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Green Tea Extract (CUMS6335) Inhibits Catecholamine Release in the Perfused Adrenal Medulla of Spontaneously Hypertensive Rats[#]

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Abstract – The aim of the present study was to examine the effects of green tea extract (CUMS6335) on the release of CA evoked by cholinergic stimulation and direct membrane-depolarization in the perfused model of the adrenal gland isolated from the spontaneously hypertensive rats (SHRs), and to establish the mechanism of action. Furthermore, it was also to test whether there is species difference between animals, and between CUMS6335 and EGCG, one of biologically the most powerful catechin compounds found in green tea. CUMS6335 (100 μg/ml), when perfused into an adrenal vein for 60 min, time-dependently inhibited the CA secretory responses evoked by ACh (5.32 mM), high K^+ (56 mM), DMPP (100 μM), and McN-A-343 (100 μM) from the isolated perfused adrenal glands of SHRs. However, CUMS6335 itself did fail to affect basal catecholamine output. Also, in adrenal glands loaded with CUMS6335 (100 μg/ml), the CA secretory responses evoked by Bay-K-8644 (10 μM) and cyclopiazonic acid (10 μM) were also inhibited in a relatively time-dependent fashion. However, in the presence of EGCG (8.0 μg/ml) for 60 min, the CA secretory response evoked by ACh, high K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid were not affected except for last period. Collectively, these results indicate that CUMS6335 inhibits the CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors as well as by direct membrane-depolarization from the perfused adrenal gland of the SHR. It seems that this inhibitory effect of CUMS6335 is exerted by blocking both the calcium influx into the rat adrenal medullary chromaffin cells and the uptake of Ca^{2+} into the cytoplasmic calcium store, which are at least partly relevant to the direct interaction with the nicotinic receptor itself. It seems likely that there is much difference in mode of the CA-releasing action between CUMS6335 and EGCG.

Keywords – Green tea extract (CUMS6335), epigallocatechin gallate (EGCG), catecholamines (CA), adrenal medulla, spontaneously hypertensive rats (SHRs)

Introduction

Recently, it has been shown that green tea extract (CUMS6335) inhibits the secretory responses of catecholamines (CA) evoked by cholinergic (nicotinic and muscarinic) stimulation and direct membrane-depolarization in the perfused adrenal medulla isolated from the rat (Lim *et al.*, 2003) and the rabbit (Lim, 2005). It is also found to cause vascular relaxation at least partly through the blockade of adrenergic α-receptors in aortic strips isolated from both normotensive rat (Lim *et al.*, 2003) and rabbit (Lim *et al.*, 2005). There are so far many reports about the effects of green tea on cardiovascular system. It has been reported that ingestion of caffeine results in a transient increase in blood pressure in subjects who have avoided caffeine for 12 h or more (Sung *et al.*, 1994; Pincomb *et al.*, 1996). Ingesting caffeine-containing tea also induces a transient increase in blood pressure (Quinlan *et al.*, 1997). However, extracts of tea (Fitzpatrick *et al.*, 1992) and flavonoids found in tea (Fitzpatrick *et al.*, 1993) have been shown to give vasodilator effects *in vitro*. The results of the few studies investigating the relationship between regular tea consumption and blood pressure have been inconsistent (Stensvold *et al.*, 1992; Bingham *et al.*, 1997; Rakic *et al.*, 1996; Abe *et al.*, 1995; Yokozawa *et al.*, 1994). In a cohort of Norwegian men and women, higher consumption of black tea was associated with lower systolic blood pressure (SBP) (Stensvold *et al.*, 1992). However, in a 4-week randomized, controlled, crossover trial in normotensive men and women, drinking six mugs of tea daily had no significant effect on clinic measured blood pressure (Bingham *et al.*,

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1997). Moreover, in older treated hypertensive subjects, the postprandial falls in SBP were attenuated by tea consumption (Rakic *et al.*, 1996), although no significant alteration in 24-h ambulatory blood pressure was observed; this outcome was possibly related to the acute pressor effects of caffeine. The effects of green tea on blood pressure have not been examined in humans. Moreover, it has been shown that (–) epicatechin also reduced arterial contraction induced by other vasoconstrictors, such as phenylephrine and endothelin-1 (Huang *et al.*, 1998). It has been also found that (–) epicatechin could act on endothelium to increase intracellular Ca^{2+} and nitric oxide release, which may account for the endothelium-dependent relaxation (Huang *et al.*, 1999) in rat isolated mesenteric arteries. It has been suggested that oxidative stress is involved in the development of raised blood pressure (Romero-Ahira & Roche, 1996), possibly via its effects on endothelial function (Briner & Luscher, 1994; Ferro & Webb, 1997; Flavahan, 1992). The main hypothesis tested in the two studies reported in this paper is that antioxidant (Rice-Evans *et al.*, 1995) and vasodilatory (Fitzpatrick *et al.*, 1993; Fitzpatrick *et al.*, 1992) polyphenolics in tea can attenuate the transient pressor effect of caffeine, and lower blood pressure during regular consumption. In contrast to these results, it has been shown that tea ingestion in the normotensive men caused larger acute increases in blood pressure than caffeine alone. However, any acute effects of tea on blood pressure did not translate into significant alterations in ambulatory blood pressure during regular tea (Hodgson *et al.*, 1999). Katayama and his co-workers (2002) have shown that EGCG can facilitate the cholinergic ganglion transmission possibly by increasing the amount of ACh released and, together with depolarizing action on myenteric neurons, may modulate the activity of the myenteric plexus of the guinea-pig ileum. Therefore, the present study was attempted to examine the effects of CUMS6335 on the CA secretion from the perfused model of the adrenal gland isolated from the spontaneously hypertensive rats (SHRs), and to compare its effect with that of EGCG.

Experimental

Experimental procedure – Mature male SHRs, weighing 200 to 350 grams, were used in the experiment. The animals were housed individually in separate cages, and food (Cheil Animal Chow) and tap water were allowed *ad libitum* for at least a week to adapt to experimental circumstances. On the day of experiment, a rat was anesthetized with thiopental sodium (40 mg/kg) intraperitoneally, and tied in supine position on fixing panel.

