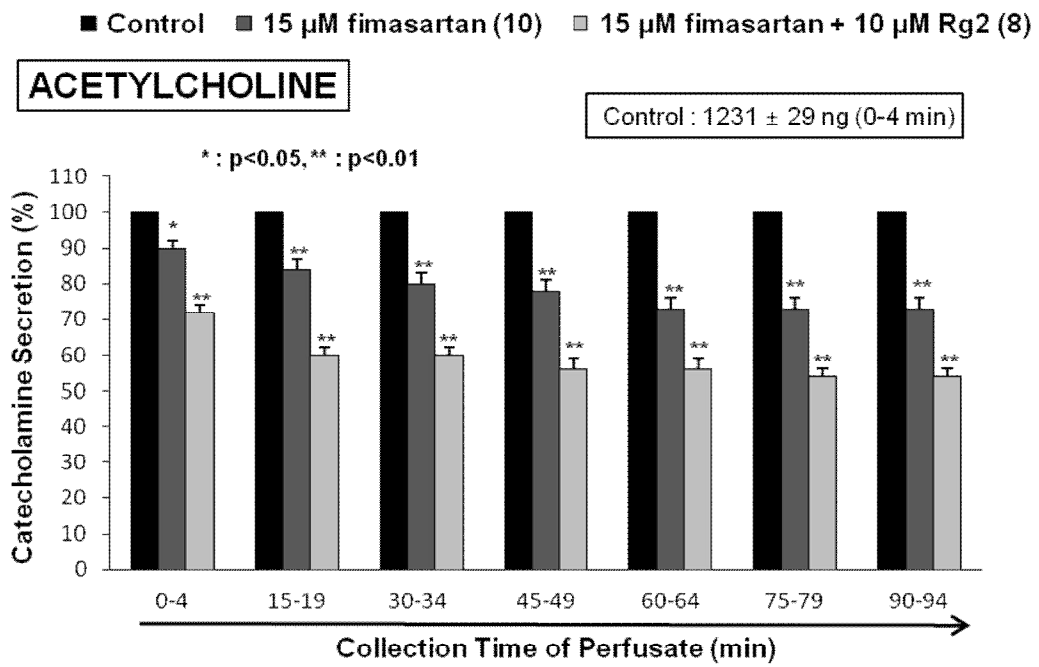

FIG. 19. Comparative time-course effects of fimasartan and ginsenoside-Rg2 plus fimasartan on acetylcholine-induced CA secretion in the perfused rat adrenal glands. The CA secretion by a single injection of ACh (5.32 mM) in a volume of 0.05 ml was evoked at 15 min intervals during perfusion of fimasartan (15 μM) or fimasartan (15 μM) plus ginsenoside-Rg2 (10 μM) for 90 min, respectively, as indicated by the arrow mark. Acetylcholine-induced perfusates were collected for 4 minutes. Other legends are the same as in Fig. 2. *: P < 0.05 or **: P < 0.01 control group vs fimasartan (15 μM)-treated group or fimasartan (15 μM) + ginsenoside-Rg2 (10 μM)-treated group.

IV. DISCUSSION

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 (an NO synthase inhibitor the CA secretory responses induced by ACh, DMPP, McN-A-343, Ang II, high K^+, Bay-K-8644, cyclopiazonic acid 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 report that, in a series of studies, ginsenoside-Rb2 depresses the CA secretion in the perfused rat adrenal medulla through increase in nitric oxide production due to activation of nNOS (Lim et al., 2014), ginsenosides reduce blood pressure via increases in production of endothelial nitric oxide (Kim et al., 1994), and that ginsenoside Rg3 is the most potent ginsenoside activating eNOS in rat aorta (Kim et al., 1999). Although several investigators have demonstrated that Rg3 induces eNOS activation in the vasculature of animal models (Kim et al., 1994; Kang et al., 1995), Hien and his co-workers (2010) have found that Rg3 activates eNOS via eNOS phosphorylation in ECV 304 human endothelial cells and increases in eNOS expression. Moreover, in this study, after loading of Rg2 into adrenal medulla, NO production was greatly elevated as shown in Fig. 18. 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, since during the simultaneous perfusion of Rg2 and L-NAME (an inhibitor of NO synthase) for 90 min, the CA secretory responses induced by ACh, DMPP, McN-A-343, Ang II, high K^+, Bay-K-8644, cyclopiazonic acid and veratridine were recovered mostly to the corresponding control secretion compared to that of Rg2-treatment alone, and also substantially, Rg2 markedly

increased NO release from rat adrenal medulla.

