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조선대학교 대학원

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고 영 권

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고 영 권

지도교수 : 박 영 봉

조선대학교 대학원 의학과

Goldstein과 Brown(1984)은 simvastatin을 비롯하여 statin류의 분자적 작용기전은 isoprenoids와 cholesterol의 전구 물질인 mevalonate의 생성을 촉매하는 HMG-CoA reductase를 억제시키는 것이라고 하였으며, Pedersen 등(2000)은 심근경색과 뇌졸증에 의한 사망율을 감소시킨다고 보고하였다. Simvastatin은 울혈성 심장기능상실증을 유발시킨 토끼에서 norepinephrine의 혈장농도를 저하시킨다고 알려져 있다(Pliquett 등, 2003). Nette 등(2005)은 simvastatin 치료를 받은 환자에서 혈장 epinephrine 농도가 약간 낮다고 발표하였다. 과량의 내인성 카테콜아민(CA)이 죽상동맥경화 발병에 대해 위험인자일 수 있지만(Westfall 및 Westfall, 2005), simvastatin이 부신속질기능에 영향을 미치는지에 대해서는 분명하지 않다. 따라서 본 연구의 목적은 statin류의 하나인 simvastatin이 흰쥐에서 분리 적출한 부신의 관류모델에서 CA분비작용에 대한 영향을 검색하고, 그 작용기전을 구명코자 본 연구를 시행하여 얻어진 연구결과는 다음과 같다.

흰쥐 적출부신의 정맥 내로 simvastatin (20~200 μM)을 90분간 관류하는 동안 아세틸콜린 (5.32 mM, ACh)의 CA 유리작용은 비교적 시간 및 용량 의존적으로 감소되었다. 또한 simvastatin (60 μM)존재 하에서 angiotesin II (Ang II, 100 nM),

McN-A-343 (100 μM, 선택성 무스카린 M_1 수용체 작용제) 및 DMPP (선택성 니코틴수용체 작용제)의 CA 유리반응 또한 시간 의존적으로 감소되었다. 그러나, simvastatin 자체는 기초 CA 분비량에 별다른 영향이 없었다. 또한, simvastatin (60 μM)존재 하에서, dihyropyridine계 전압의존적 L형 칼슘통로 활성화제인 Bay-K-8644 (10 μM), 직접 막탈분극제인 고칼륨 (56 mM), 선택성 전압의존적 나트륨통로 활성화제인 veratridine (50 μM) 및 세포질 내 내형질세망막의 Ca^{2+}-ATPase 억제제로 알려진 cyclopiazonic acid (10 μM)에 의한 CA 유리효과가 역시 시간 의존적으로 억제되었다. 흥미롭게도, NO Synthase 억제제인 L-NAME (30 μM) 와 simvastatin (60 μM)을 함께 90분간 관류하는 동안 ACh, Ang II, McN-A-343, DMPP, high K^+, veratridine, cyclopiazonic acid 및 Bay-K-8644의 CA 유리효과가 simvastatin (60 μM) 단독처치 시의 억제효과에 비교하여 거의 상응하는 대조치의 수준으로 회복하였다. 또한 NO 분비량이 simvastatin (60 μM) 관류 후에 실제로 기초분비량에 비교하여 현저히 증가되었다. 또한, Ang II AT_1 수용체길항제인 fimasartan (15 μM) 과 simvastatin (60 μM)을 동시 관류하는 동안 ACh의 CA 유리에 대한 억제작용이 simvastatin (60 μM) 이나 fimasartan (15 μM)을 단독 투여 시의 CA 유리 감소작용에 비교하여 뚜렷이 증강되였다. 또한 simvastatin의 CA 유리 억제작용은 HMG-CoA의 첫 대사물인 mevalonate의 동시 존재 하에서 영향을 받지 않았다. 그러나 또 다른 statin 유도체인 pravastatin (60 μM)은 ACh의 CA분비효과에 별다른 영향을 미치지 못하였다. 반면에 atorvastatin (60 μM)은 ACh의 CA분비효과에 대한 유의한 억제반응을 나타내었다.