Isolation of adrenal glands – The adrenal gland was isolated by the 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 removed, but pushed over to the right side and covered by saline-soaked gauge pads and urine in bladder was removed in order to obtain enough working space for tying blood vessels and cannulations. A cannula, used for perfusion of the adrenal gland, was inserted into the distal end of the renal vein after all branches of adrenal vein (if any), vena cava and aorta were ligated. Heparin (400 IU/ml) was injected into vena cava to prevent blood coagulation before ligating vessels and cannulations. A small slit was made into the adrenal cortex just opposite entrance of adrenal vein. Perfusion of the gland was started, making sure that no leakage was present, and the perfusion fluid escaped only from the slit made in adrenal cortex. Then the adrenal gland, along with ligated blood vessels and the cannula, was carefully removed from the animal and placed on a platform of a leucite chamber. The chamber was continuously circulated with water heated at 37 ± 1°C.

Perfusion of adrenal gland – The adrenal glands were perfused by means of peristaltic pump (Isco, St. Lincoln, NE, U.S,A.) at a rate of 0.31 ml/min. The perfusion was carried out with Krebs-bicarbonate solution of following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11.7. The solution was constantly bubbled with 95 % O_2 + 5% CO_2 and the final pH of the solution was maintained at 7.4 ~ 7.5. The solution contained disodium EDTA (10 μg/ml) and ascorbic acid (100 μg/ml) to prevent oxidation of catecholamines.

Drug administration – The perfusions of DMPP (10^{-4} M) for 2 minutes and/or a single injection of ACh (5.32×10^{-3} M) and KCl (5.6×10^{-2} M) in a volume of 0.05 ml were made into perfusion stream via a three-way stopcock, respectively. McN-A-343 (10^{-4} M), Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were also perfused for 4 min, respectively. In the preliminary experiments, it was found that upon administration of the above drugs, secretory responses to ACh, KCl, McN-A-343, Bay-K-8644 and cyclopiazonic acid returned to preinjection level in about 4 min, but the responses to DMPP in 8 min.

Collection of perfusate – As a rule, prior to stimulation with various secretagogues, the perfusate was collected for 4 min to determine the spontaneous secretion of CA

(background sample). Immediately after the collection of the background sample, collection of the perfusates was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Stimulated sample's was collected for 4 to 8 min. The amounts secreted in the background sample have been subtracted from that secreted from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures.

To study the effect of CUMS6335 or EGCG on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing CUMS6335 or EGCG for 60 min, and then the perfusate was collected for a certain period (background sample). Then the medium was changed to the one containing the stimulating agent or along with CUMS6335 or EGCG, and the perfusates were collected for the same period as that for the background sample. The adrenal gland's perfusate was collected in chilled tubes.

Measurement of catecholamines – The content of perfusate was measured directly by the fluorometric method of Anton and Sayre (Anton & Sayre, 1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using fluoro-spectrophotometer (Kontron Co., Milano, Italy). A volume of 0.2 ml of the perfusate was used for the reaction. The CA content in the perfusate of stimulated glands by secretagogues used in the present work was high enough to obtain readings several folds greater than the reading of control samples (unstimulated). The sample blanks were also lowest for perfusates of stimulated and non-stimulated samples. The content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

Preparation of green tea extract – Dry leaves of *Thea sinensis* were collected from green tea farm at Boseong County, Cheollanamdo Province, South Korea. Powdered green tea leaves (100 g) were extracted at 100 °C for one hour, and after cooling at 4 °C for 12 hours the precipitate was removed by centrifugation at 5000 × g for 30 min. Evaporation of the filtrate was made in the dryer and then grinded into powder. Finally, this powder was shaken with ether for 10 hours, and then after removing ether layer the supernatant was vaporized in the spray-dryer to give dried water-soluble fraction into powdered form (9.1 g). The working solution of this crude extract (CUMS 6335) was prepared by dissolving in 0.9% NaCl solution on the day of each experiment and filtered before administration.

Statistical analysis – The statistical difference between the control and pretreated groups was determined by the Student's *t* and ANOVA tests. A P-value of less than 0.05 was considered to represent statistically significant changes unless specifically noted in the text. Values given in the text refer to means and the standard errors of the mean (S.E.M.). The statistical analysis of the experimental results was made by computer program described by Tallarida and Murray (1987).

Drugs and their sources – The following drugs were used: CUMS6335 (gift from Dongsuj Pharmacutical Co., Seoul, Korea), acetylcholine chloride, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), norepinephrine bitartrate, potassium chloride (KCl), epigallocatechin-3-gallate (EGCG), methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-carboxylate (BAY-K-8644) (Sigma Chemical Co., U.S.A.), and cyclo-piazonic acd, (3-(m-cholro-phenyl-carbamoyl-oxy)-2-but-ynyltrimethylammonium chloride [McN-A-343] (RBI, U.S.A.). Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except Bay-K-8644, which was dissolved in 99.5% ethanol and diluted appropriately with Krebs-bicarbonate solution (final concentration of alcohol was less than 0.1%). Concentrations of all drugs used are expressed in terms of molar base.

Results

Effects of CUMS6335 and epigallocatechin 3-gallate (EGCG) on CA secretion evoked by ACh and high K^+ in the perfused adrenal gland of the SHRs – After the initial perfusion with oxygenated Krebs-bicarbonate solution for 1 hr, basal CA release from the isolated perfused adrenal glands of the SHRs amounted to 21 ± 2.2 ng/2 min (n = 10). It has been shown that CUMS6335 in a dose-dependent fashion inhibited the contractile responses by phenylephrine or high potassium in the isolated aortic strips of rats (Lim *et al.*, 2003) and rabbits (Lim *et al.*, 2004). Therefore, it was decided initially to examine the effects of CUMS6335 on CA secretion evoked by cholinergic receptor stimulation as well as membrane depolarization from perfused adrenal glands of the SHRs. Secretagogues were given at 15 min-intervals, and CUMS 6335 was introduced for 60 min after obtaining the control secretory response of each secretagogue. In the present study, it was found that CUMS6335 itself did not affect basal CA output (data not shown).

