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# Inhibitory Mechanism of Chlorogenic Acid, an Active Component of Coffee, on Adrenal Catecholamine Secretion

조선대학교 대학원

의 학 과

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지도교수 김 상 훈

이 논문을 의학 박사학위신청 논문으로 제출함

2019 년 10 월

조선대학교 대학원

의 학 과

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# 박정현의 박사학위논문을 인준함

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2019 년 12 월

## 조선대학교 대학원

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박 정 현

지도교수 : 김 상 훈

조선대학교 대학원 의학과

Chlorogenic acid, resveratrol 및 flavonoids를 포함한 상이한 천연물이 건강에 대한 여러 유익한 효과를 가지고 있다고 알려져 있다. 또한 푸른 커피 추출물에서 유래된 chlorogenic acid가 혈압강하에 효과적인 약물이며, 또한 경증의 고혈압환자에서도 신뢰성이 보고되어 있다. 무작위 이중맹검시험에서 커피콩을 복용한 그룹이 어떠한 부작용도 없이 혈압을 현저하게 감소시켰다. 식물에서 유래된 페놀화합물이 강력한 혈관확장작용을 가지고 있으며, 고혈압관련 질환에도 도움이 될 것이라고 보고하였다.

이와 같은 여러 결과에도 불구하고, 자율신경계 특히 부신수질의 카테콜아민(CA) 분비에 대한 커피성분에 대한 효과에 대해서는 아직까지 알려진 바가 없다. 따라서 본 연구에서는 커피의 유효 주성분으로 알려진 chlorogenic acid 가 흰쥐 적출부신의 관류모델에서 CA 에 미치는 효과를 탐색하고, 그 작용의 본태를 구명하고자 본 연구를 이행하여 얻은 연구결과는 다음과 같다.

Chlorogenic acid (30~300 μM)를 90분간 부신수질 내로 관류하는 동안 acetylcholine (5.32 mM, ACh)의 CA 분비를 비교적 시간과 농도 의존적으로 감약시켰다. 또한 100 μM chlorogenic acid를 역시 90분간 관류하는 동안 100 μM DMPP (선택적

니코틴수용체 작용제), 100 ηM angiotesin II (Ang II, 안지오테신 수용체 작용제) 및 100 μM McN-A-343 (선택적 무스카린 $M_1$수용체 작용제)의 CA 분비반응은 시간 경과에 따른 억제반응을 나타내었다. 그러나, chlorogenic acid 자체는 기초 CA 분비작용에 영향을 미치지 않았다. 또한, 100 μM chlorogenic acid를 관류시, 직접적인 세포막 탈분극제인 56 mM 고칼륨, 선택적 전압의존적 나트륨통로 활성화제인 50 μM veratridine, L-형 dihyropyridine계 전압의존적 칼슘통로 활성화제인 10 μM Bay-K-8644 및 세포질 내 내형질세망막의 $Ca^{2+}$-ATPase 억제제인 10 μM cyclopiazonic acid의 CA 유리작용이 역시 시간 의존적인 감소반응을 나타내었다. 흥미롭게도, 100 μM chlorogenic acid 와 산화질소 (NO)의 합성효소 억제제인 30 μM L-NAME을 동시에 90분간 관류하는 동안 Ang II 및 ACh의 CA유리반응이 100 μM chlorogenic acid 단독투여에 의한 분비억제효과와 비교한 결과 거의 대조치에 상응하는 수준으로 회복됨을 나타내었다. 또한 100 μM chlorogenic acid 관류 후에 실제로 유리된 NO 함량이 기초 NO 유리량에 비해 뚜렷이 증가하였다.

이상과 같은 연구결과를 종합하면, chlorogenic acid는 흰쥐 부신수질의 관류모델에서 ACh 수용체, 즉, 니코틴 및 무스카린 수용체 및 안지오텐신II 수용체 활성화에 따른 CA 분비반응에 대해 뚜렷한 감약반응을 나타내었다. 이러한 chlorogenic acid의 억제반응은 흰쥐 부신수질에서 NO 합성효소의 활성화에 의해 부신수질의 크롬친화세포 내로 나트륨통로와 칼슘통로로 이들 이온의 유입억제와 세포 내 칼슘저장고로부터 칼슘유리 억제효과에 기인하며, 이와 같은 작용은 아세틸콜린 수용체 및 안지오텐신II 수용체 봉쇄와 관련이 있는 것 같다. 이러한 연구결과를 바탕으로 보건데 chlorogenic acid는 부신수질세포에서 CA반응을 차단하여, 결국 순환혈액에서 CA농도를 감소시킴으로써 고혈압 이나 협심증 같은 심혈관계 질환의 경감, 치료 및 예방에 임상적으로 유익할 것으로 생각된다.

# I. INTRODUCTION

Chlorogenic acid (IS,3R,4R.5R)-3-{[(2Z)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic acid) is an ester formed from caffeic acid and quinic acid (Fig. 1) and works as an intermediate in lignin biosynthesis (Abrankó and Clifford, 2017). Chlorogenic acid is one of the most abundant polyphenol compounds in the human diet, which is an important component of coffee. It has the capacity to manipulate the taste of coffee by modifying astringent, sweet, and sour tastes, which change with the concentration (Tajik et al., 2017).

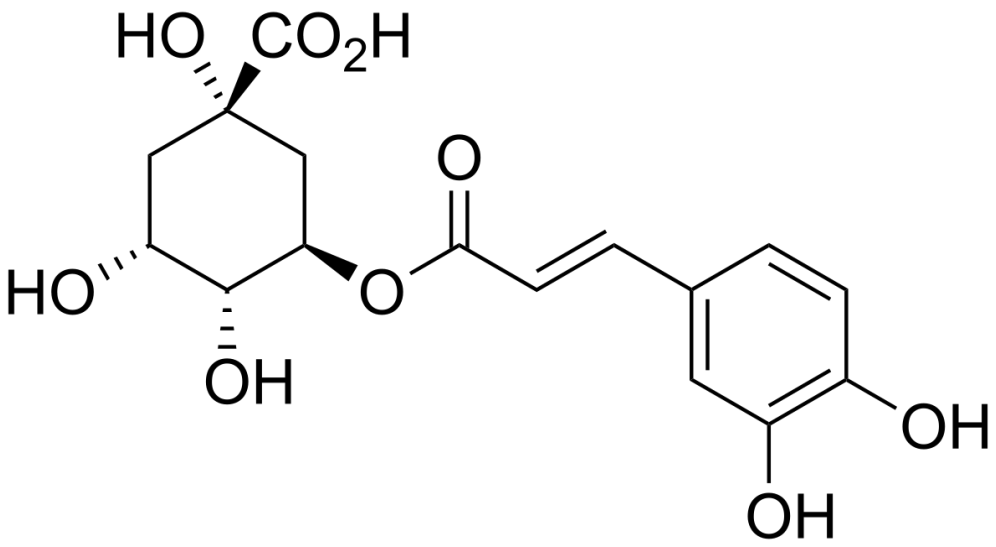


**Fig. 1.** Chemical structure of chlorogenic acid

Chlorogenic acid has a broader range of potential biological properties for health benefits, which might provide nonpharmacological and non-invasive hepatoprotective, antioxidant, anti-diabetic, antimicrobial, anticarcinogenic, anti-inflammatory, and anti-obesity strategies (Maalik et al., 2016; Santana-Gálvez et al., 2017; Tošović et al., 2017; Naveed et al., 2018). It has been postulated that different natural products involving polyphenols such as chlorogenic acid, resveratrol, and

flavonoids have several beneficial effects on health (Panche et al., 2016).

Chlorogenic acids from green coffee bean extract at a dosage of 140 mg/day lowered blood pressure in spontaneously hypertensive humans as determined by randomized clinical trial (Watanabe et al., 2006). In the contrary, its vasorelaxant effect of cynarin was not described; however, this polyphenol possesses an antioxidant effect (Sloley et al., 2001). The relationship between the antioxidant effect of phenolic compounds and their vasorelaxant effect is well established (Duarte et al., 2001).

It has been largely accepted that chlorogenic acid has several health advantages as an anti-hypertension agent (Suzuki et al., 2002b). In another study, it was reported that chlorogenic acid derived from green coffee extract was the most effective agent in lowering blood pressure and it was also trusty for patients with mild hypertension as well (Wan et al., 2013). During one randomized and double-blind clinical trial among 117 individuals with hypertension, results indicated that consuming group of coffee bean extract could significantly reduce the blood pressure without having any adverse effects compared to placebo group (Kozuma et al., 2005). The presence of phenolic compounds, (such as chlorogenic acid) that are derived from the plant extract, which has a strong vasorelaxant effect may be helpful in the hypertensive related disorders (Hakkou et al., 2017). Coffee and its derivatives can be a major source of dietary chlorogenic acid for humans. A regular cup of Arabica coffee (*Coffea arabica*) contains between 70 and 200mg of chlorogenic acid, and a cup of Robusta coffee (*Coffea canephora*) contains between 70 and 300mg of chlorogenic acid (Clifford, 1999). Daily chlorogenic acid intake in heavy coffee drinkers is about 0.5–1.0 g, whereas in coffee abstainers the daily intake can be 100 mg (Olthof et al., 2001). Thus, dietary chlorogenic acid intake can be heavily influenced by coffee consumption.