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 (Torres et al., 1994) and also that sodium nitroprusside (SNP) inhibits ACh-induced CA secretion in bovine chromaffin cells (Uchiyama et al., 1994). 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 (Torres et al., 1994), suggesting that not only 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, among the ginsenosides of the protopanaxatriol and protopanaxadiol groups, ginsenoside Rg3 is known to be the most potent vasodilator (Kim et al., 1999; Kim et al., 2003). Previously, it has been found that Rg3 inhibits calcium-induced vascular contraction (Kim et al., 1999) as well as phenylephrine-induced vasocontraction as a consequence of NO production (Kim et al., 2003).

In contrast, it has been reported that L-NAME inhibits ACh-induced CA secretion in bovine chromaffin cells (Uchiyama et al., 1994), and also that the NO donor SNP enhances nicotine-induced CA secretion in cultured bovine chromaffin cells (O'Sullivan and Burgoyne, 1990). 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 (Breslow et al., 1992; 1993).

Generally, the adrenal medulla has been used as a good model system to research many cellular activities including not merely noradrenergic nerve cells but also neurons. During neurogenic acivation of the adrenal medulla, ACh is released from splanchnic nerve endings and stimulates cholinergic receptors on the chromaffin cell membrane (Viveros, 1975). This stimulation starts a series of incidents known as stimulus-secretion coupling, eventuating in the exocytotic secretion of CA and other constituents of the releasing vesicles into the extracellular gap. Mostly, two

mechanisms are included in the release of adrenomedullary hormones. Upon stimulation of splanchnic nerves, ACh is liberated from the nerve terminals, and then stimulates nicotinic receptors and the CA secretion. Based on this fact, the present findings demostrated that Rg2 inhibits the CA secretory responses induced by stimulation of cholinergic (nicotinic and muscarinic) receptors as well as AT_1 receptors in the adrenal medulla. These results appear to support the fact that ginsenosides isolated from *Panax ginseng* can reduce the blood pressure in both experimental animals and hypertensive patients (Kim et al., 1994; Han et al., 1998; Jeon et al., 2000b; Sung et al., 2000). These experimental results indicate that Rg2-induced inhibitory effects on the CA secretory response induced by stimulation of cholinergic receptors as well as AT_1 receptors may contribute partly to its hypotensive mechanism. ACh, the physiological presynaptic transmitter at the adrenal medulla, which is released by depolarizing splanchnic nerve terminals and then stimulates nicotinic receptors, secrets the CA, and induces dopamine β-hydroxylase by calcium-dependent secretory process (Dixon et al., 1975; Viveros et al., 1968). In view of this finding, the present results demonstrate that Rg2 may reduce CA secretion induced by nicotinic activation from the splanchnic nerve terminal via the antagonism of neuronal nicotinic receptors. The CA secretion from the adrenal medullary cells in response to splanchnic nerve activation or nicotinic agonist is exerted by activation of nicotinic receptors located on the chromaffin cells. The exocytotic CA release from the chromaffin cells seems to be essentially similar to that occurring in noradrenergic axons (Douglas, 1968; Sorimachi and Yoshida, 1979). ACh-induced CA secretion has shown to be caused through stimulation of both nicotinic and muscarinic receptors in guinea-pig adrenal gland (Nakazato et al., 1988) as well as in the perfused rat adrenal glands (Lim and Hwang, 1991). In support of this idea, it has been found that the ginseng saponins, ginsenoside Rg2, a panaxatriol (Tachikawa et al., 1995) as well as ginsenoside Rg3, a panaxadiol (Tachikawa et al., 2001) block the nicotinic ACh receptors or the receptor-operated Na^+ channels (but not voltage-sensitive Na^+ and Ca^{2+} channels), inhibit Na^+ influx through the channels and consequently reduce both Ca^{2+} influx and the CA secretion in bovine adrenal chromaffin cells.