When ACh (5.32×10^{-3} M) in 0.05 ml volume was injected into the perfusion stream, the amount of CA secreted was 373 ± 26 ng for 4 min. However, in 6 adrenal glands, the pretreatment with CUMS6335 (100 μg/ml) for

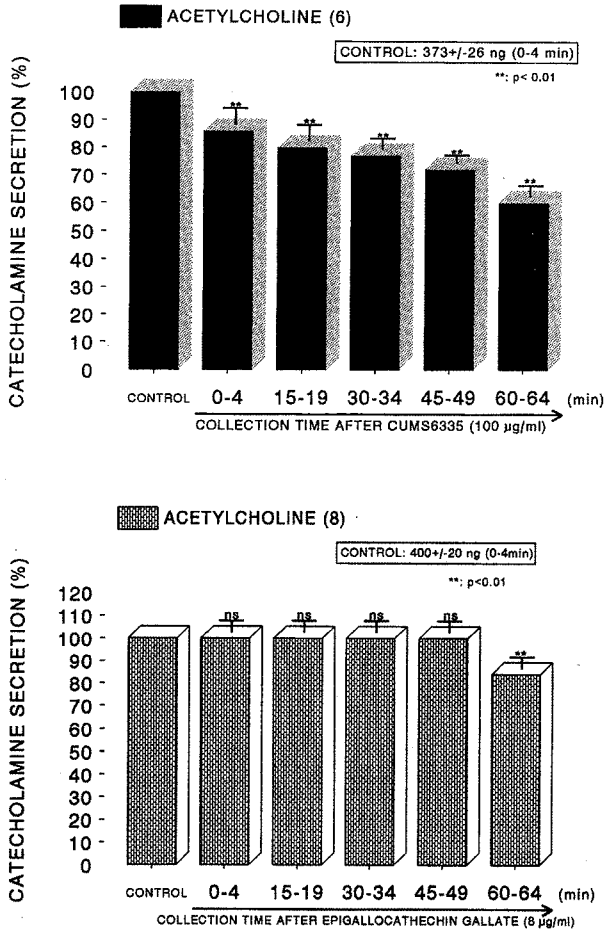

Fig. 1. Effects of CUMS6335 (green tea extract, **upper**) and epigallocatechin gallate (EGCG, **lower**) on the secretory responses of catecholamines (CA) evoked by acetylcholine from the isolated perfused adrenal glands of the spontaneously hypertensive rats (SHRs). CA secretion by a single injection of ACh (5.32×10^{-3} M) was induced "BEFORE" and "AFTER" preloading with CUMS6335 (100 μg/ml) or EGCG (8 μg/ml) for 60 min. Number in the parenthesis indicates the number of experimental adrenal glands. Vertical bars represent the standard error of the mean (S.E.M.). Ordinate: the amounts of CA secreted from adrenal gland (% of the control). Abscissa: collection time (min). Statistical difference was obtained by comparing the corresponding "BEFORE" (control) with each period "AFTER" the initiation of CUMS6335 perfusion. Perfusates were collected for 4 minutes at 15 min intervals. **: $P < 0.01$, ns: Statistically not significant.

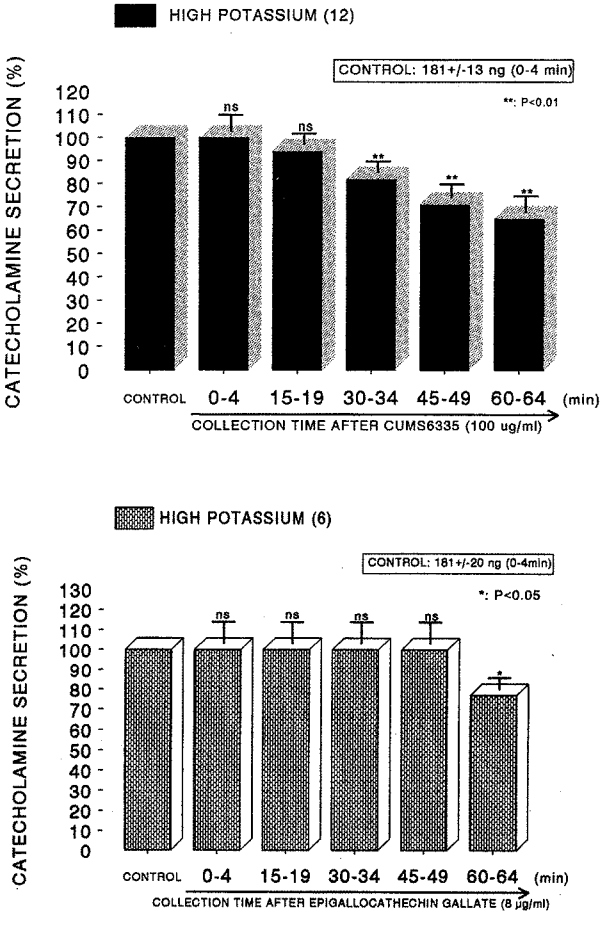

Fig. 2. Effects of CUMS6335 (green tea extract, **upper**) and epigallocatechin gallate (EGCG, **lower**) on the secretory responses of catecholamines (CA) evoked by high potassium from the isolated perfused adrenal glands of SHRs. CA secretion by a single injection of high potassium (5.6×10^{-2} M) was induced "BEFORE (control)" and "AFTER" preloading with CUMS6335 (100 μg/ml) or EGCG (8 μg/ml) for 60 min. Perfusates were collected for 4 minutes at 15 min intervals. Other legends are the same as in Fig. 1 *: $P < 0.05$, **: $P < 0.01$, ns: Statistically not significant.

60 min significantly inhibited ACh-stimulated CA secretion to 60% (224 ± 14 ng for 60 - 64 min) of the control response in a time-dependent manner (Fig. 1-Upper). However, in the presence of EGCG (8 μg/ml) for 60 min, ACh-stimulated CA secretion was not affected by comparing to the control release of CA (400 ± 20 ng for 0 - 4 min), except for last period (336 ± 20 ng for 60 - 64 min), as shown in Fig. 1 (Lower). Also, it has earlier been found that depolarizing agent such as KCl stimulates sharply CA secretion. In the present work, excess K^+ (5.6×10^{-2} M)-stimulated CA secretion in the presence of CUMS 6335 (100 μg/ml) was significantly inhibited to 65% (118 ± 20 ng for 60 - 64 min) of the corresponding control secretion (181 ± 20 ng for 0 - 4 min) from 6 glands (Fig. 2-Upper). However, it was not changed even in the presence of EGCG (8 μg/ml) for 60 min compared to the corresponding release of CA (162 ± 4 ng for 0 - 4 min) except for the last period (139 ± 11 ng for 60 - 64 min), as depicted in Fig. 2 (Lower).