상기한 연구결과를 종합적으로 보면, 흰쥐에서 적출한 부신속질의 관류모델에서 simvastatin은 안지오텐신 AT_1 수용체 및 콜린수용체(니코틴 및 무스카린 수용체) 활성화에 의한 CA 유리작용에 대하여 현저한 억제반응을 보였다. 이러한 simvastatin의 억제효과는 흰쥐 부신속질에서 NO Synthase의 활성화에 의한 NO 생산증가로 인하여 부신속질 크롬친화세포 내로 Na^+ 및 Ca^{2+}통로를 통한 이들 이온의 유입억제와 세포 내 Ca^{2+}저장고로부터 Ca^{2+}유리 감소작용에 기인되며, 이러한 작용은 안지오텐신 AT_1 수용체 와 니코틴수용체의 길항작용과 연관이 있는 것으로 사료된다. 또한

fimasartan 과 simvastatin 함께 투여 시 CA 유리 감소효과가 증강되는 점은 임상적으로 고혈압과 같은 심혈관계 질환 치료에 유용할 것으로 생각된다. 또한 본 연구결과를 기반으로 보면, 부신속질에서 simvastatin의 CA 분비억제작용은 HMG-CoA reductase 억제와는 직접적인 연관은 없는 것으로 사료된다.

I. INTRODUCTION

Simvastatin is known to be a drug widely used for the treatment of cardiovascular diseases (Kjekshus et al., 1997; Pedersen et al., 2000; De Sotomayor et al., 2000). Simvastatin acts as an inhibitor of 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, which is a rate-determining enzyme in the biosynthesis of cholesterol (Goldstein and Brown, 1984), and reduces the plasma levels of low-density lipoprotein (LDL) (Mauro et al., 1991). Clinical studies have shown that treatment with simvastatin markedly decreased the incidence of cardiovascular events (Gryn and Hegele, 2015). While the lipid-lowering effect is a major mechanism of action of simvastatin against cardiovascular diseases, increasing obvious evidences have demonstrated that other mechanisms, namely, their so-called pleiotropic effects, are involved, including reduction of oxidative stress (Stoll et al., 2005) and vascular inflammation (Ridker et al., 1998, 2001), improvement of endothelial function (Laufs et al., 1998), inhibiting the thrombogenic response (Notarbartolo et al., 1995), and enhancement of the stability of atherosclerotic plaques (Fukumoto et al., 2001; Robinson, 2007). In addition, independent of these pleiotropic effects, simvastatin induced vascular relaxation in the rat aorta and inferior mesenteric artery (De Sotomayor et al., 2000) as well as in the rat superior mesenteric arterial rings (Chen et al., 2016), through an endothelium-dependent pathway. Moreover, simvastatin protected the vascular endothelium against damage induced by LDL or oxidized LDL, and relaxed the thoracic aorta in rats (Jiang et al., 2004). In mesenteric arteries of high fat diet-fed rats, both enhanced adrenergic and diminished nitrergic components contributed to increased vasoconstrictor responses to electric field stimulation. All these changes were reversed by rosuvastatin, indicating novel mechanisms of statins in neural regulation of vascular tone (Blanco-Rivero et al., 2011).

Furthermore, Pliquett and his coworkers (2003) reported that simvastatin lowered the plasma concentration of norepinephrine in rabbits associated with congestive heart failure. Plasma epinephrine concentration was slightly lower in patients treated with simvastatin therapy (Nette et al., 2005). It has been reported that simvastatin inhibits acetylcholine (ACh)-induced Na^+ influx and Ca^{2+} influx and subsequently reduces secretion and synthesis of catecholamines (CA) in

cultured bovine adrenal medullary cells in a fashion independent of cholesterol synthesis (Matsuda et al., 2008). However, the mechanisms have remained to be fully elucidated. Although an endogenous excess of CA may be a risk factor for the development of atherosclerosis (Westfall and Westfall, 2005), and these many studies on various statins are reported, it is still unclear whether simvastatin affects the function of the adrenomedullary CA secretion. Thus, the present study is the first attempt to determine whether simvastatin influences several secretagogues-evoked CA secretion from the perfused model of the isolated rat adrenal gland, and to establish its mechanism of action.

Ⅱ. MATERIALS AND METHODS

Experimental procedure

Mature Sprague-Dowley male rats (DAMOOL SCIENCE, Daejeon, Korea), weighing 180 to 250 grams, were used in this study. The experimental rats were individually housed in separate cages, and food (DAMOOL SCIENCE, Daejeon, Korea) and tap water were allowed freely for one week before experiment starts. On the day of experiment, the rat was anesthetized with intraperitoneal thiopental sodium (50 mg/kg), and tied with supine position on fixing platform.