In this study, Rg2 inhibited the CA secretory responses induced by ACh, McN-A-343, Ang II, and DMPP. This finding suggests that Rg2 can produce the similar effect as in adrenal medulla of

the normotensive rats (Hong et al., 1999) and also in bovine adrenal chromaffin cells (Tachikawa et al., 1995; 2001). In previous study, ginsenoside-Rg_3 inhibited both ACh-induced Ca^{2+} and Na^{+} influxes in a concentration-dependent manner similar to that observed with the ACh-induced CA secretion (Tachikawa et al., 2001). However, it had no or only a slight effect on the CA secretion and Ca^{2+} influx induced by high K^{+} concentration or veratridine, an activator of the voltage-sensitive Ca^{2+} or Na^{+} channels (Kudo et al., 1992; Tachikawa et al., 1995). These findings robustly implied that ginsenoside-Rg_3 behaves on the nicotinic ACh receptor-gated cation channels but not on the voltage-dependent Ca^{2+} or Na^{+} channels. Furthermore, the ginsenoside-Rg_3-induced inhibitory effect was not overcome by increasing the external ACh and Ca^{2+} concentrations (Tachikawa et al., 2001), indicating that the inhibitory effect of ginsenoside-Rg_3 is distinct from that of the competitive antagonists of the nicotinic ACh receptors, such as trimethaphan (Rang, 1982; Weaver et al., 1994), and that of blockers of the L-type voltage-sensitive Ca^{2+} channels, which are competitive with external Ca^{2+} concentrations, such as diltiazem (Wada et al., 1983). In fact, the mode of the ginsenoside-Rg_3 antagonism was non-competitive with nicotine (Tachikawa et al., 2001). Anyway, these results seem to be quite different from those of the present study that Rg2 significantly inhibited the CA secretory responses induced by cholinergic nicotinic stimulation as well as an activator of the voltage-sensitive Ca^{2+} or Na^{+} channels in the perfused model of rat adrenal medulla. The discrepancy is attributed to the experimental employment of different methodology and different components between previous and the present studies.

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 (Garcia et al., 1984; Schramm et al., 1983), as well as by high K+, a direct membrane depolarizer. 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 (Fisher et al., 1981; Yanagihara et al., 1979). It has also been known that the stimulation of nicotinic receptors facilitates the CA secretion by increasing Ca^{2+} entry through receptor-linked and/or voltage-dependent Ca^{2+} channels in both perfused rat adrenal glands (Lim and Hwang, 1991;

Wakade and Wakade, 1983) and isolated bovine adrenal chromaffin cells (Wakade and Wakade, 1983; Kilpatrick et al., 1981; 1982; Knight and Kesteven, 1983). 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 (Wada et al., 1985b). In the present study, the result 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 this inhibitory effect of Rg2 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 (Kidokoro and Ritchie, 1980), 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 (Burgoyne, 1984; Oka et al., 1979). 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.

The present study has also shown that Rg2 inhibits the CA secretion induced by cyclopiazonic acid. Cyclopiazonic acid is known to be a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Goeger and Riley, 1989; Seidler et al., 1989) and a valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic currents regulated by intracellular Ca^{2+} (Suzuki et al., 1992). Thus, 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 (Uyama et al., 1992). It has been found that cyclopiazonic acid easily penetrates into the cytoplasm through the plasma membrane and reduces Ca^{2+}-ATPase activity in sarcoplasmic/endoplasmic reticulum, resulting in an increase in the subsequent Ca^{2+} release from those storage sites (Suzuki et al., s coworkers (1992). 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 (Cheek et al., 1989; Challiss et al., 1991). 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 (an AT_1 antagonist) were perfused in combination, their inhibitory effect on ACh-induced CA release was enhanced. In support of this idea, the losartan (an AT_1 antagonist)-enalapril (an angiotensin-converting enzyme inhibitor) combination is more effective in decreasing blood pressure and increasing plasma active renin than doubling of the enalapril dose in normotensive male volunteers (Azizi et al., 1997). 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 20, 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 via 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 such as angina pectoris, heart failure and hypertension.