Moreover, results from a meta-analysis also indicated that chlorogenic acid can significantly reduce both systolic and diastolic blood pressure (Onakpoya et al., 2015). One study by Tom et al. (2016) stated that chlorogenic acid can alter the level of nitric oxide and therefore have a relaxing effect on vasodilation of the rat vessel.

On the other hand, it has been reported that caffeine, trigonelline, N-methylpyridinium, chlorogenic acid, catechol, pyrogallol and 5-hydroxytryptamides increased calcium signaling and

dopamine release in pheochromocytoma cells (PC-12 cells), although with different efficacies (Walker et al., 2012). Ina and his colleagues (2004) found that, in experimental menopausal model rats, chlorogenic acid decreased the ACTH level increased by ether stress more markedly than other compounds. Furthermore, chlorogenic acid significantly increased the dopamine level decreased by ether stress.

Despite of these contradictory studies, there is still little known about the functional effects of chlorogenic acid on the catecholaminergic system, especially adrenal CA release. Therefore, the present study was aimed to scrutinize the function of chlorogenic acid on the CA release in the isolated perfused model of the rat adrenal medullae, and to define its action mechanism.

# Ⅱ. MATERIALS AND METHODS

## *Experimental manipulation*

Male mature Sprague-Dowley rats (DAMOOL SCIENCE, International Customer Service, Seoul, South Korea), weighing 180 to 250 grams, were employed in the present study. The experimental animals were separately housed in individual cages. Food (Cheil Animal Chow, Seoul, Korea) and tap water were permitted freely for about 7 to 14 days to adapt to lab circumstances. The rat, on the day of the experiment, was anesthetized by intraperitoneal administration with thiopental sodium at a dose of 50 mg/kg, and fastened in supine position on the operation platform.

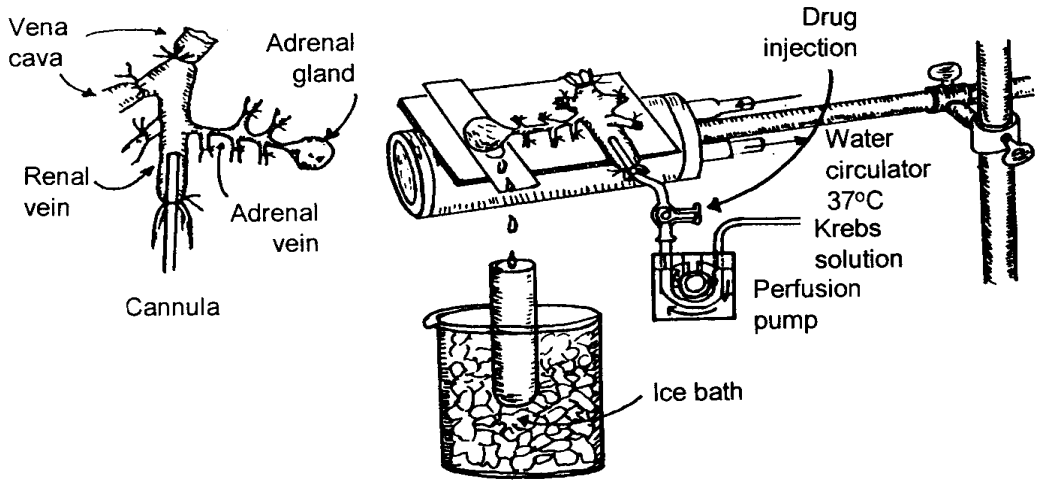


**Fig. 2.** Schematic figure of the preparation used to perform the CA secretion in the isolated perfused model of the rat adrenal medulla.

***Isolation of the adrenal gland:*** The adrenal medulla was isolated by some modification of previous methodology (Wakade, 1981). Through incision along the midline, the abdomen was opened, and usually using the three-hook retractors, the left adrenal gland with its surrounding area

were exposed The portions of the stomach, intestine and the liver are not subduced, but shunt to the right side and enclosed with 0.9% saline-soaked gauge pads, and urine in the bladder is removed in order to secure enough working space for ligating the blood vessels and cannulations. A cannula, employed for perfusion of the rat adrenal gland, is intromited into the distal portion of the renal vein after the tight tying of all branches of adrenal vein (if any), vena cava and aorta. Prior to tying of vessels and cannulations, heparin (400 IU/mL) is administered into vena cava to block the blood clotting. To make a small slit into just opposite side of adrenal vein, the adrenal cortex is cut down. The adrenal medulla is started to infuse, checking for no leakage, and the perfusion stream flowed out only from the small slit on the adrenal cortex. Then the adrenal medulla together with the cannula and the tied blood vessels, was prudently separated from the anesthetized rat and fixed on the platform of a leucite chamber. The chamber was continually circulated at a constant speed with water heated at 37 ±1℃ (Fig. 2).

### *Perfusion of the adrenal gland*

The perfusion of the adrenal medullae was performed by means of peristaltic pump (Isco, St. Lincoln, NE, U.S.A.) at a rate of 0.31 mL/min. The adrenal perfusion was made with Krebs-bicarbonate solution including the following composition (mM): KCl, 4.7; $KH_2PO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; glucose, 11.7; NaCl, 118.4; $MgCl_2$, 1.18. The perfusion solution was bubbled continually with 95 % $O_2$ + 5 % $CO_2$ and the pH of the Krebs-bicarbonate solution was steadily adjusted to 7.4 ~ 7.5. Ascorbic acid (100 μg/mL) and disodium EDTA (10 μg/mL) to blockade oxidation of the CA were added into the Krebs-bicarbonate solution.

### *Medication of drugs*

Adrenal Perfusion od angiotensin II (100 nM) and DMPP (100 μM) for 2 minutes and/or a single injection of KCl (56 mM) and ACh (5.32 mM) in a volume of 50 μL were administered into infusion stream via a three-way stopcock, respectively. Veratridine (50 μM), cyclopiazonic acid (10 μM), McN-A-343 (100 μM), and Bay-K-8644 (10 μM) were also given by infusion for 4 min, respectively.

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

### *Collection of the perfusate*

In usual, the perfusate collection before secretagogue administration was performed for 4 min to assay the basal CA release (background sample). Immediately following the background sample was collected, the perfusates were continuously gathered in another tube as fast as the perfusion solution including the facilitator secretagogue got to the adrenal medulla. The perfusate of the stimulated sample was collected for 4 or 8 min. The CA quantity secreted in the background sample was subtracted from that secreted from the stimulated sample to gain the net CA release, which is described in all of the figures.

Just before check the impact of chlorogenic acid on the basal and the produced CA secretion, the infusion into the adrenal medulla was made with normal Krebs-bicarbonate solution for 90 min, and then the perfusate collection was done for a special period (background sample). Then the perfusion solution was switched with the one containing the stimulatory secretagogue or with chlorogenic acid, and the perfusate collection was made for the same period as that for the background sample. The perfusate collection from the adrenal medulla was performed in chilled tubes.

### *Assay of catecholamines*

The amount of CA, together with epinephrine, norepinephrine and dopamine in the perfusate was quickly determined by the fluorometric method of Anton and Sayre (1962) without the intermediate purification with alumina for the reasons described earlier (Wakade, 1981) employing fluorospectrophotometer (Kontron Co., Milano, Italy).

The perfusate solution in a volume of 0.2 mL was utilized for the assay reaction. The CA amount in the perfusate solution of stimulated medulla by secretagogues utilized in this work was quite

enough to gain readings several folds much morer than them of unstimulated samples (control). The CA amount in the perfusate solution was described in terms of norepinephrine (base) equivalents.

### *Assay of NO release*

The NO-selective microelectrode (ami700, Innovative Instruments Inc., Tampa, FL, USA) and an amplifier (inNo meter, Innovative Instruments Inc., Tampa, FL, USA) were utilized for measurement of NO liberated from the perfused adrenal medulla. NO liberated from adrenal medulla was assayed as the integrated signal detected by the microelectrode after perfusion of chlorogenic acid into rat adrenal medulla, as previously described (McVeigh et al., 2002). The value of electrode was calibrated by the approved standard levels of NO in 0.5% (wt/vol) KI in 0.1 Mol/L $H_2SO_4$ from $NaNO_2$ standards. NO liberation was assayed as the current concentration detected at the electrode after infusion of chlorogenic acid into adrenal medulla. The net NO release was described as picomole in the figure.