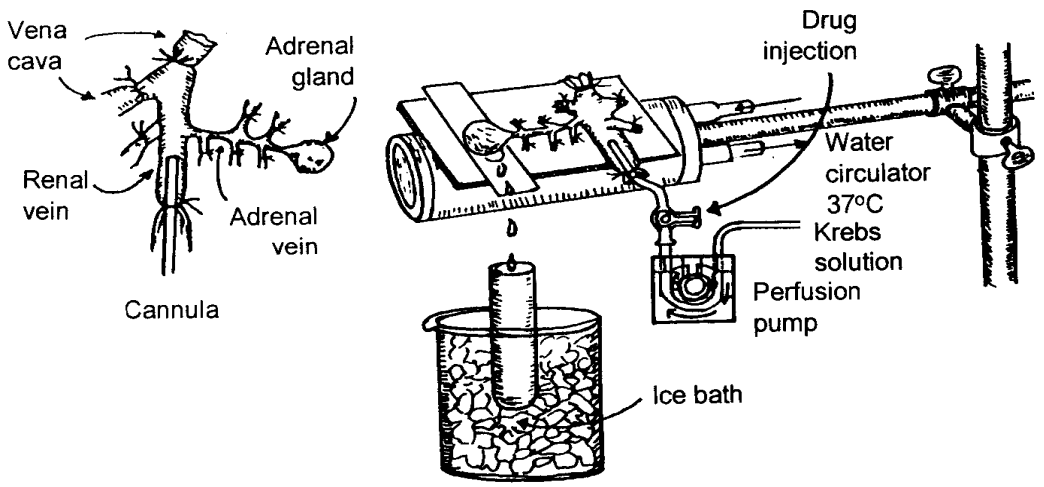

Fig. 1. Schematic drawing of the preparation used to study the CA secretion in the isolated perfused rat adrenal gland.

Isolation of adrenal glands: The adrenal medulla was isolated by some modification of previous method (Wakade, 1981). The abdomen was exposed by a midline incision, and the left adrenal gland and surrounding area were opened by the placement of three-hook retractors. The stomach, portion of the liver, and intestine were not removed out, but pushed over to the right side

and pulled up by saline-soaked gauge pads, and urine in bladder was drained in order to consolidate enough working space for tying blood vessels and cannulations. Prior to ligating vessels and cannulations, heparin (400 IU/mL) was given into vena cava to avoid blood coagulation. A cannula, employed for perfusion of the adrenal medulla, was placed into the distal end of the renal vein following all branches of adrenal vein (if any), vena cava and aorta were ligated. The adrenal cortex was cut down to make a small slit into just opposite side of adrenal vein. The adrenal gland was started to perfuse, checking up there is no leakage, and the perfusion fluid flowed out only from the slit made in adrenal cortex. Then the adrenal gland, including ligated blood vessels and the cannula, was carefully removed from the rat and placed on a platform of a leucite chamber. The chamber was constantly circulated with water heated at 37 ±1℃ (Fig. 1).

Perfusion of adrenal gland

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

Drug administration

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

In the preliminary studies, upon injection or perfusion of these drugs, secretory responses to ACh, KCl, McN-A-343, angiotensin II, veratridine, Bay-K-8644 and cyclopiazonic acid turned

back to pre-injection level in about 4 min, but the responses to DMPP in 8 min.

Collection of perfusate

Before stimulation with various secretagogues, the collection of perfusate was perfused for 4 min to determine the spontaneous CA release (background sample). Immediately following the collection of the background sample, the perfusates were collected continuously in another tube as soon as the perfusion solution containing the secretagogue reached the adrenal medulla. Stimulated sample's perfusate was collected for 4 or 8 min. The amounts secreted in the background sample have been subtracted from that secreted from the stimulated sample to get the net CA release, which is described in all of the figures.

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

Measurement of catecholamines

The content of CA (all of epinephrine, norepinephrine and dopamine) in perfusate was assayed directly by the fluorometric method of Anton and Sayre (1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using fluorospectrophotometer (Kontron Co., Milano, Italy).

0.2 mL volume of the perfusate was used for the measurement reaction. The CA content in the perfusate of stimulated adrenal medulla by secretagogues used in the present study was enough to get readings several folds greater than the reading of unstimulated samples (control). The sample blanks were also lowest for perfusates of stimulated and non-stimulated samples. The CA level in the perfusate was depicted in terms of norepinephrine (base) equivalents.