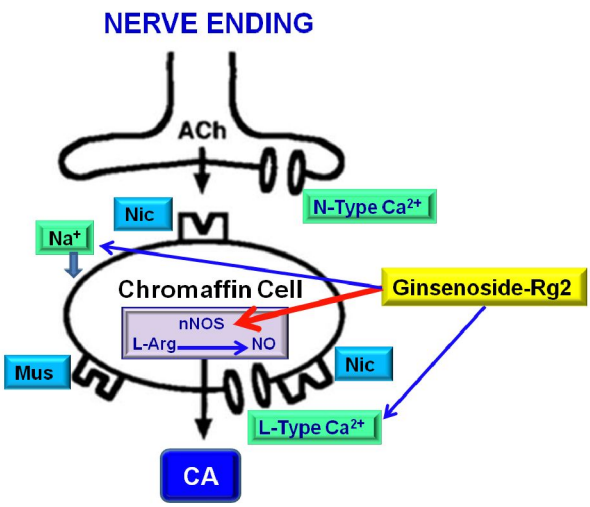

Fig. 20. Schematic diagram of possible action site of ginsenoside-Rg2 (Rg2) in the rat adrenal medulla.

V. SUMMARY

Recently, it has been reported that ginsenoside-Rb2, one of panaxadiol type saponins, suppresses secretion of catecholamines (CA) from the isolated perfused rat adrenal glands (Lim et al., 2014). Moreover, total ginseng saponins (TGS) rather inhibit the CA secretion from the isolated perfused rat adrenal glands (Hong et al., 1999) and SHRs (Jang et al, 2013). However, it has previously been reported that panaxadiol-type saponin rather causes the increased secretion of CA from the isolated perfused rabbit adrenal glands (Lim et al., 1988) and more recently gintonin greatly enhances the CA secretion in the perfused rat adrenal medulla (Na et al., 2016). Thus the present study was aimed to examine the characteristics of ginsenoside-Rg2 (Rg2), one of the panaxatriol saponins isolated from Korean ginseng root, on the CA release in the perfused model of the rat adrenal medulla, and also to clarify its mechanism of action. Rg2 (3~30 μM), administered into an adrenal vein for 90 min, depressed ACh (5.32 mM)-induced CA secretion in a dose- and time-dependent manner. Rg2 (10 μM) also time-dependently inhibited the CA secretion induced by McN-A-343 (100 μM, a selective muscarinic M_1 receptor agonist), DMPP (100 μM, a selective neuronal nicotinic receptor agonist), and angiotensin II (100 nM). Rg2 itself failed to influence spontaneous CA release (data not shown). Also, During the perfusion of Rg2 (10 μM) for 90 min, the CA secretory responses induced by high K^+ (56 mM, a direct membrane depolarizer), veratridine (a voltage-dependent Na^+ channel activator (50 μM), cyclopiazonic acid (a cytoplasmic Ca^{2+}-ATPase inhibitor, 10 μM), Bay-K-8644 (an L-type dihydropyridine Ca^{2+} channel activator, 10 μM) were greatly inhibited, respectively. Interestingly, under the simultaneous existence of Rg2 (10 μM) and L-NAME (30 μM, an inhibitor of NO synthase), the CA secretory responses induced by ACh, DMPP, McN-A-343, Ang II, high K^+, Bay-K-8644, cyclopiazonic acid and veratridine was restored nearly to the extent of their corresponding control level, respectively, compared to those of the inhibitory effects of Rg2 (10 μM)-treatment alone. Virtually, the NO release in adrenal medulla following theperfusion of Rg2 (10 μM) was significantly enhanced in comparison to the corresponding spontaneous release. Also, in the coexistence of Rg2 (10 μM) and fimasartan (15