### *Statistical analysis*

The statistical difference between the control and the drug-treated group was evaluated by employing the Student's *t*-test. A P-value of less than 0.05 was regarded statistically to elicit significant changes unless specifically described in the text. The values described in this text expressed in the means and the standard errors of the mean (S.E.M.). All data obtained in this study were statistically evaluated by the computer program (Tallarida and Murray, 1987).

### *Drugs and their sources*

The following drugs were used: chlorogenic acid, ascorbic acid, cyclopiazonic acid, angiotensin II, norepinephrine bitartrate, calcium chloride, acetylcholine chloride, veratridine hydrochloride, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), sodium bicarbonate, potassium chloride (KCl), $N^{\omega}$-nitro-L-arginine methyl ester hydrochloride (L-NAME), potassium phosphate, sodium

chloride, glucose, 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.), and 3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride [McN-A-343] (RBI, U.S.A.). Drugs were melted in distilled water (stock) and then put into the normal Krebs-bicarbonate solution. Extraordinarily, Bay-K-8644 was dissolved in 99.5 % (stock) ethanol, and then diluted properly with the perfusion solution (final concentration of ethanol was less than 0.1 %). Concentrations of all drugs utilized in this study are depicted in terms of their molar base.

# III. RESULTS

## *Effects of chlorogenic acid on the CA secretion caused by angiotensin II, ACh, DMPP, and McN-A-343 from the perfused adrenal medullae*

Since chlorogenic acid revealed the powerful inhibitory effect on Ach-evoked CA secretion, it was initially attempted to determine the effect of chlorogenic acid itself on CA secretion from the perfused adrenal medullae. However, in the present study, chlorogenic acid itself did not impact on the spontaneous CA secretion in the perfused adrenal medullae (data not described). Therefore, it was decided to investigate effects of chlorogenic acid on the CA secretory responses produced by activation of angiotensinergic receptors as well as cholinergic receptors. Secretagogues used in the present experiment injected or perfused at 15 to 20 min-intervals. Chlorogenic acid was given for 90 minutes after the substantiation of the control CA release.

When 5.32 mM ACh in a volume of 50 μL was injected into the isolated adrenal medullae, the secreted CA content was 1343±42 ng for 4 min. But, in the simultaneous existence of chlorogenic acid in the range of 30 ~ 300 μM for 90 min, ACh-caused CA release was strikingly attenuated in comparatively concentration- and time-dependent fashion. As illustrated in Fig. 3, under the coexistence of chlorogenic acid, the CA secretory responses were depressed maximally to 47% of the corresponding control secretion (100%).

DMPP (100 μM, a selective neuronal nicotinic [$N_n$] receptor agonist in autonomic sympathetic ganglia) caused a fast and steep elevation in the CA secretion (1232±48 ng for 0-8 min). However, as depicted in Fig. 4, DMPP-produced CA secretion in the presence of chlorogenic acid (100uM) for 90 min was greatly reduced to 65% of the control secretion.

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 (690±32 ng for 0-4 min). However, under the existence of chlorogenic acid (100 μM), McN-A-343-produced CA release was greatly decreased to 65% of the corresponding control secretion as shown in Fig. 5.

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 chlorogenic acid on Ang II-produced CA secretion. Ang II (100 nM) greatly elevated the CA secretion (676±34 ng for 0-4 min), however in the presence of chlorogenic acid (100 μM), Ang II-produced CA secretion was markedly inhibited to 67% of the corresponding control secretion (Fig. 6).

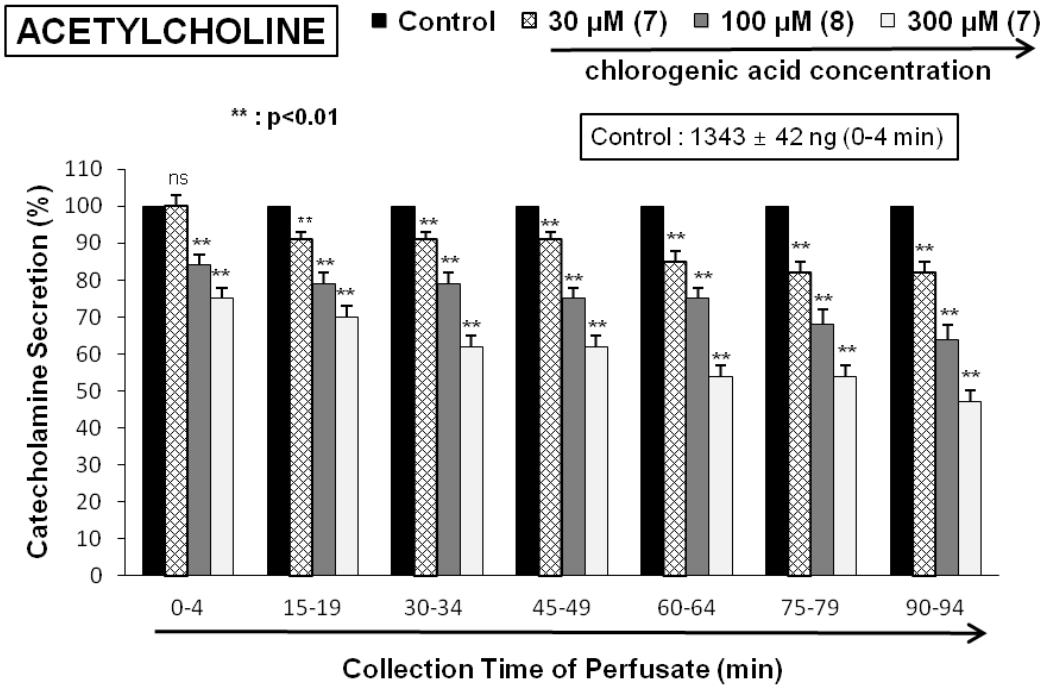


**Fig. 3.** Dose-dependent effects of chlorogenic acid on acetylcholine (ACh)-caused secretory responses of catecholamines (CA) in the isolated adrenal medullae. The CA release produced by a single injection of ACh (5.32 mM) in a volume of 50 μL was induced at 15 min intervals during the perfusion of 30, 100 and 300 μM of chlorogenic acid for 90 min as designated by the arrow marks, respectively. The number in each parenthesis displays number of adrenal medulla. T-type bar on each column indicates standard error of the mean (S.E.M.). Ordinate: the quantities of CA released in adrenal medulla (% of control). Abscissa: collecting time of the perfusate (min). Statistical significant difference was derived from comparison of the corresponding control value with individual dose-treatment group of chlorogenic acid. The perfusate following ACh-injection was collected for 4 minutes.

**: $p<0.01$, ns: Not statistically significant.

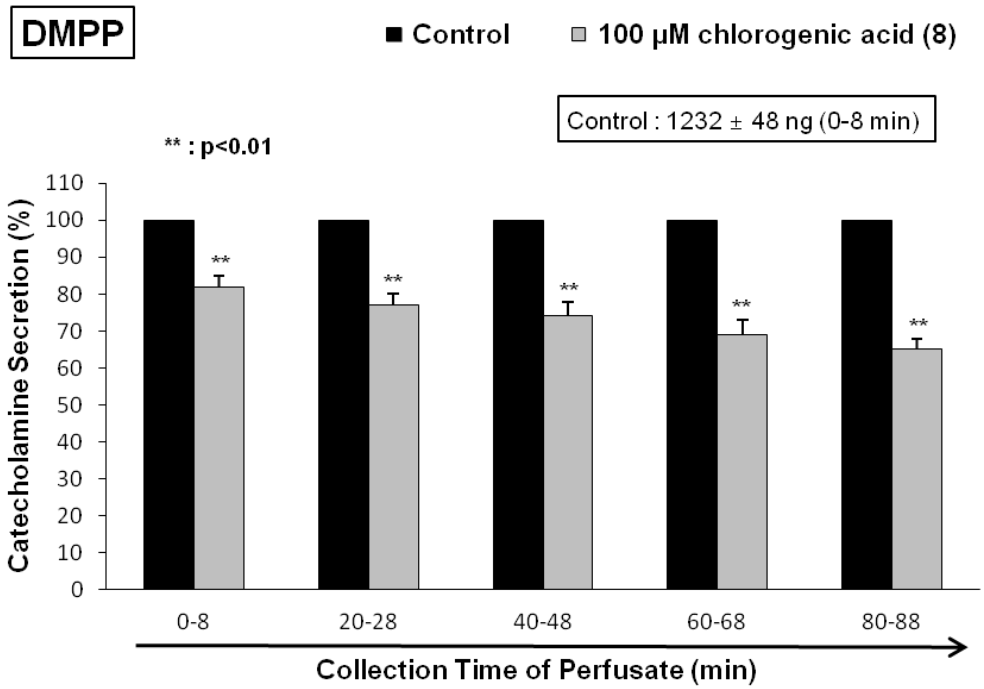


**Fig. 4.** Time-course effects of chlorogenic acid on DMPP-caused CA secretion from the isolated adrenal medullae. The CA release produced by infusion of DMPP (100 μM) for 2 min was made at 20 min interval during perfusion of 100 μM chlorogenic acid for 90 min. The perfusate following infusion of DMPP was collected for 8 minutes. Other legends are the same as in Fig. 3. **: $p<0.01$.