Measurement of NO release

An amplifier (inNo meter, Innovative Instruments Inc) and the NO-selective microelectrode (ami700, Innovative Instruments Inc) were utilized for assay of NO released from the perfused adrenal medulla. NO production released from adrenal medulla was quantified as the integrated signal detected by the microelectrode after perfusion of simvastatin into rat adrenal medulla, as previously described (McVeigh et al., 2002). The level of electrode was calibrated by founding standardized levels of NO in 0.5% (wt/vol) KI in 0.1 Mol/L H_2SO_4 from $NaNO_2$ standards. NO release was measured as the current detected at the electrode after perfusion of simvastatin into adrenal medulla. The net NO release was calculated as picomoles.

Statistical analysis

The difference between the drug-treated group and the control group was statistically analyzed by the Student's *t* and ANOVA tests. Unless specifically described in the text, a P-value of less than 0.05 was statistically regarded to express significant changes. Values described in the text refer to means ± the standard errors of the mean (S.E.M.). The experimental data were statistically assayed by computer program made by Tallarida and Murray (1987).

Drugs and their sources

The following drugs were used: simvastatin (a gift from Sam-A Pharmaceutical company, Seoul, Korea), 3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride [McN-A-343] (RBI, U.S.A.), fimasartan (a gift from Boryung Pharmaceutical Company, Seoul, Korea), cyclopiazonic acid, acetylcholine chloride, norepinephrine bitartrate, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), veratridine hydrochloride, potassium chloride (KCl), calcium chloride, sodium bicarbonate, angiotensin II, sodium chloride, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), glucose, potassium phosphate, ascorbic acid, magnesium chloride disodium EDTA, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoro-methyl -phenyl)-pyridine-5-carboxylate [Bay–K-8644], (Sigma Chemical Co., U.S.A.). Drugs were

usually dissolved in distilled water (stock) and added to the normal Krebs-bicarbonate solution, Exceptionally, Bay-K-8644 was dissolved in 99.5 % (stock) ethanol and then diluted adequately with Krebs-bicarbonate solution (final concentration of ethanol was less than 0.1 %). Concentrations of all drugs used in the present study are described in terms of their molar base.

III. RESULTS

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

After the perfusion of oxygenated Krebs-bicarbonate solution for 60 min, the basal CA secretion from the perfused model of the isolated rat adrenal medulla was 22±2 ng for 2 min (n=12). Since it has been shown that simvastatin inhibits ACh-induced CA secretion and through blocking Na^+ and Ca^{2+} influx in cultured bovine adrenal medullary (Matsuda et al., 2008), it was tried initially to examine the effects of simvastatin itself on CA secretion from the perfused model of the rat adrenal medulla. However, in the present study, simvastatin itself did not affect basal CA secretion in the perfused model of the rat adrenal medullary chromaffin cells (data not shown). Therefore, it was attempted to determine the effects of simvastatin on the CA secretion evoked by activation of angiotensin II receptors and cholinergic receptors. Secretagogues were administered at 15 or 20 min-intervals. Simvastatin was infused for 90 minutes after the corroboration of the control secretion.

When ACh (5.32 mM) in a volume of 50 μL was given into the perfusion stream, the amount of CA secretion was 1295±53 ng for 4 min. However, during the perfusion of simvastatin in the range of 20 ~ 200 μM for 90 min, ACh-evoked CA release was markedly inhibited in relatively time- and concentration-dependent manners. As shown in Fig. 2, during loading of simvastatin, the ACh-evoked CA secretion was suppressed maximally to 42% of the control CA secretion (100%).

DMPP (100 μM), which is a selective agonist of neuronal nicotinic receptor in autonomic sympathetic ganglia, produced a sharp and rapid rise in the CA release (1296±35 ng for 0-8 min). However, as in Fig. 3, DMPP-induced CA secretion during the presence of simvastatin (60 μM) for 90 min was vastly diminished to 62% of the control secretion.