Effects of CUMS6335 and epigallocatechin 3-gallate (EGCG) on CA secretion evoked DMPP and McN-A-343 in the perfused adrenal gland of the SHRs – As shown in Fig. 2, it is suggested that CUMS6335 possesses

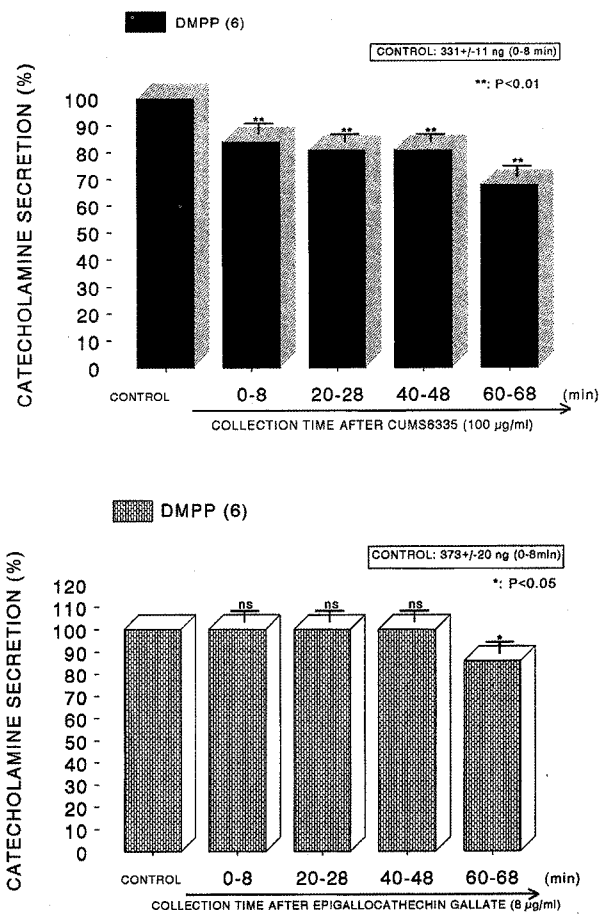

Fig. 3. Effects of CUMS6335 (green tea extract, **upper**) and epigallocatechin gallate (EGCG, **lower**) on the secretory responses of catecholamines (CA) evoked by DMPP from the isolated perfused adrenal glands of SHRs. CA secretion by the perfusion of DMPP (10^{-4} M) for 2 min was induced "BEFORE" and "AFTER" preloading with CUMS6335 (100 μg/ml) or EGCG (8 μg/ml) for 60 min. Perfusates were collected for 8 minutes at 20 min intervals. Other legends are the same as in Fig. 1. *: $P < 0.05$, **: $P < 0.01$, ns: Statistically not significant.

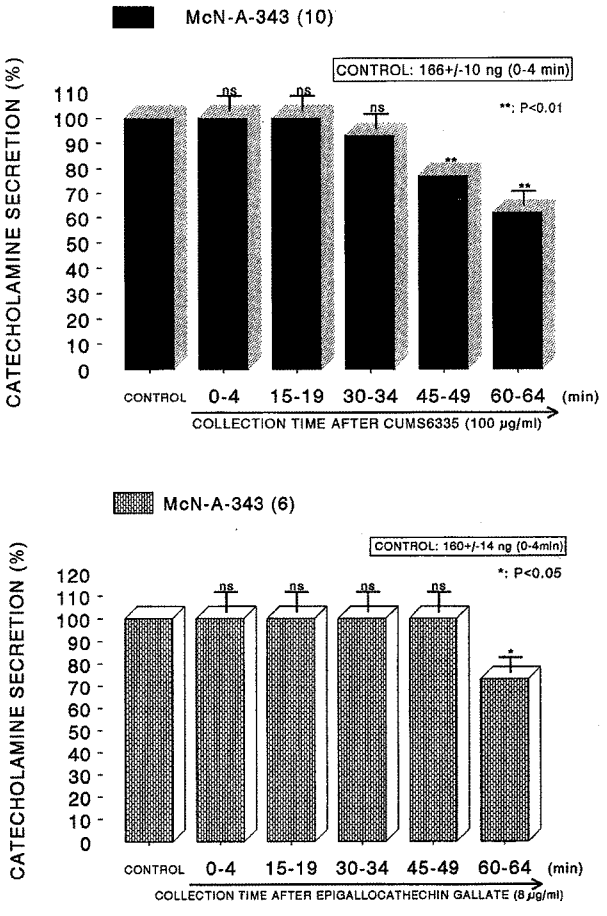

Fig. 4. Effects of CUMS6335 (green tea extract, **upper**) and epigallocatechin gallate (EGCG, **lower**) on the secretory responses of catecholamines (CA) evoked by McN-A-343 from the isolated perfused adrenal glands of SHRs. CA secretion by the perfusion of McN-A-343 (10^{-4} M) for 4 min was induced "BEFORE" and "AFTER" preloading with CUMS6335 (100 μg/ml) or EGCG (8 μg/ml) for 60 min. Perfusates were collected for 4 minutes at 15 min intervals. Other legends are the same as in Fig. 1. *: $P < 0.05$, **: $P < 0.01$, ns: Statistically not significant.

the antagonist effect of neuronal cholinergic nicotinic receptors in adrenal medulla of SHRs. Therefore, it was tried to examine the effects of CUMS6335 and EGCG on DMPP- or McN-A-343-evoked CA releasing responses in the perfused adrenal gland of the SHRs. DMPP (10^{-4} M), a selective nicotinic receptor agonist in autonomic sympathetic ganglia, when perfused through the adrenal gland of SHR, evoked a sharp and rapid increase in CA secretion. As shown in Fig. 3 (Upper), DMPP (10^{-4} M)-stimulated CA secretion following the loading with CUMS6335 (100 μg/ml) was depressed by 71% of the corresponding control secretion (331 ± 11 ng/0 - 8 min). However, in the presence of EGCG (8.0 μg/ml) for 60 min, DMPP (10^{-4} M)-evoked CA secretory response was not changed in comparison with the corresponding release of CA (373 ± 20 ng for 0 - 8 min) except for the last period (331 ± 20 ng for 60 - 64 min), as shown in Fig. 3 (Lower). McN-A-343 (10^{-4} M), which is a selective muscarinic M_1-agonist (Hammer & Giachetti, 1982), when perfused into an adrenal gland for 4 min caused an increased CA secretion (166 ± 10 ng for 0 - 4 min) from 10 glands. However, McN-A-343-stimulated CA secretion in the presence of CUMS6335 (100 μg/ml) was markedly depressed to 62% (103 ± 10 ng for 60 - 64 min) of the corresponding control secretion (100%) as depicted in Fig. 4 (Upper). However, in the presence of EGCG (8 μg/ml) for 60 min, McN-A-343-stimulated CA secretion was not affected by comparing to the control release of CA (160 ± 14 ng for 0 - 4 min), except for the last period (117 ± 11 ng for 60 - 64 min) from 6 glands, as shown in Fig. 4 (Lower).