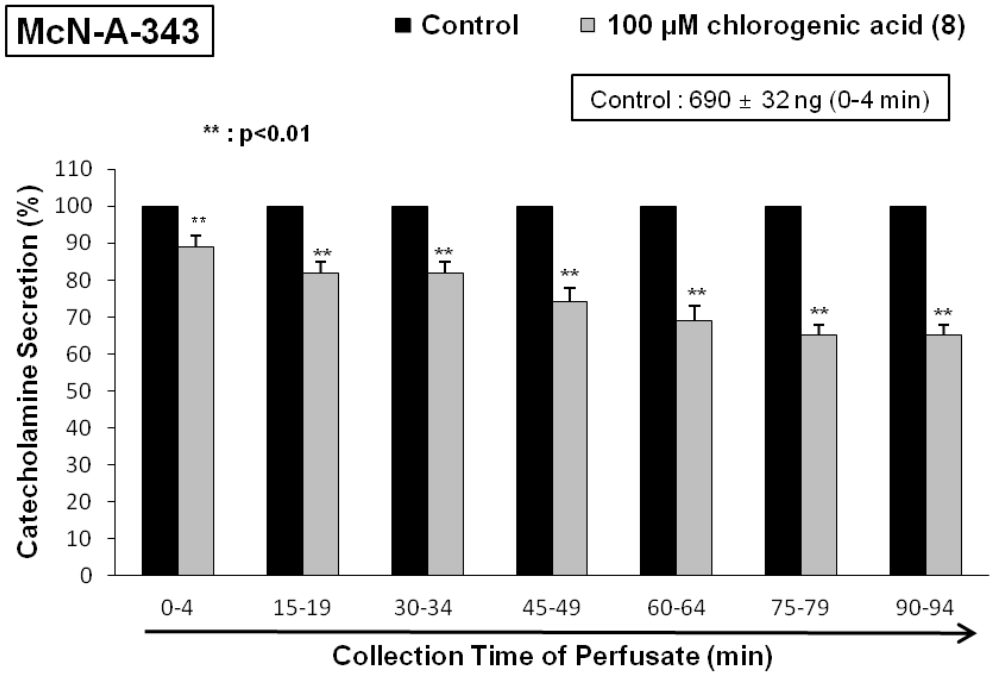


**Fig. 5.** Time-course effects of chlorogenic acid on McN-A-343-caused CA secretion from the isolated adrenal medullae. The CA release produced by infusion of McN-A-343 (100 μM) for 4 min was produced at 15 min interval during the loading of 100 μM chlorogenic acid for 90 min. The perfusate following the loading of McN-A-343 was collected for 4 minutes. Other legends are the same as in Fig. 3. **: p<0.01.

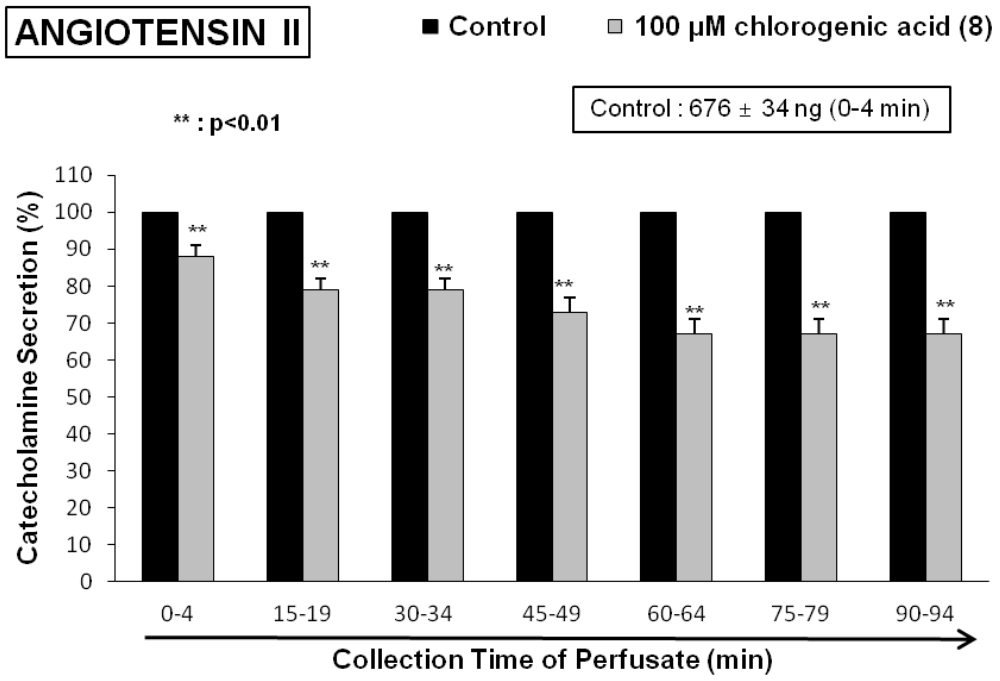


**Fig. 6.** Time-course effects of chlorogenic acid on angiotensin II-caused CA secretion from the isolated adrenal medullae. The CA release produced by angiotensin II (100 nM) infused into an adrenal medulla for 1 min was made at 15 min intervals during the loading of chlorogenic acid (100 μM) for 90 min. The perfusate following the infusion of angiotensin II was gathered for 4 minutes. Other legends are the same as in Fig. 3. **: $p<0.01$.

### *Effects of chlorogenic acid on the CA secretion caused by high $K^+$, Bay-K-8644, cyclopiazonic acid, and veratridine from the perfused adrenal medullae*

High KCl, a depolarizing agent, significantly elevated the CA release (732±36 ng for 0-4 min). However, in the presence of chlorogenic acid (100 μM) for 90 min, high $K^+$ (56 mM)-caused CA secretion was maximally suppressed to 63% of the corresponding control at 90~94 min periods, as in Fig. 7.

Bay-K-8644, a calcium channel activator, is known to enhance the basal $Ca^{2+}$ uptake (Garcia et al., 1984) and CA release (Lim et al., 1992), it was of interest to check the impacts of chlorogenic acid on Bay-K-8644-caused CA release in the perfused rat adrenal medulla. Bay-K-8644 (10 μM)-caused CA release during loading with chlorogenic acid (100 μM) was diminished to 67% of the control release excluding the early 15 min period in comparison with the control level (721±32 ng for 0-4 min) from 5 rat adrenal medullas, as illustrated in Fig. 8.

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 chlorogenic acid on cyclopiazonic acid-caused CA released was attained as depicted in Fig. 9. In 7 rat adrenal glands, in the existence of chlorogenic acid (100 μM) for 90 min, cyclopiazonic acid ($10^{-5}$ μM)-caused CA release was also depressed to 68% of the control release (604±27 ng for 0-4 min), even though it was not impacted only for the first period (0-4 min).

It has been found that veratridine-produced $Na^+$ 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. 10, veratridine (50 μM) sharply increased the CA release (825±42 ng for 0-4 min). In 8 rat adrenal medullae, chlorogenic acid (100 μM) also attenuated veratridine-produced CA secretion to 64% of the corresponding control release.