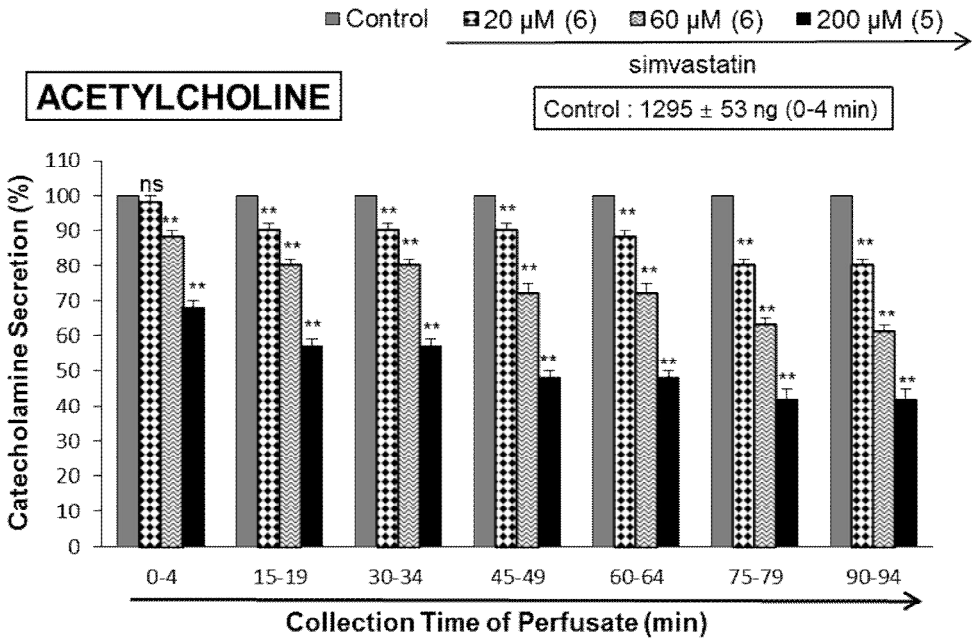

Fig. 2. Dose-dependent effects of simvastatin on acetylcholine (ACh)-evoked secretion of catecholamines (CA) from the perfused rat adrenal medulla. The CA secretion by a single injection of ACh (5.32 mM) in a volume of 50 μL was produced at 15 min intervals during perfsusion with 20, 60 and 200 μM of simvastatin for 90 min as denoted by the arrow marks, respectively. The numbers in parentheses denote the number of adrenal medulla used in this experiment. Vertical bars on the columns describe the standard error of the mean (S.E.M.). Ordinate: the amounts of CA released from the adrenal medulla (% of control). Abscissa: collection time of perfusate (min). Statistical difference was analyzed by comparing the control group with each concentration-treated group of simvastatin. ACh-evoked perfusate was collected for 4 minutes. **: $p<0.01$. ns: Not statistically significant.

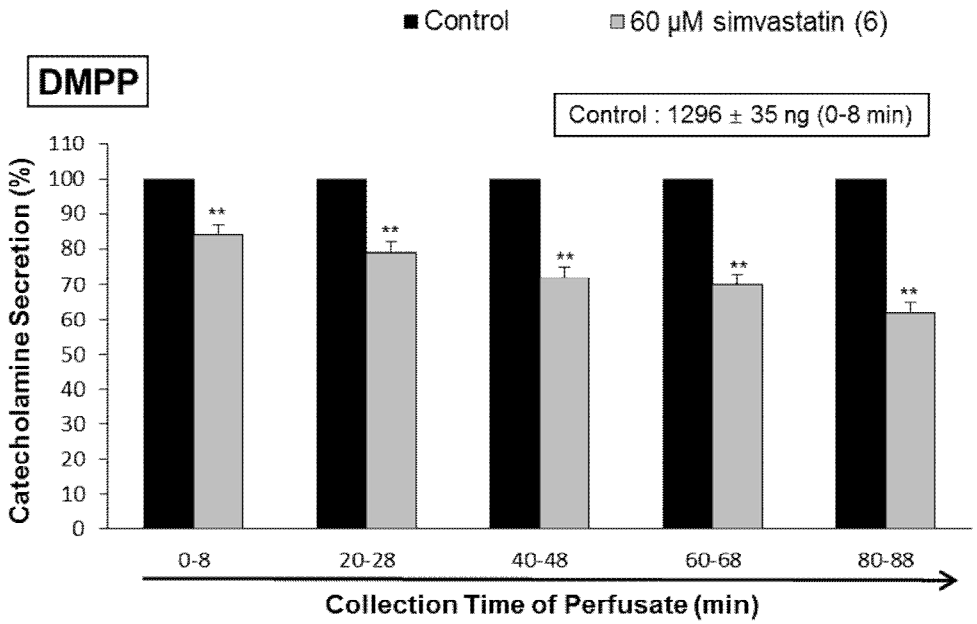

Fig. 3. Time-course effects of simvastatin on DMPP-evoked CA release in the perfused rat adrenal medulla. The CA release by perfusion of DMPP (100 μM) for 2 min was made at 20 min interval during infusion of 60 μM simvastatin for 90 min. DMPP-evoked perfusate was collected for 8 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$.