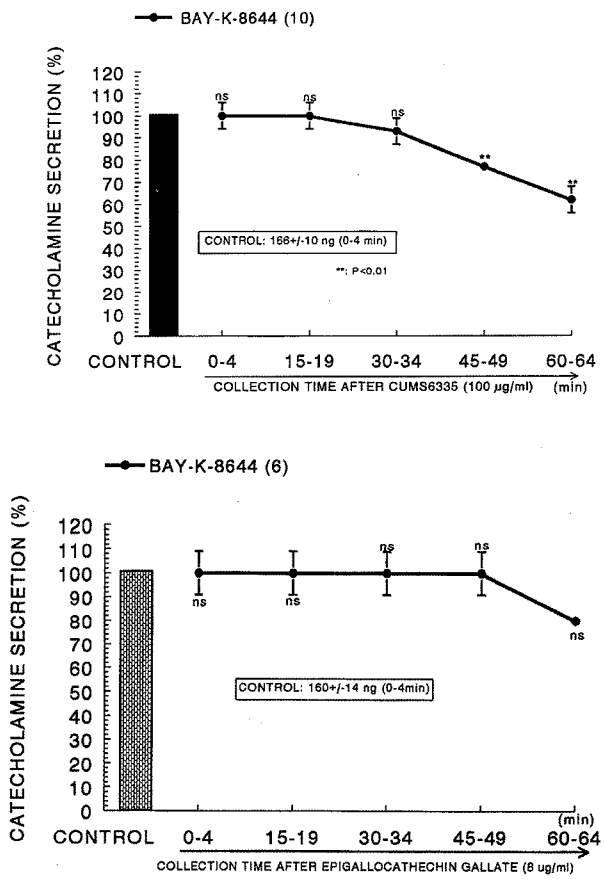

Fig. 5. Effects of CUMS6335 (green tea extract, **upper**) and epigallocatechin gallate (EGCG, **lower**) on the secretory responses of catecholamines (CA) evoked by Bay-K-8644 from the isolated perfused adrenal glands of SHRs. CA secretion by a single injection of Bay-K-8644 (10^{-5} M) was induced "BEFORE" and "AFTER" preloading with CUMS6335 (100 μg/ml) or EGCG (8 μg/ml) for 60 min. Perfusates were collected for 4 minutes at 15 min intervals. Other legends are the same as in Fig. 1. *: $P < 0.05$, **: $P < 0.01$, ns: Statistically not significant.

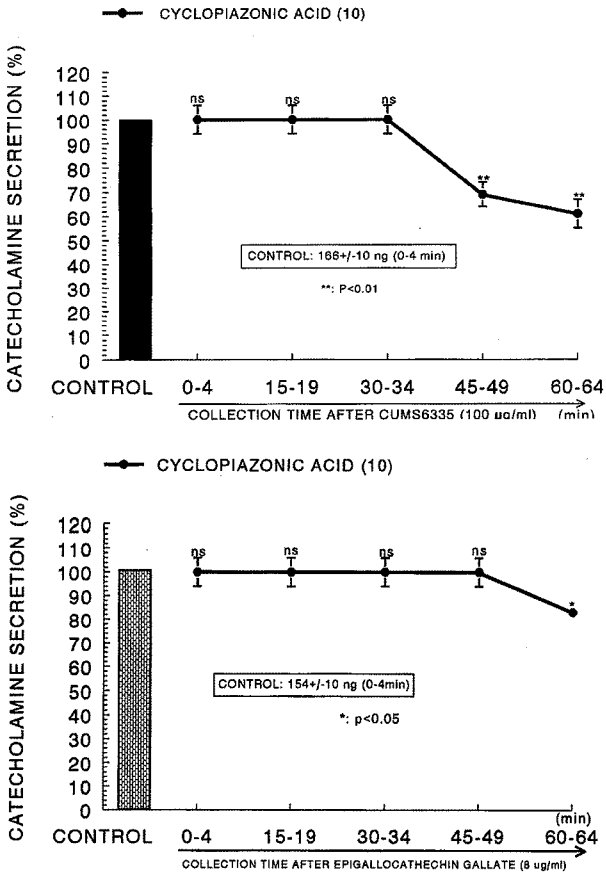

Fig. 6. Effects of CUMS6335 (green tea extract, **upper**) and epigallocatechin gallate (EGCG, **lower**) on the secretory responses of catecholamines (CA) evoked by cyclopiazonic acid from the isolated perfused adrenal glands of SHRs. CA secretion by a single injection of cyclopiazonic acid (10^{-5} M) was induced "BEFORE" and "AFTER" preloading with CUMS6335 (100 μg/ml) or EGCG (8 μg/ml) for 60 min. Perfusates were collected for 4 minutes at 15 min intervals. Other legends are the same as in Fig. 1. *: $P < 0.05$, **: $P < 0.01$, ns: Statistically not significant.

Effects of CUMS6335 and epigallocatechin 3-gallate (EGCG) on CA secretion evoked by Bay-K-8644 and cyclopiazonic acid in the perfused adrenal gland of the SHRs – It has been found that Bay-K-8644 is a calcium channel activator that causes positive inotropy and vasoconstriction in isolated tissues and intact animals (Schramm *et al.*, 1983; Wada *et al.*, 1985a), and enhances basal Ca^{2+} uptake (Garcia *et al.*, 1984) and CA release (Lim *et al.*, 1992). Therefore, it was of interest to examine the effects of CUMS6335 on Bay-K-8644-evoked CA secretion from the isolated perfused adrenal glands of the SHRs. Fig. 10 shows the inhibitory effect of CUMS6335 on Bay-K-8644-evoked CA secretory responses. In the absence of CUMS6335, Bay-K-8644 (10^{-5} M) given into the perfusion stream evoked CA secretion of 166 ± 10 ng (0 - 4 min) from 10 rat adrenal glands (Fig. 5-Upper). However, in the presence of CUMS6335 (100 μg/ml), Bay-K-8644-stimulated CA secretion was inhibited to 62% (103 ± 10 ng for 0 - 8 min) of the corresponding control release. However, in the presence of EGCG (8 μg/ml) for 60 min, Bay-K-8644 (10^{-5} M)-stimulated CA secretion was not affected, excepting the last period, as compared to the control release of CA (160 ± 14 ng for 0 - 4 min) as shown in Fig. 5 (Lower).