μM), ACh-induced CA secretion was markedly diminished compared to that of fimasartan-treated alone. Taken together, these results demonstrate that Rg2 greatly suppresses the CA secretory responses induced by activation of cholinergic receptors as well as AT_1-receptors from the perfused model of the rat adrenal gland. This Rg2-induced inhibitory effect seems to be exerted by reducing both the influx of Na^+ and Ca^{2+} through their ionic channels into the adrenomedullary chromaffin cells as well as by suppressing the Ca^{2+} release from the cytoplasmic calcium store, at least through the elevated NO release by the activation of NO synthase, which is associated to the blockade of neuronal cholinergic receptors 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 medullary cells and consequent decreased CA level in the circulation. When Rg2 and fimasartan were used in combination, their inhibitory effects were enhanced, which may also be clinically of benefit in treating cardiovascular diseases, including hypertension and angina pectoris.

REFERENCES

Anton AH, Sayre DF. A study of the factors affecting the aluminum oxide trihydroxy indole procedure for the analysis of catecholamines. *J Pharmacol Exp Ther.* 1962;138:360-375.

Azizi M, Guyene TT, Chatellier G, Wargon M, Ménard J. Additive effects of losartan and enalapril on blood pressure and plasma active renin. *Hypertension.* 1997;29:634-640.

Breslow MJ, Tobin JR, Bredt DS, Ferris CD, Snyder SH, Traystman RJ. Nitric oxide as a regulator of adrenal blood flow. *Am J Physiol.* 1993;264:H464-H469.

Breslow MJ, Tobin JR, Bredt DS, Ferris CD, Snyder SH, Traystman RJ. Role of nitric oxide in adrenal medullary vasodilation during catecholamine secretion. *Eur J Pharmacol.* 1992;210:105-106.

Burgoyne RD. Mechanism of secretion from adrenal chromaffin cells. *Biochem Biophys Acta.* 1984;779:201-216.

Chai H, Zhou W, Lin P, Lumsden A, Yao Q, Chen C. Ginsenosides block HIV protease inhibitor ritonavir-induced vascular dysfunction of porcine coronary arteries Am. *J Physiol Heart Circ Physiol.* 2005;288:H2965-2971.

Challiss RA, Jones JA, Owen PJ, Boarder MR. Changes in inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate mass accumulations in cultured adrenal chromaffin cells in response to bradykinin and histamine. *J Neurochem.* 1991;56:1083-1086.

Cheek TR, O'Sullivan AJ, Moreton RB, Berridge MJ, Burgoyne RD. Spatial localization of the stimulus-induced rise in cytosolic Ca^{2+} in bovine adrenal chromaffin cells: Distinct nicotinic and muscarinic patterns. *FEBS Lett.* 1989;247:429-434.

Dixon Wr, Garcia AG, Kirkekar SM. Release of catecholamines and dopamine-beta-hydroxylase from the rat adrenal gland of the cat. *J Physiol.* 1975;244:805-824.

Douglas WW. Stimulus-secretion coupling: The concept and clues from chromaffin and other cells. *Br J Pharmacol.* 1968;34:451-474.

Fisher SK, Holz RW, Agranoff BW. Muscarinic receptors in chromaffin cell culture mediate enhanced phospholipid labeling but not catecholamine secretion. *J Neurochem.*

1981;37:491-487.

Garcia AG, Sala F, Reig JA, Viniegra S, Frias J, Fonteriz R, Gandia L. Dihydropyridine Bay-K-8644 activates chromaffin cell calcium channels. *Nature.* 1984;309:69-71.

Goeger DE, Riley RT. Interaction of cyclopiazonic acid with rat skeletal muscle sarcoplasmic reticulum vesicles. Effect on Ca^{2+} binding and Ca^{2+} permeability. *Biochem Pharmacol.* 1989;38:3995-4003.

Hammer R, Giachetti A. Muscarinic receptor subtypes: M_1 and M_2 biochemical and functional characterization. *Life Sci.* 1982;31:2992-2998.

Han KH, Choe SC, Kim HS, Sohn DW, Nam KY, Oh BH, Lee MM, Park YB, Choi YS, Seo JD, Lee YW. Effect of red ginseng on blood pressure in patients with essential hypertension and white coat hypertension. *Am J Chin Med.* 1998;26:199–209.