McN-A-343 (100 μM), a selective muscarinic M_1-receptor agonist (Hammer and Giachetti, 1982), when given into an adrenal gland for 4 min, also increased the CA secretion (643±24 ng for 0-4 min). However, under the existence of simvastatin (60 μM), McN-A-343-evoked CA release was greatly inhibited to 64% of the control release as shown in Fig. 4.

Since it has been found that Ang II increases epinephrine release from the adrenal medulla via the AT_1 receptors (Hano et al., 1994), it was attempted to examine the effect of simvastatin on Ang

II-induced CA secretion. Ang II (100 nM) markedly increased the CA release (585±26 ng for 0-4 min), whereas under the existence of simvastatin (60 μM) for 90 min, Ang II-evoked CA release was significantly inhibited to 62% of the control secretion (Fig. 5).

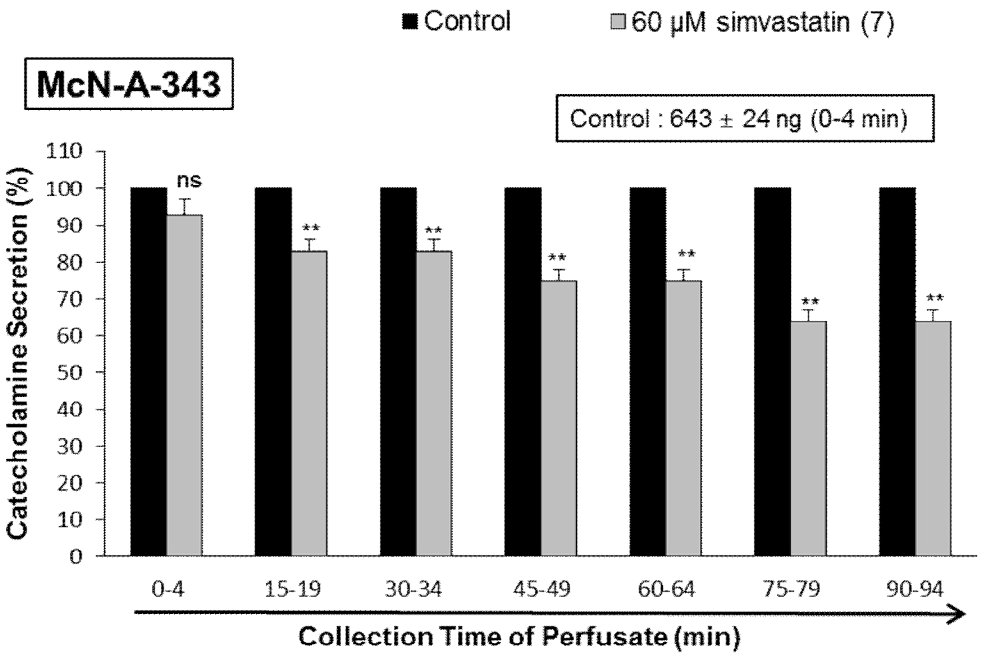

Fig. 4. Time-course effects of simvastatin on McN-A-343-evoked CA secretion from the perfused rat adrenal gland. The CA release by perfusion of McN-A-343 (100 μM) for 4 min was produced at 15 min interval during perfusion of 60 μM simvastatin for 90 min. McN-A-343-evoked perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