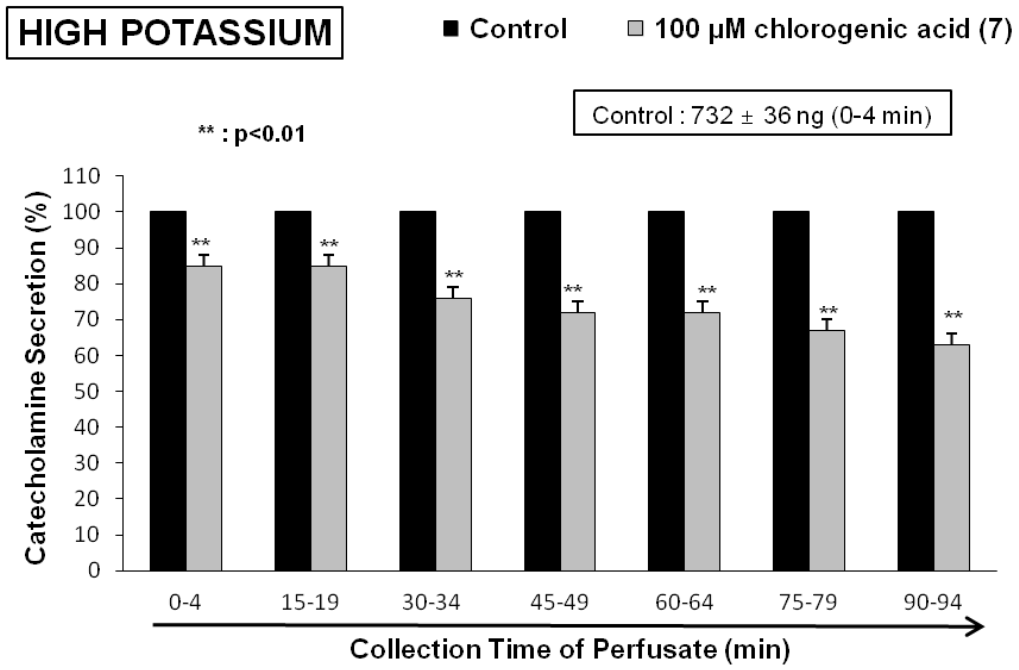


**Fig. 7** Time-course effects of chlorogenic acid on high potassium-caused CA secretion from the isolated adrenal medullae. The CA secretion produced by a single injection of $K^+$ (56 mM) in a volume of 0.05 mL was made at 15 min intervals during the loading of 100 μM chlorogenic acid for 90 min as designated by the arrow marks. The perfusate following perfusion of high $K^+$ was gathered for 4 minutes. Other legends are the same as in Fig. 3. **: $p<0.01$.

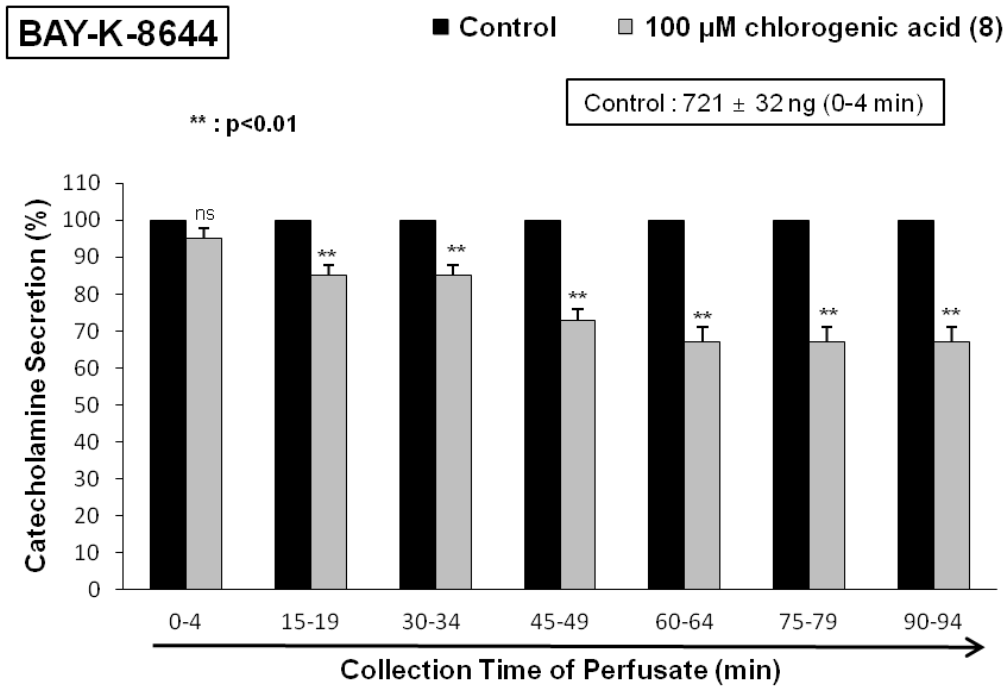


**Fig. 8.** Time-course effects of chlorogenic acid on Bay-K-8644-caused CA secretion from the isolated adrenal medullae. The CA release produced by Bay-K-8644 (10 μM) infused into an adrenal vein for 4 min was made at 15 min intervals during the perfusion of chlorogenic acid (100 μM) for 90 min. The perfusate following infusion of Bay-K-8644- was gathered for 4 minutes. Other legends are the same as in Fig. 3. **: $p<0.01$, ns: Not statistically significant.

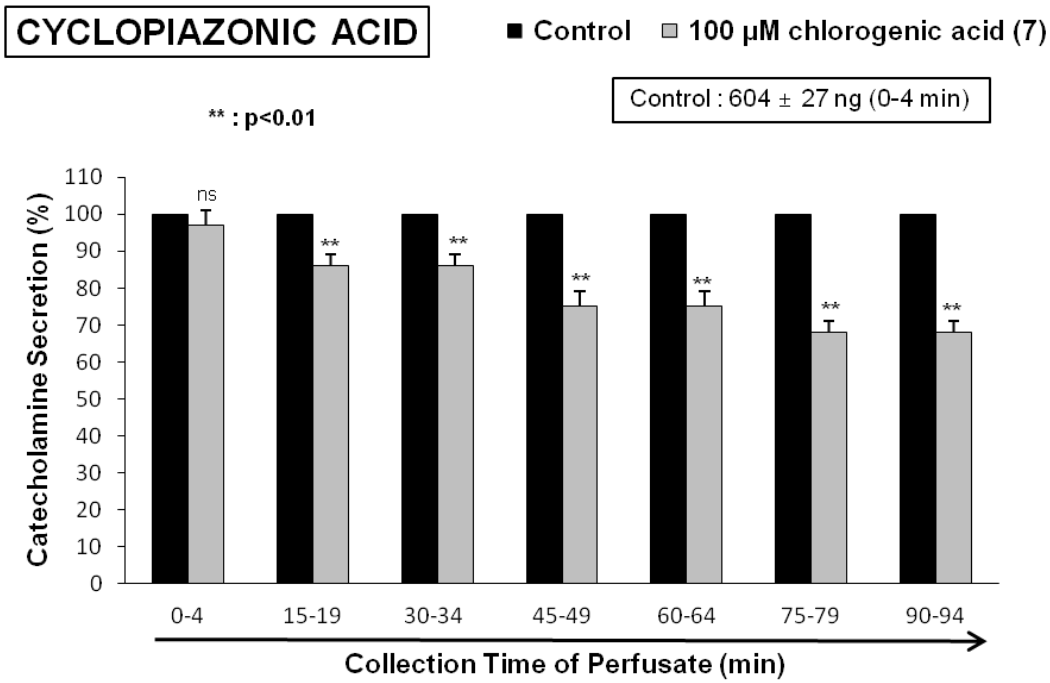


**Fig. 9.** Time-course effects of chlorogenic acid on cyclopiazonic acid-caused CA secretion from the isolated adrenal medullae. The CA release produced by cyclopiazonic acid (10 μM) infused into an adrenal medulla for 4 min was made at 15 min intervals during the infusion of chlorogenic acid (100 μM) for 90 min. The perfusate following infusion of cyclopiazonic acid was gathered for 4 minutes. Other legends are the same as in Fig. 3. **: $p<0.01$, ns: Not statistically significant.

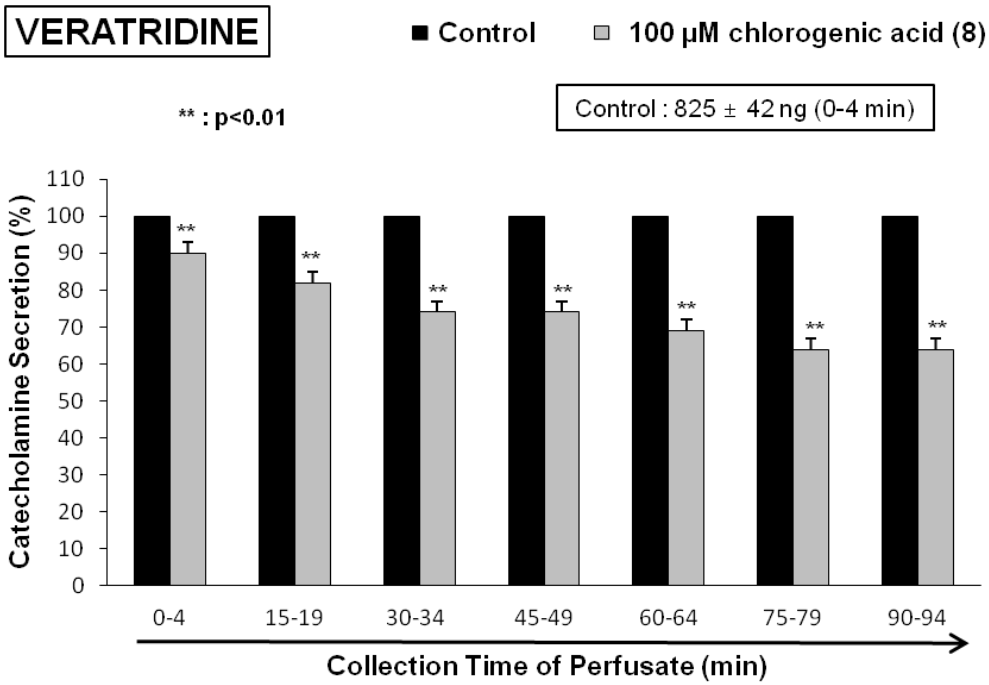


**Fig. 10.** Time-course effects of chlorogenic acid on veratridine-caused CA secretion from the isolated adrenal medullae. The CA release produced by veratridine (50 μM) given into an adrenal medulla for 4 min was made at 15 min intervals during perfusing with chlorogenic acid (100 μM) for 90 min. The perfusate following the infusion with veratridine was gathered for 4 minutes. Other legends are the same as in Fig. 3. **: $p<0.01$.