Han K, Shin IC, Choi KJ, Yun YP, Hong JT, Oh KW. Korea red ginseng water extract increases nitric oxide concentrations in exhaled breath. *Nitric Oxide.* 2005;12:159-162.

Hano T, Mizukoshi M, Baba A, Nakamura N, Nishio I. Angiotensin II subtype 1 receptor modulates epinephrine release from isolated rat adrenal gland. *Blood Press.* 1994;5:S105-108.

Han SW, Kim H. Ginsenosides stimulate endogenous production of nitric oxide in rat kidney. *Int J Biochem Cell Biol.* 1996;28:573-580.

Hien TT, Kim ND, Pokharel YR, Oh SJ, Lee MY, Kang KW. Ginsenoside Rg3 increases nitric oxide production via increases in phosphorylation and expression of endothelial nitric oxide synthase: Essential roles of estrogen receptor-dependent PI3-kinase and AMP-activated protein kinase. *Toxicol Appl Pharmacol.* 2010;246:171-183.

Hong SP, Chi H, Cho SH, Lee YK, Woo SC, Kim IS, Oh SH, Yang WH, Lim DY. Influence of total Ginseng saponin on nicotinic stimulation-induced catecholamine secretion from the perfused rat adrenal gland. *Korean J Hypert.* 1999;5:1-10.

Jang SJ, Lim HJ, Lim DY. Inhibitory Effects of Total Ginseng Saponin on Catecholamin Secretion from the Perfused Adrenal Medulla of SHRs. *J Ginseng Res*. 2011;35:176-190.

Jeon BH, Kim CS, Kim HS, Park JB, Nam KY, Chang SJ. Effect of Korean red ginseng on

blood pressure and nitric oxide production. *Acta Pharmacol Sin.* 2000a;21:1095-1100.

Jeon BH, Kim CS, Park KS, Lee JW, Park JB, Kim KJ, Kim SH, Chang SJ, Nam KY. Effect of Korea red ginseng on the blood pressure in conscious hypertensive rats. *Gen Pharmacol.* 2000b;35(3):135-141.

Kang SY, Schini-Kerth VB, Kim ND. Ginsenosides of the protopanaxatriol group cause endothelium-dependent relaxation in the rat aorta. *Life Sci.* 1995;56:1577–1586.

Kidokoro Y, Ritchie AK. Chromaffin cell action potentials and their possible role in adrenaline secretion from rat adrenal medulla. *J Physiol.* 1980;307:199-216.

Kilpatrick DL, Slepetis RJ, Corcoran JJ, Kirshner N. Calcium uptake and catecholamine secretion by cultured bovine adrenal medulla cells. *J Neurochem.* 1982;38:427-435.

Kilpatrick DL, Slepetis RJ, Kirshner N. Ion channels and membrane potential in stimulus-secretion coupling in adrenal medulla cells. *J Neurochem.* 1981;36:1245-1255.

Kim JH, Lee JH, Paik SH, Kim JH, Chi YH. Fimasartan, a novel angiotensin II receptor antagonist. *Arch Pharm Res*. 2012;35:1123-1126.

Kim ND, Kang SY, Park JH, Schini-Kerth VB. Ginsenoside Rg3 mediates endothelium-dependent relaxation in response to ginsenosides in rat aorta: role of K^+ channels. *Eur J Pharmacol.* 1999;367:41–49.

Kim ND, Kang SY, Schini VB. Ginsenosides evoke endothelium-dependent vascular relaxation in rat aorta. *Gen Pharmacol.* 1994;25:1071–1077.

Kim ND, Kim EM, Kang KW, Cho MK, Choi SY, Kim SG. Ginsinoside Rg3 inhibits phenylephrine-induced vascular contraction through induction of nitric oxide synthase. *Br J Pharmacol.* 2003;140:661-670.

Knight DE, Kesteven NT. Evoked transient intracellular free Ca^{2+} changes and secretion in isolated bovine adrenal medullary cells. *Proc R Soc Lond Biol Sci.* 1983;218:177-199.