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Georger & Riley, 1989; Seidler *et al.*, 1989). It may be extremely valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic current regulated by intracellular calcium (Suzuki *et al.*, 1992). As shown in Fig. 6 (Upper), in the presence of

CUMS6335 (100 μg/ml) in 10 adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was inhibited by 61% of the control response (166 ± 10 ng for 0 - 4 min). However, in the presence of EGCG (8 μg/ml), cyclopiazonic acid (10^{-5} M)-evoked CA secretion was not affected, excepting the last period, as compared to the control release of CA (154 ± 10 ng for 0 - 4 min) as shown in Fig. 6 (Lower).

Discussion

The present experimental results have suggested that CUMS6335 inhibits the CA secretory responses from the isolated perfused adrenal gland of the SHRs evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization. It seems that this inhibitory effect of CUMS6335 is exerted by blocking both the calcium influx into the adrenal medullary chromaffin cells of the SHRs and the uptake of Ca^{2+} into the cytoplasmic calcium store, which are at least partly relevant to the direct interaction with the nicotinic receptor itself.

In general, the adrenal medulla has been employed as a model system to study numerous cellular functions involving not only noradrenergic nerve cells but also neurons. During neurogenic stimulation of the adrenal medulla, ACh is released from splanchnic nerve endings and activated cholinergic receptors on the chromaffin cell membrane (Viveros, 1975). This activation initiates a series of events known as stimulus-secretion coupling, culminating in the exocytotic release of CA and other components of the secretory vesicles into the extracellular space. Usually, two mechanisms are involved in the secretion of adrenal medullary hormones. Upon excitation of splanchnic nerves, ACh is released from the nerve terminals, which then activates nicotinic secretion of CA. Based on this fact, the present findings that CUMS6335 inhibited the CA secretory responses evoked by nicotinic receptor stimulation as well as by membrane depolarization in the rat adrenal medulla seem to support the fact that it causes vasodilatation through Ca^{2+} antagonism in the isolated aorta from rats (Lim *et al.*, 2003) and rabbits (Lim *et al.*, 2004)

These experimental results indicate that CUMS6335-induced inhibitory activity of CA secretory response evoked by stimulation of nicotinic receptors might contribute at least partly to its hypotensive mechanism. ACh, the physiological presynaptic transmitter at the adrenal medulla, which is released by depolarizing splanchnic nerve terminals and then activates nicotinic receptors, releases CA and dopamine β-hydroxylase by calcium-dependent secretory process (Dixon *et al.*, 1975; Viveros *et al.*, 1968). In the light of this fact, the present results suggest that CUMS6335 may inhibit CA secretion evoked by nicotinic stimulation from the splanchnic nerve ending through the blockade of nicotinic receptors. The release of epinephrine from the adrenal medulla in response to splanchnic nerve stimulation or nicotinic agonist is mediated by activation of nicotinic receptors located on the chromaffin cells. The exocytotic CA release from the chromaffin cells appears to be essentially similar to that occuring in noradrenergic axons (Douglas, 1968; Sorimachi & Yoshida, 1979). ACh-evoked 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 & Hwang, 1991).

In support of this idea, recently, it has been found that, in the adrenal medulla isolated from the rat (Lim *et al.*, 2003) and the rabbit (Lim, 2005), CUMS6335 inhibits the secretory responses of CAs evoked by ACh, DMPP, McN-A-343 and high K^+. It suggests that CUMS6335 can produce the same effects in adrenal medulla of the SHRs as in adrenal medulla of the normotensive rats and rabbits.

Tannins contained in green tea are also found to induce the depressor effect in rat with renal hypertension (Yokozawa *et al.*, 1994). Extracts of tea (Fitzpatrick *et al.*, 1992) and flavonoids found in tea (Fitzpatrick *et al.*, 1993) have been shown to give vasodilator effects. In a cohort of Norwegian men and women, higher consumption of black tea was associated with lower SBP (Stensvold *et al.*, 1992). In terms of these findings, the results of the present study seem likely that CUMS6335 can cause the depressor effect by the inhibition of CA secretion from the adrenal medulla. The present findings appeared to contribute at least partly to the facts that extracts of tea (Fitzpatrick *et al.*, 1992) and flavonoids found in tea (Fitzpatrick *et al.*, 1993) produced vasodilator effects, but not to the fact that tea ingestion in the normotensive men caused larger acute increases in blood pressure than caffeine alone (Hodgson *et al.*, 1999). In the aortic strips isolated from the rat (Lim *et al.*, 2003) and the rabbit (Lim *et al.*, 2004), CUMS6335 has been found to inhibit the contractile responses induced by phenylephrine and high potassium. Moreover, it also diminished the pressor responses evoked by intravenous norepinephrine in these animals.

In the present study, CUMS6335 also depressed greatly CA secretory response evoked by Bay-K-8644, which is known to activate L-type voltage-dependent Ca^{2+} channels

(Garcia *et al.*, 1984; Schramin *et al.*, 1983). This result indicates that CUMS6335 may inhibit Ca^{2+} influx to 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 secretion of CA (Fisher *et al.*, 1981; Yanagihara *et al.*, 1979). It has been also known that the activation of nicotinic receptors stimulates CA secretion by increasing Ca^{2+} entry through receptor-linked and/or voltage-dependent Ca^{2+} channels in both perfused rat adrenal glands (Wakade & Wakade, 1983; Lim & Hwang, 1991) and isolated bovine adrenal chromaffin cells (Kilpatrick *et al.*, 1981; 1982; Knight & Kesteven, 1983). Wada and his coworkers (1985b) have found that the adrenomedullary chromaffin cells have (i) nicotinic receptor-associated 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, 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. In the present study, the finding that high potassium-induced CA secretory response was markedly depressed by pretreatment with CUMS6335 indicates strongly that this inhibitory effect of CUMS6335 is exerted through the direct inhibition of calcium influx into the rat adrenal chromaffin cells. Furthermore, slight elevation in the extracellular potassium concentration increases both the frequency of spontaneous action potentials and the secretion of CA (Kidokoro & Ritchie, 1980), suggesting that the influx of calcium that occurs during action potentials is directly linked to the rate of secretion.