### *Impact of simultaneous perfusion with chlorogenic acid and L-NAME on ACh- and angiotensin II-caused CA release in the isolated adrenal medullae*

It was found that, in this study, chlorogenic acid meaningfully repressed the CA liberating response formed by activation of angiotensinergic receptors along with cholinergic receptors in the perfused adrenal medullae. Thus, in order to explore the association between NO release and chlorogenic acid-produced inhibitory effect on the CA release in the adrenal medullae, the impact of L-NAME on chlorogenic acid-induced repression of CA release caused by ACh and Ang II was examined.

In the present work, during the concurrent infusion of L-NAME (30 μM) and chlorogenic acid (100 μM) for 90 min in 8 rat adrenal medullae, ACh (5.32 mM)-produced CA release was largely recovered to 100~87% of the corresponding control level (1334±46 ng for 0-4 min) as compared with that of chlorogenic acid (100 μM)-treated alone, as shown in Fig. 11.

Moreover, in 7 rat adrenal medullae, during the simultaneous presence of L-NAME (30 μM) and chlorogenic acid (100 μM), the Ang II (100 nM)-produced CA secretion returned to 100~86% of the control release (665±30 ng for 0-4 min in comparison with the inhibitory effect of chlorogenic acid-treatment alone, as illustrated in Fig. 12.

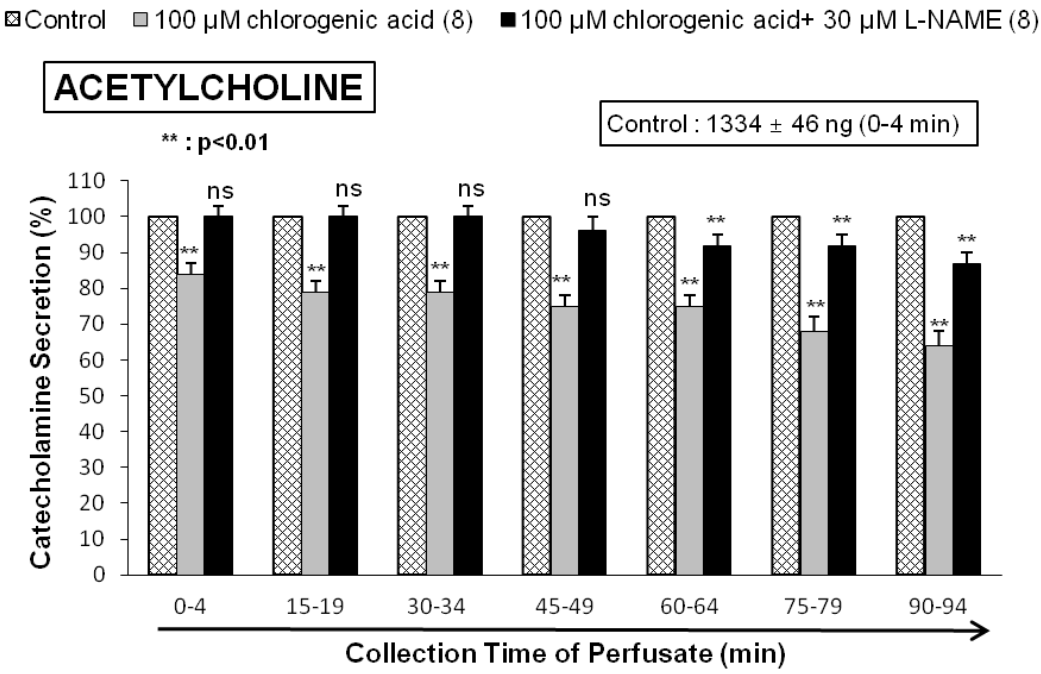


**Fig. 11.** Impact of simultaneous perfusion with chlorogenic acid and L-NAME on acetylcholine-caused CA release in the isolated adrenal medullae. The CA secretion produced by a single injection of ACh (5.32 mM) in a volume of 50 μL was made at 15 min intervals during concurrent perfusion with chlorogenic acid (100 μM) and L-NAME (30μM) for 90 min. Statistical significant difference was attained by comparison of the control value with chlorogenic acid-treated alone group or group co-treated with chlorogenic acid plus L-NAME. The perfusate following the injection of acetylcholine was gathered for 4 minutes. Other legends are the same as in Fig. 3. **: $p<0.01$, ns: Not statistically significant.

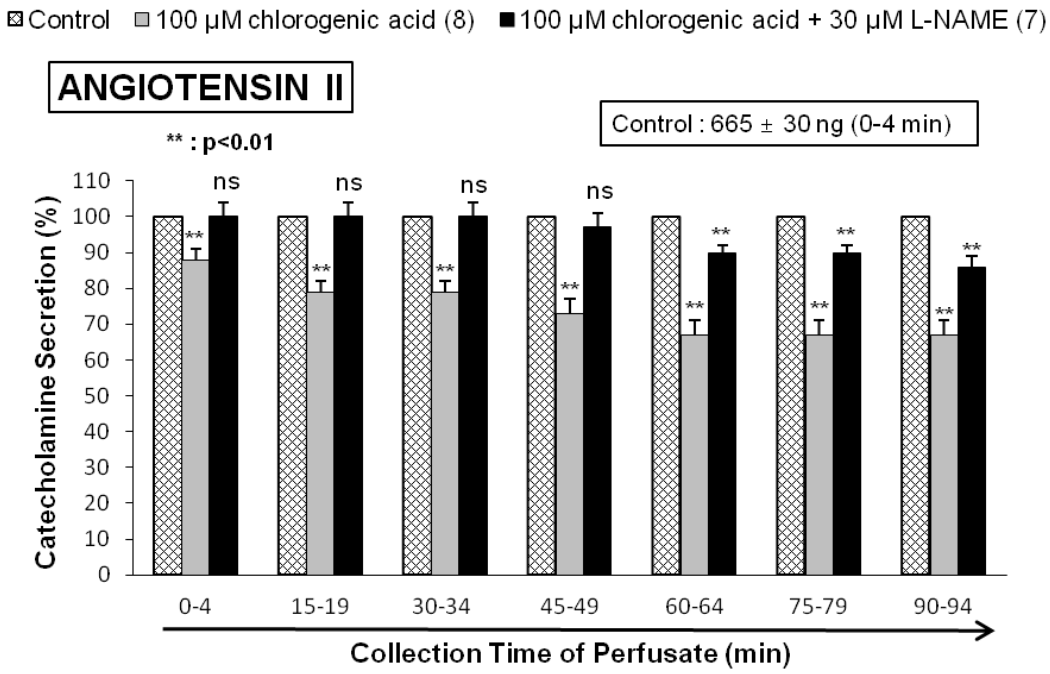


**Fig. 12.** Impact of simultaneous perfusion with chlorogenic acid and L-NAME on angiotensin II-caused CA release in the isolated adrenal medullae. The CA secretion produced by the perfusion with angiotensin II (100 nM) for 1 min was made at 15 min intervals during concurrent perfusion with chlorogenic acid (100 μM) and L-NAME (30μM) for 90 min. The perfusate following the perfusion with angiotensin II was gathered for 4 minutes. Other legends are the same as in Fig. 3. **: $p<0.01$, ns: Not statistically significant.

### *Influence of chlorogenic acid on the liberated content of nitric oxide in the isolated adrenal medullae*

As illustrated in Fig. 11~12, the CA-releasing responses produced by ACh and Ang II were restored mostly to their control secretions under the simultaneous existence of chlorogenic acid and L-NAME. Thus, it was attempted directly to assay the content of NO liberated from the perfused rat adrenal medullae during the perfusion of chlorogenic acid.