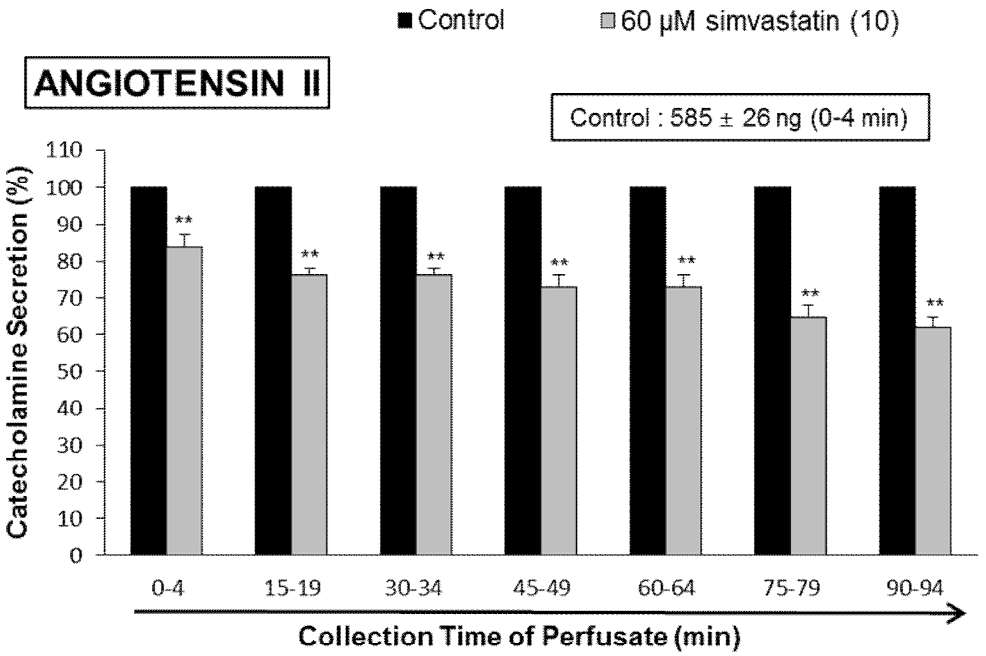

Fig. 5. Time-course effects of simvastatin on angiotensin II-evoked CA release in the perfused rat adrenal medulla. Angiotensin II (100 nM) was given into an adrenal vein for 2 min at 15 min intervals during perfusion of 60 μM simvastatin for 90 min. Angiotensin II-evoked perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$.

Influence of simvastatin on the CA release evoked by high K^+, Bay-K-8644, cyclopiazonic acid and veratridine from the perfused rat adrenal medulla

Also, high KCl, a depolarizing agent, greatly increased the CA release (789±39 ng for 0-4 min). High K^+ (56 mM)-evoked CA release during perfusion of simvastatin (60 μM) for 90 min was significantly inhibited to 65% of the control in 75~94 min periods, as shown in Fig. 6.

Since Bay-K-8644 is known to be a calcium channel activator, which enhances basal Ca^{2+} uptake (Garcia et al., 1984) and CA release (Lim et al., 1992), it was of interest to determine the effects of simvastatin on Bay-K-8644-induced CA secretion from the perfused rat adrenal medulla. Bay-K-8644 (10 μM)-evoked CA release in the presence of simvastatin (60 μM) was markedly diminished to 64% of the control compared to the control release (614±17 ng for 0-4 min) from 6 rat adrenal medullas, as shown in Fig. 7.

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Goeger and Riley, 1989; Seidler et al., 1989). The inhibitory action of simvastatin on cyclopiazonic acid-evoked CA secretion was shown as in Fig. 8. In 8 rat adrenal glands, under the existence of simvastatin (60 μM) for 90 min, cyclopiazonic acid (10 μM)-evoked CA secretion was also reduced maximally to 62% of the control release (574±21 ng for 0-4 min).

It has been found that veratridine-induced Na^{+} influx mediated through voltage-dependent Na^{+} channels increased Ca^{2+} influx via activation of voltage-dependent Ca^{2+} channels and produced the exocytotic CA secretion in cultured bovine adrenal medullary cells (Wada et al., 1985a). Veratridine (50 μM) sharply increased the CA release (845±21 ng for 0-4 min), as shown in Fig. 9. In 7 rat adrenal medullae, simvastatin (60 μM) also reduced veratridine-induced CA secretion to 61% of the control secretion.