However, in the present study, the pretreatment with EGCG failed to affect the secretion of CA evoked by ACh and high K^+ as well as by Bay-K-8644. EGCG is well known to be a major component of various catechins found in green tea, excepting the response only for last period (60 - 64 min). This finding suggests that CUMS 6335-induced inhibitory action of the CA secretion is unlikely mediated at least by polyphenols found in green tea. Moreover, the result obtained from the present study is consistent with the previous finding that EGCG did not affect phenylephrine- or high potassium-induced contractile response of the isolated rat aorta. It supports that the inhibitory effect of CUMS6335 on CA secretion is not associated with the effects of catechins including EGCG contained in green tea.

In contrast, it has been shown that (–) epicatechin also concentration-dependently relaxed U46619-contracted arteries without the functional endothelium. It is unlikely that (–) epicatechin acts as an antagonist at prostaglandin receptors to cause relaxation since it reduced arterial contraction induced by other vasoconstrictors, such as phenylephrine and endothelin-1 (Huang *et al.*, 1998). The endothelium-independent relaxation induced by (–) epicatechin may be partly mediated through inhibition of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in vascular smooth muscle cells because (–) epicatechin significantly reduced the high K^+-induced contraction in the same preparation (Huang *et al.*, 1998). Recently, it has been also found that (–) epicatechin could act on endothelium to increase intracellular Ca^{2+} and nitric oxide release, which may account for the endothelium-dependent relaxation (Huang *et al.*, 1999). In addition, (–) epicatechin-induced relaxation in endothelium-intact tissues may be also mediated by nitric oxide-dependent activation of iberiotoxin-sensitive K^+ channels. These mechanisms may be associated with a beneficial effect of green tea epicatechins on vascular system (Huang *et al.*, 1999). Recently, it has been shown that (–)-EGCG can facilitate the cholinergic ganglion transmission possibly by increasing the amount of ACh released and, together with its previously described depolarizing action on myenteric neurons, may modulate the activity of the myenteric plexus of the guinea-pig ileum (Katayama *et al.*, 2002). However, these (–) epicatechin's effects are not in agreement with the present result that EGCG failed to alter the CA secretory responses evoked by ACh and high potassium in the isolated perfused rat adrenal medulla. Moreover, these results was in agreement with the recent finding that EGCG did not affect the contractile responses induced by phenylephrine and high potassium in the isolated aortic strips of the rat (Lim *et al.*, 2003) and the rabbits (Lim *et al.*, 2004). The effects of various catechins remain to be investigated in the future.

In conclusion, these results of the present study have suggested that CUMS6335 inhibits CA secretions by stimulation of cholinergic nicotinic receptors as well as by membrane depolarization in the isolated perfused adrenal glands of the SHRs evoked whereas EGCG does not affect them. It seems that this inhibitory effect of CUMS 6335 is exerted by blocking both the calcium influx into the rat adrenal medullary chromaffin cells and the uptake of Ca^{2+} into the cytoplasmic calcium store, which are at least partly relevant to the direct interaction with the nicotinic receptor itself. These experimental results may contribute at least partly to the hypotensive effect of CUMS6335 components, through inhibition of CA secre-

tion from adrenal medullary chromaffin cells and consequent reduction of the CA level in the circulation. It seems likely that there is much difference in mode of the CA-releasing action between CUMS6335 and EGCG, but no species difference between the rat, rabbit and SHR.

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References

Abe, Y., Umemura, S., Sugimoto, K., Hirawa, N., Kato, Y., Yokoyama, N., Yokoyama, T., Iwai, J., and Ishii, M., Effect of green tea rich in gamma-aminobutynic acid on blood pressure of Dahl salt-sensitive rats. *Am. J. Hyperten.* **8**, 74-79 (1995).

Anton, A.H. and Sayre, D.F., A study of the factors affecting the aluminum oxide trihydroxy indole procedure for the analysis of catecholamines. *J. Pharmacol. Exp. Ther.* **138**, 360-375 (1962).

Bingham, S.A., Vorster, H., Jerling, J.C., Magee, E., Mulligan, A., Runswick, S.A., and Cummings, J.H., Effect of tea drinking on blood lipids, blood pressure and aspects of bowel habit. *Br. J. Nutr.* **78**, 41-45 (1997).

Briner, V.A. and Luscher, T., Role of vascular endothelial abnormalities in clinical medicine: atherosclerosis, hypertension, diabetes and endotoxaemia. *Adv. Int. Med.* **39**, 910-915 (1994).

Dixon, Wr., Garcia, A.G., and Kirkekar, S.M., Release of catecholamines and dopamine-beta-hydroxylase from the rat adrenal gland of the cat. *J. Physiol.* **244**, 805-824 (1975).

Douglas, W.W., Stimulus-secretion coupling: The concept and clues from chromaffin and other cells. *Br. J. Pharmacol.* **34**, 451-474 (1968).

Ferro, C.J. and Webb, D.J., Endothelial dysfunction and hypertinsion. *Drugs* **53**, 30-41 (1997).

Fitzpatrick, D.F., Hirschfield, S.L., and Coffey, R.G., Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am. J. Physiol.* **265**, H77-78 (1993).

Fisher, S.K., Holz, R.W., and Agranoff, B.W., Muscarinic receptors in chromaffin cell culture mediate enhanced phospholipid labeling but not catecholamine secretion. *J. Neurochem.* **37**, 491-487 (1981).

Fitzpatrick, D.F., Hirschfield, S.L., Ricci, T., Jantzen, P., and Coffey, R.G., Endothelium-dependent vasorelaxation caused by various plant extracts. *J. Cardiovasc. Pharmacol.* **26**, 90-95 (1992).

Flavahan, N.A., Atheroscterosis or Epoprotein-induced endothelial dysfunction: potential mechanisms underlying reduction in EDRF/nitric oxide activity. *Circulation* **85**, 1927-1938 (1992).

Garcia, A.G., Sala, F., Reig, J.A., Viniegra, S., Frias, J., Fonteriz, R., and Gandia, L., Dihydropyridine Bay-K-8644 activates chromaffin cell calcium channels. *Nature* **309**, 69-71 (1984).

Goeger, D.E. and Riley, R.T., Interaction of cyclopiazonic acid with rat skeletal muscle sarcoplasmic reticulum vesicles. Effect on Ca^{2+} binding and Ca^{2+} permeability. *Biochem. Pharmacol.* **38**, 3995-4003 (1989).

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

Hodgson, J.M., Puddey, I.B., Byrke, V., and Beilin, L.J., Jordan N: Effects on blood pressure of drinking green and black tea. *J. Hypert.* **17**, 457-463 (1999).