In 4 adrenal medullae, spontaneously liberated content of NO in the adrenal medulla just before the perfusion of chlorogenic acid was 37.4±6 picomoles. However, 8 min after loading with chlorogenic acid (100 μM) it was greatly elevated to 66.1±11 picomoles ($p<0.01$), which was 177% of the basal liberation, as shown in Fig. 13.

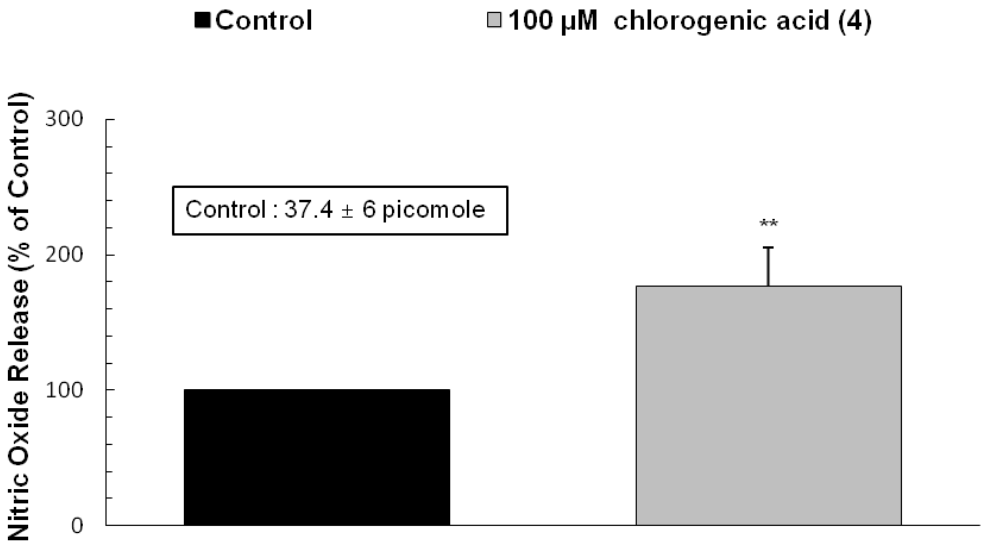


**Fig. 13.** Impact of chlorogenic acid on the liberated content of nitric oxide in the isolated adrenal medullae. After the perfusion with chlorogenic acid (100 μM) at a rate of 0.31 mL/min, perfusate sample was gathered for 8 min. Ordinate: the liberated NO content in the adrenal gland (% of control). Abscissa: Before and after treatment with chlorogenic acid. Statistical significant difference was analyzed by comparison of the control value with chlorogenic acid-treated group. **: $p<0.01$.

# IV. DISCUSSION

The results of this experimental study are the first evidence demonstrating that chlorogenic acid greatly suppresses the CA secretion produced by the activation of acetylcholinergic receptors as well as angiotensinergic receptors from the isolated perfused model of the rat adrenal medullae. This chlorogenic acid-produced reduction of CA release appears to be attributed by depressing influx of both $Na^+$ and $Ca^{2+}$ ions through their channels into the adrenal chromaffin cells and also by reducing $Ca^{2+}$ release in the cytoplasmic calcium pool partly via the enhanced NO formation through neuronal NO synthase activation, which is relevant to the blockade of angiotensinergic and neuronal cholinergic receptors.

In this study, during the simultaneous existence of chlorogenic acid and L-NAME (an NO synthase inhibitor), the CA secretory action elicited by ACh and Ang II were mostly recovered to their control secretion as compared with their inhibitory action of chlorogenic acid-treatment alone. This is well accordance with the finding that, in as series of studies, ferulic acid of the chlorogenic acid metabolites had greatest effect on BP reduction (Suzuki et al., 2002a; Suzuki et al., 2002b). Interestingly, ferulic acid contained an ortho-methoxy-substituted-catechol structure and had been implicated to work as a non-selective NAD(P)H oxidase antagonist by reacting with sulfhydryl groups (Johnson et al., 2002; Kanegae et al., 2007). In aortic rings pre-contracted with phenylephrine (an alpha-adrenergic neurotransmitter agonist), the administration of ferulic acid greatly increased NO bioavailability and enhanced acetylcholine induced endothelial-dependent vasodilation (Suzuki et al., 2007), but had no effect on sodium nitroprusside (a direct NO donor)-induced endothelium-independent vasodilation (Suzuki et al., 2006a; Suzuki et al., 2007), signifying that vascular integrity (in particular intact endothelium) is essential for chlorogenic acids to lower BP. In vivo, chlorogenic acids significantly increased levels of urinary NO metabolites (that is, the nitrite/nitrate ratio) in spontaneously hypertensive rats (Suzuki et al., 2006a; Suzuki et al., 2008), but when non-selective nitric oxide synthase inhibitor No-nitro-L-arginine methyl ester hydrochloride (L-NAME) was administered simultaneously, the hypotensive effect of chlorogenic acids disappeared (Suzuki et al., 2006a; Suzuki et al., 2006b). Moreover, in this study, after loading

of chlorogenic acid into adrenal medulla, formation of NO was portentously escalated as illustrated in Fig. 13. Taking account of these results, in this work, it looks that chlorogenic acid depresses the CA secretory effect revealed by cholinergic secretagogues as well as Ang II via escalated NO formation in adrenal medullary chromaffin cells, since, during concurrent infusion of chlorogenic acid and L-NAME (an inhibitor of NO synthase) for 90 min, ACh- and Ang II-caused CA secretory responses were nearly returned to the control value in comparison to that of chlorogenic acid-treatment alone, and also substantially, chlorogenic acid obviously escalated NO liberation in the rat adrenal medullae.

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-produced 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 $K^+$-produced or the nicotinic receptor agonist DMPP-produced 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. Based on previously reported studies, these experimental data strongly suggest that chlorogenic acid can trigger neuronal NO synthase (nNOS) in the adrenal chromaffin cells, producing suppression of the CA release through escalated NO formation, moreover the direct suppressive action on the CA secretion. In supporting of this finding, previous studies in mammals suggest that NO inhibits CA secretion by promoting a cascade of events beginning with activation of soluble guanylyl cyclase (sGC) and leading to phosphorylation of $Ca^{2+}$ channels and an attenuation of the inward $Ca^{2+}$ flux in response to stimulation (Schwarz et al., 1998; Ferrero et al., 2000; Hirooka et al., 2000; Vicente et al., 2002). In the previous study, the sGC inhibitor, ODQ was used to prevent $Ca^{2+}$ channel phosphorylation during electrical stimulation. In the presence of ODQ, there was a pronounced increase in stimulus-evoked CA secretion, suggesting that the activation of sGC and the downstream events are important factors leading to the decrease in CA secretion from the perfused posterior cardinal vein (PCV) preparation (McNeil and Perry, 2005). In the present study, the finding that chlorogenic acid decreased the CA

outflow from the perfused adrenal medullae through the increased production of NO, suggesting that the activation of sGC by NO would seem to be the primary mechanism. Recently, it has been known that sGC is the intracellular receptor of NO (Xiao et al., 2019). This activation of sGC produces the conversion of GTP to the secondary messenger cGMP. cGMP regulates a series of downstream cascades through activating many effectors. NO-sGC-cGMP pathway plays important roles in many physiological processes in body, including platelet aggregation, smooth muscle relaxation and neurotransmitter delivery (Xiao et al., 2019).

In contrast, it has been reported that L-NAME inhibits ACh-produced CA secretion in bovine chromaffin cells (Uchiyama et al., 1994), and also that the NO donor sodium nitroprusside (SNP) enhances nicotine-produced CA secretion in cultured bovine chromaffin cells (O'Sullivan and Burgoyne, 1990). These findings indicate that NO may enhance cholinergic agonist-produced 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).

Commonly, the adrenal medulla has been utilized as a good system to explore many cellular actions, involving noradrenergic nerve cells along with neurons. During neurogenic activation of the adrenal medulla, ACh is released from splanchnic nerve endings and stimulates cholinergic receptors on the chromaffin cell membrane (Viveros, 1975). This stimulus twitchs series of actions as stimulus-secretion coupling, causing the exocytosis of CA and other components from synaptic vesicles into the extracellular space. Ordinarily, two mechanisms are linked to the liberationof adrenal medullary hormones. When splanchnic nerves are stimulated, ACh is liberated from the nerve terminals, and then this liberated ACh stimulates AChergic cholinoceptors (nicotinic receptors) and evokes the release of CA. In accordance with this mechanism, the results of the present study validated that chlorogenic acid represses the CA secretion caused by stimulating acetylcholinergic (nicotinic and muscarinic) receptors as well as angiotensinergic receptors in the adrenal medulla. These present data imply that this chlorogenic acid-caused suppressive action on the CA release can contribute partly to blood pressure-lowering 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 the light of this result, the present data validate that chlorogenic acid can diminish CA secretion caused by nicotinic receptor activation of the splanchnic nerve terminal, which is mediated by the blockade of neuronal nicotinic ($N_n$) receptors. The CA secretion produced by AChergic nicotinic agonist or the splanchnic activation is thought to be mediated by activation of $N_n$ receptors of the adrenomedullary 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-produced 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 the present work, chlorogenic acid suppressed the CA release caused by DMPP, ACh, McN-A-343, and Ang II. Based on this findings, it has been forcefully suggested that chlorogenic acid can affect neuronal nicotinic receptor-gated cation channels.