Kudo K, Akasaka Y, Miyate Y, Takahashi E, Tachikawa E, Kashimoto T. Effects of red ginseng fractions on catecholamine secretion from bovine adrenal medullary cells. *J Med Pharm Soc.* 1992;9:236–239.

Kudo K, Tachikawa E, Kashimoto T, Takahashi E. Properties of ginseng saponins of catecholamine secretion in bovine adrenal chromaffin cells. *Eur J Pharmacol.* 1998;341:139–144.

Leung KW, Cheng YK, Mak NK, Chan KK, Fan TP, Wong RN. Signaling pathway of ginsenoside-Rg1 leading to nitric oxide production in endothelial cells. *FEBS Lett.* 2006;580:3211-3216.

Lim DY, Choi CH, Kim CD, Kim KH, Kim SB, Lee BJ, Chung MH. Influnce of Panaxatriol-type saponin on secretion of catecholamine from isolated perfused rabbit adrenal gland. *Arch Pharm Res.* 1989;12:166-175.

Lim DY, Hwang DH. Studies on secretion of catecholamines evoked by DMPP and McN-A-343 in the rat adrenal gland. *Korean J Pharmacol.* 1991;27:53-67.

Lim DY, Kim CD, Ahn KW. Influence of TMB-8 on secretion of catecholamines from the perfused rat adrenal glands. *Arch Pharm Res.* 1992;15:115-125.

Lim DY, Park KB, Kim KH, Choi CH, Bae JW, Kim MW. Studies on secretion of catecholamines evoked by panaxadiol in the isolated rabbit adrenal gland. *Korean J Pharmacol.* 1988;24:31-42.

Lim DY, Park KB, Kim KH, Moon JK, Kim YH. Influence of total ginseng saponin on secretion of catecholamines in the isolated adrenal gland of rabbits. *Korean Biochem J.* 1987;20:230-238.

Lim HJ, Lee HY, and Lim DY. Inhibitory Effects of Ginsenoside-Rb2 on Nicotinic Stimulation Evoked Catecholamine Secretion. *Korean J Physiol Pharmacol*, 2014;18(5): 431-439.

McVeigh GE, Hamilton P, Wilson M, Hanratty CG, Leahey WJ, Devine AB, Morgan DG, Dixon LJ, McGrath LT. Platelet nitric oxide and superoxide release during the development of nitrate tolerance: effect of supplemental ascorbate. *Circulation.* 2002;106:208-213.

Nakazato Y, Ohga A, Oleshansky M, Tomita U, Yamada Y. Voltage-independent catecholamine release mediated by the activation of muscarinic receptors in guinea-pig adrenal glands. *Br J Pharmacol.* 1988;93:101-109.

Na SY, Kim KH, Choi MS, Ha KS and Lim DY. Gintonin facilitates catecholamine secretion from the perfused adrenal medulla. *Korean J Physiol Pharmacol.* 2016;20: 629-639.

Oka M, Isosaki M, Yanagihara N. Isolated bovine adrenal medullary cells: studies on regulation of catecholamine synthesis and release. In: Usdin E, Kopin IJ, Brachas J, ed. Catecholamines: Basic and Clinical frontiers. Oxford: Pergamon Press; 1979. 70-72 p.

O'Sullivan AJ, Burgoyne RD. Cyclic GMP regulates nicotine-induced secretion from cultured bovine adrenal chromaffin cells: effects of 8-bromo-cyclic GMP, atrial natriuretic peptide, and nitroprusside (nitric oxide). *J Neurochem.* 1990;54:1805-1808.

Rang HP. The action of ganglionic blocking drugs on the synaptic responses of rat submandibular ganglion cells. *Br J Pharmacol.* 1982;75:151–168.

Schramm M, Thomas G, Towart R, Franckowiak G. Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels. *Nature.* 1983;303:535-537.

Seidler NW, Jona I, Vegh N, Martonosi A. Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J Biol Chem.* 1989;264:17816-17823.

Sorimachi M, Yoshida K. Exocytotic release of catecholamines and dopamine-beta-hydroxylase from the perfused adrenal gland of the rabbit and cat. *Br J Pharmacol.* 1979;65:117-125.