Huang, Y., Zhang, A.Q., Lau, C.W., and Chen, Z.Y., Vasorelaxant effect of purified green tea epicatechin derivatives in rat mes-enteric artery. *Life Sci.* **63**, 275-283 (1998).

Huang, Y., Chan, N.W.K., Lau, C.W., Yao, X.Q., Chan, F.L., and Chen, Z.Y., Involvement of endothelium/nilvicoxide in vasorelaxation induced by purified green tea (−) epicatechin. *Biochim. Biophys. Acta.* **1427**, 322-328 (1999).

Katayama, Y., Homma, T., Hara, Y., and Hirai, K., Tea catechin, (−)-epigallocatechin gallate, facilitates cholinergic ganglion transmission in the myenteric plexus of the guinea-pig small intestine. *Neurosci. Lett.* **319(2)**, 63-66 (2002).

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

Kilpatrick, D.L., Slepetis, R.J., Corcoran, J.J., and Kirshner, N., Calcium uptake and catecholamine secretion by cultured bovine adrenal medulla cells. *J. Neurochem.* **38**, 427-435 (1982).

Kilpatrick, D.L., Slepetis, R.J., and Kirshner, N., Ion channels and membrane potential in stimulus-secretion coupling in adrenal medulla cells. *J. Neurochem.* **36**, 1245-1255 (1981).

Knight, D. E. and Kesteven, N.T., Evoked transient intracellular free Ca^{2+} changes and secretion in isolated bovine adrenal medullary cells. *Proc. R. Soc. Lond. Biol. Sci.* **218**, 177-199 (1983).

Lim, D.Y., Comparison of Green Tea Extract and Epigallocatechin Gallate on Secretion of Catecholamines from the Rabbit Adrenal Medulla. *Arch. Pharm. Res.* **28**(8), 914-922 (2005).

Lim, D.Y., Baek, Y.J., and Lee, E.B., Green Tea Extract (CUMC6335), not Epigallocatechin Gallate, Carses Vascular Relaxation in Rabbits. Natural Product. *Sciences* **10**(5), 228-236 (2004).

Lim, D.Y. and Hwang, D.H., Studies on secretion of catecholamines evoked by DMPP and McN-A-343 in the rat adrenal gland. *Korean J. Pharmacol.* **27**(1), 53-67 (1991).

Lim, D.Y., Kim, C.D., and Ahn, K.W., Influence of TMB-8 on secretion of catecholamines from the perfused rat adrenal glands. *Arch. Pharm. Res.* **15**(2), 115-125 (1992).

Lim, D.Y., Lee, E.S., Park, H.G., Kim, B.C., Hong, S.P., and Lee, E.B., Comparison of Green Tea Extract and Epigallocatechin gallate on Blood Pressure and Contractile Responses of Vascular Smooth Muscle of Rats. *Arch. Pharm. Res.* **26**(3), 214-223 (2003).

Lim, D.Y., Park, H.G., and Lee, B.R., Green Tea Extract, not Epigallocatechin gallate Inhibits Catecholamine Release From the Rat Adrenal Medulla. *J. Applied. Pharmacol.* **11**(1), 33-40 (2003).

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

Pincomb, G.A., Lovallo, W.R., Mckey, B.S., Sung, B.H., Passey, R.B., and Everson, S.A., Acute blood pressure elevations with caffeine in men with borderline systemic hypertension. *Am. J. Cardiol.* **77**, 270-274 (1996).

Quinlan, P., Lane, J., and Aspinal, L., Effects of hot tea, coffee and water ingestion on physiological responess and mood: role of caffeine, water and beverage type. *Psychopharmacology* **134**, 164-173 (1997).

Rakic, V., Beilin, L.J., and Burke, v., Effect of coffee and tea drinking on postprandial hypotension in older men and women. *Clin. Exp. Pharmacol. Physiol.* **23**, 559-563 (1996).

Ricc-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M., and Pridham, J.B., The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Rad. Res.* **22**, 375-383 (1995).

Romero-Ahira, D. and Roche, E., High blood pressure orgeniradicals and antioxidants: enological relationships. *Med. Hypoth.* **46**, 414-420 (1996).

Schramm, M., Thomas, G., Towart, R., and Franckowiak, G., Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels. *Nature* **303**, 535-537 (1982).

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

Shalleck, J., Tea. Viking Press, New York, 1996, pp. 7.

Sorimachi, M., Nishimura, S., and Yamagami, K., Possible occurence of Na^{+}-dependent Ca^{2+} influex mechanism in isolated bovine chromaffin cells. *Brain Res.* **208**, 442-446 (1981).

Sorimachi, M. and Yoshida, K., Exocytotic release of catecholamines and dopamine-beta-hydroxylase from the perfused adrenal gland of the rabbit and cat. *Br. J. Pharmacol.* **65**(1), 117-125 (1979).

Stensvold, I., Tverdal, A., and Solvoll, K., Foss OP: Tea consumption. Relationship to cholesterol, blood pressure, and coronary and total mortality. *Prev. Med.* **21**, 546-553 (1992).

Sung, B.H., Whitsett, T.L., Lovallo, W.R., Absi, M., Pincomv, G.A., and Wilson, M.F., Prolonged increase in blood pressure by a single orial dose of caffeine in mildly hypertensive men. *Am. J. Hypertens.* **7**, 755-756 (1994).

Suzuki, M., Muraki, K., Imaizumi, Y., and 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.* **107**, 134-140 (1992).

Tallarida, R.J. and Murray, R.B., Manual of pharmacologic calculation with computer programs. 2nd Ed. New York, Speringer-Verlag, 1987, pp. 132.

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

Viveros, O.H., Arqueros, L.C., and Kirshner, N., Release of catecholamines and dopamine beta-hydroxylase from the adrenal medulla. *Life Sci.* **7**, 609-618 (1968).

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

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

Wakade, A.R., Studies on secretion of catecholamines evoked by acetylcholine or transmural stimulation of the rat adrenal gland. *J. Physiol.* **313**, 463-480 (1981).

Wakade, A.R. and Wakade, T.D., Contribution of nicotinic and muscarinic receptors in the secretion of catecholamines evoked by endogenous and exogenous acetylcholine. *Neuroscience* **10**, 973-978 (1983).

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

Yokozawa, T., Oura, H., Sakanaka, S., Ishigaki, S., and Kim, M., Depressor effect of tannin in green tea on rats with renal hypertension. *Biosci. Biotech. Biochem.* **58**, 855-858 (1994).

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