In the present study, chlorogenic acid also time-dependently suppressed the CA secretory response produced 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 chlorogenic acid can block $Ca^{2+}$ entry via voltage-dependent $Ca^{2+}$ channels into the rat adrenomedullary 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-produced $Na^+$ influx, (ii) voltage-dependent $Na^+$ channels, responsible for veratridine-produced $Na^+$ 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 findings that the CA secretion produced by not merely high $K^+$ but also by Bay-K-8644 were markedly attenuated during the perfusion of chlorogenic acid indicates that this chlorogenic acid-produced inhibitory effect is mediated by the direct suppression of $Ca^{2+}$ entry via VDCC into the adrenochromaffin 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 chlorogenic acid diminished the CA secretion produced by Bay-K-8644 and by high $K^+$ suggest that chlorogenic acid can inhibit 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 chlorogenic acid on the CA secretion produced by ACh, DMPP, veratridine and Bay-K-8644 may be mediated by blocking $Ca^{2+}$ entry through voltage-dependent $Ca^{2+}$ channels by activation of $N_n$ receptor-gated ionic channels, liable for carbachol-produced $Na^+$ entry, as well as of voltage-dependent $Na^+$ channels, liable for veratridine-produced $Na^+$ entry.

The present study has also shown that chlorogenic acid 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 chlorogenic acid-produced suppressive effect on cyclopiazonic acid-caused CA release might be related to the mobilization of $Ca^{2+}$ from the cytoplasmic $Ca^{2+}$ pool into cytoplasm. 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., 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). Thus, it can be inferred that chlorogenic acid-produced suppressive action on McN-A-343-caused CA secretion seems to be associated to the $Ca^{2+}$ mobilization from the cytoplasmic $Ca^{2+}$ pool into cytoplasm. This implies that chlorogenic acid has suppressive action on the $Ca^{2+}$ release produced by activation of muscarinic receptors, which might be marginally responsible for the adrenal CA secretion. These data suggest that chlorogenic acid-produced repression of the CA secretion caused by cyclopiazonic acid and McN-A-343 can be exerted by the blockade of $Ca^{2+}$ release produced by stimulating muscarinic ACh receptors from the intracellular store. But in this study, it is not sure whether chlorogenic acid-produced suppressive action on $Ca^{2+}$ mobilization from intracellular store isascribed to the indirect action on the PI pathway or its direct action. In near future, it is necessary to verify the perfect property of these data.

Conclusively, as illustrated in Figure 14, the data of the present work have validated that chlorogenic acid unquestionably represses the CA release produced by stimulating acetylcholinergic nicotinic receptors along with angiotensinergic receptors in the perfused model of the isolated rat adrenal glands. It appears that this chlorogenic acid-produced suppressive action is revealed not merely by blocking the entry of $Na^{+}$ and $Ca^{2+}$ via their ionic channels into the adrenal chromaffin cells but also by oppressing the $Ca^{2+}$ release from the intracellular $Ca^{2+}$ pool partly via the escalated NO formation by triggering neuronal NO synthase. In view of these data, the consumption of chlorogenic acid can be beneficial to alleviate or protect the cardiovascular diseases, such as angina pectoris and hypertension, through attenuation of CA release in adrenal medullary cells and subsequently lessened CA concentration in the blood stream of the body.

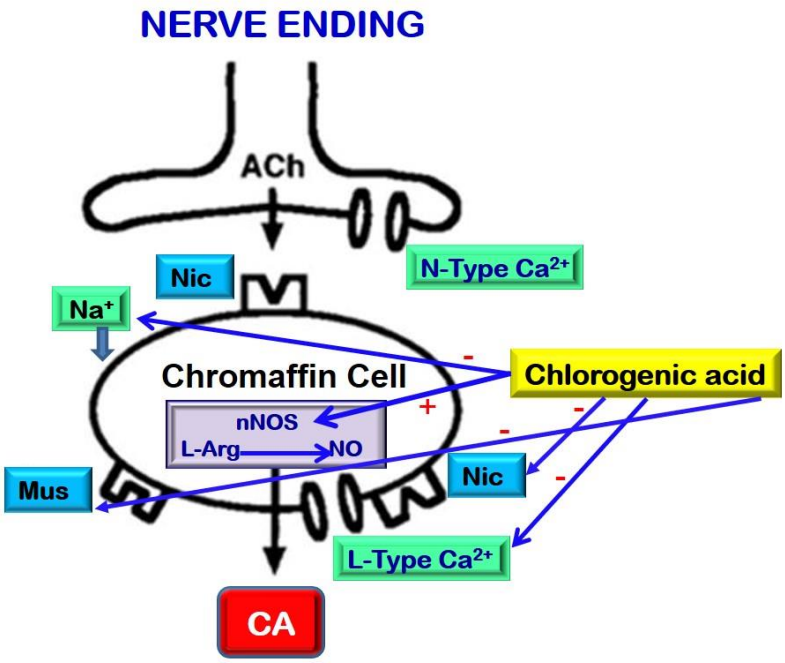


**Fig. 14.** Schematic illustration of probable action site of chlorogenic acid in the rat adrenal medulla. +: activation, -: Inhibition.

# V. SUMMARY

It has been known that different natural products involving polyphenols such as chlorogenic acid, resveratrol, and flavonoids have several beneficial effects on health. It was also reported that chlorogenic acid derived from green coffee extract was the most effective agent in lowering blood pressure and it was also trusty for patients with mild hypertension as well. Results of one randomized and double-blind clinical trial indicated that consuming coffee bean extract could significantly reduce the blood pressure without having any adverse effects. The presence of phenolic compounds that are derived from the plant extract, which has a strong vasorelaxant effect may be helpful in the antihypertensive related disorders. The present study was aimed to investigate the characteristics of chlorogenic acid, one of several components isolated from coffee, on the CA secretion from the perfused model of the isolated rat adrenal medulla, and also to clarify its mechanism of action.

Chlorogenic acid (30~300 μM), administered into an adrenal vein for 90 min, dose- and time-dependently suppressed the ACh (5.32 mM)-caused CA secretion. Chlorogenic acid (10 μM) also time-dependently repressed the CA secretion caused by angiotensin II (100 nM) and DMPP (100 μM, a selective neuronal nicotinic receptor agonist). Chlorogenic acid failed to affect basal CA output.

Furthermore, in adrenal glands loaded with chlorogenic acid (100 μM), the CA secretory responses evoked by high $K^+$ (56 mM, a direct membrane depolarizer), Bay-K-8644 (10 μM, an activator of L-type $Ca^{2+}$ channels), cyclopiazonic acid (10 μM, an inhibitor of cytoplasmic $Ca^{2+}$-ATPase), and veratridine (100 μM, an activator of $Na^+$ channels), were prominently suppressed.

Excitingly, in the concurrent presence of chlorogenic acid (100 μM) and L-NAME (an inhibitor of NO synthase, 30 μM), the CA secretion caused by ACh and angiotensin II substantially returned back to the extent of their control level compared with the suppressive activity of chlorogenic acid-treatment alone.

Virtually, the content of NO liberated from adrenal medulla following the treatment of chlorogenic acid (100 μM) was significantly escalated in comparison with the basal liberated level.

Taken together, these results are the first evidences showing that chlorogenic acid inhibits the CA secretion evoked by stimulation of both AChergic- and angiotensinergic-receptors from the perfused rat adrenal medulla. It seems that this suppressive effect of chlorogenic acid is revealed by repressing both the influx of $Ca^{2+}$ and $Na^{+}$ into the adrenomedullary chromaffin cells as well as by diminishing the release of $Ca^{2+}$ from the cytoplasmic calcium store, at least through the enhanced NO formation due to triggering nitric oxide synthase, which is pertinent to the blockade of angiotensinergic receptors and neuronal cholinergic receptors.

These results also suggest that chlorogenic acid might be benficial to avert or relieve cardiovascular diseases, such as hypertension and angina pectoris, and that chlorogenic acid has the prospective potentiality in developing a new drug, which is efficacious to treat or prevent the cardiovascular diseases.

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