Sung J, Han KH, Zo JH, Park HJ, Kim CH, Oh BH. Effects of red ginseng upon vascular endothelial function in patients with essential hypertension. *Am J Chin Med.* 2000;28:205–216.

Suzuki M, Muraki K, Imaizumi Y, Watanabe M. Cyclopiazonic acid, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-pump, reduces Ca^{2+}-dependent K^+ currents in guinea-pig smooth muscle cells. *Br J Pharmacol.* 1992;107:134-140.

Tachikawa E, Kudo K, Kashimoto T, Takahashi E. Ginseng saponins reduce acetylcholine-evoked Na^+ influx and catecholamine secretion in bovine adrenal chromaffin cells. *J Pharmacol Exp Ther.* 1995;273:629–636.

Tachikawa E, Kudo K, Nunokawa M, Kashimoto T, Takahashi E, Kitagawa S. Characterization of ginseng saponin ginsenoside-Rg(3) inhibition of catecholamine secretion in bovine adrenal chromaffin cells. *Biochem Pharmacol.* 2001;62:943-951.

Tallarida RJ, Murray RB. Manual of pharmacologic calculation with computer programs. 2nd ed. New York: Speringer-Verlag; 1987. 132 p.

Torres M, Ceballos G, Rubio R. Possible role of nitric oxide in catecholamine secretion by chromaffin cells in the presence and absence of cultured endothelial cells. *J Neurochem.* 1994;63:988-996.

Uchiyama Y, Morita K, Kitayama S, Suemitsu T, Minami N, Miyasako T, Dohi T. Possible involvement of nitric oxide in acetylcholine-induced increase of intracellular Ca^{2+} concentration and catecholamine release in bovine adrenal chromaffin cells. *Jpn J Pharmacol.* 1994;65:73-77.

Uyama Y, Imaizumi Y, Watanabe M. Effects of cyclopiazonic acid, a novel Ca^{2+}-ATPase inhibitor on contractile responses in skinned ileal smooth muscle. *Br J Pharmacol.* 1992;106:208-214.

Viveros OH, Arqueros LC, Kirshner N. Release of catecholamines and dopamine beta-hydroxylase from the adrenal medulla. *Life Sci.* 1968;7:609-618.

Viveros OH. Mechanism of secretion of catecholaminies from adrenal medulla. In handbook of physiology, Endocrinology. Vol VI, Sect 7, The adrenal gland. Washington DC: American physiological society; 1975. 389-426 p.

Wada A, Takara H, Izumi F, Kobayashi H, Yanagihara N. Influx of 22Na through acetylcholine receptor-associated Na channels: relationship between 22Na influx, 45Ca influx and secretion of catecholamines in cultured bovine adrenal medullary cells. *Neuroscience.* 1985b;15:283-292.

Wada A, Yanagihara N, Izumi F, Sakurai S, Kobayasi H. Trifluoperazine inhibits $^{45}Ca^{2+}$ uptake and catecholamine secretion and synthesis in adrenal medullary cells. *J Neurochem.* 1983;40:481–486.

Wada Y, Satoh K, Taira N. Cardiovascular profile of Bay-K-8644, a presumed calcium channel activator in the dog. *Naunyn-Schmiedebergs Arch Pharmacol.* 1985a;328:382-387.

Wakade AR. Studies on secretion of catecholamines evoked by acetylcholine or transmural stimulation of the rat adrenal gland. *J Physiol.* 1981;313:463-480.

Wakade AR, Wakade TD. Contribution of nicotinic and muscarinic receptors in the secretion of catecholamines evoked by endogenous and exogenous acetylcholine. *Neuroscience.* 1983;10:973-978.

Weaver WR, Wolf KM, Chiappinelli, VA. Function heterogeneity of nicotinic receptors in the avian lateral spiriform nucleus detected with trimethaphan. *Mol Pharmacol.* 1994;46:993–1001.

Yanagihara N, Isosaki M, Ohuchi T, Oka M. Muscarinic receptor-mediated increase in cyclic GMP level in isolated bovine adrenal medullary cells. *FEBS Lett.* 1979;105:296-298.