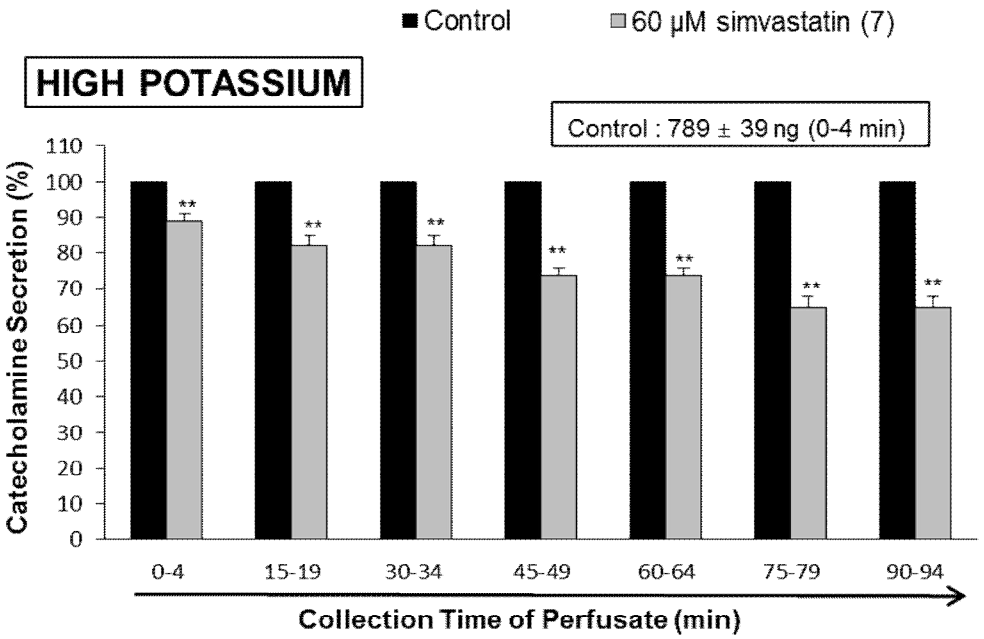

Fig. 6. Time-course effects of simvastatin on the high K^+-evoked CA release in the perfused rat adrenal medulla. The CA release by a single injection of K^+ (56 mM) in a volume of 50 μL was made at 15 min intervals during perfusion of 60 μM simvastatin for 90 min as nenoted by the arrow marks. High K^+-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$.

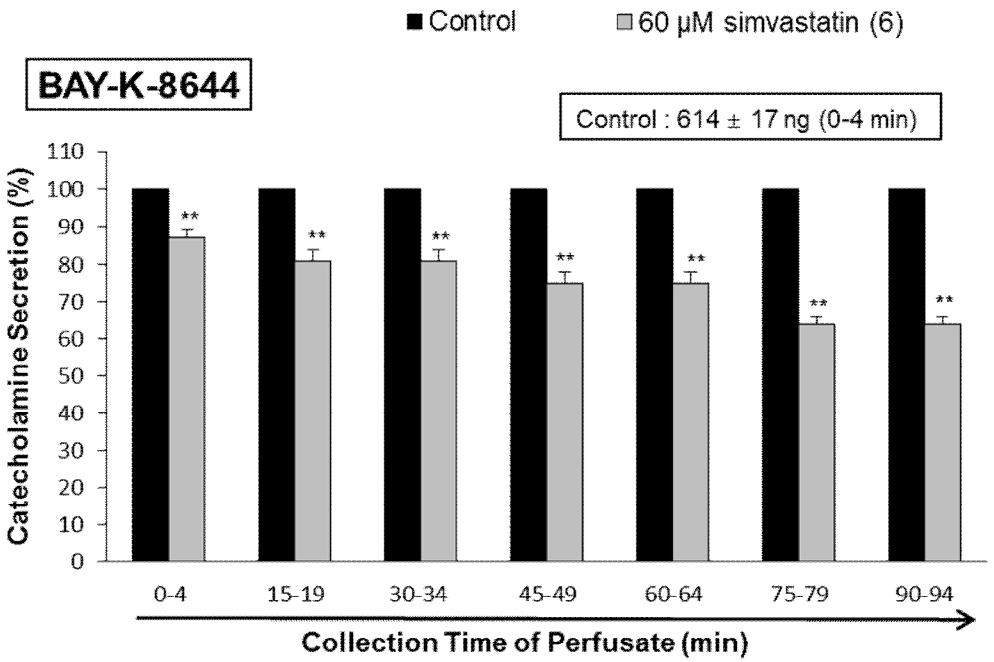

Fig. 7. Time-course effects of simvastatin on Bay-K-8644-evoked CA release in the perfused rat adrenal medulla. Bay-K-8644 (10 μM) was administered into an adrenal vein for 4 min at 15 min intervals during loading with simvastatin (60 μM) for 90 min. Bay-K-8644-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

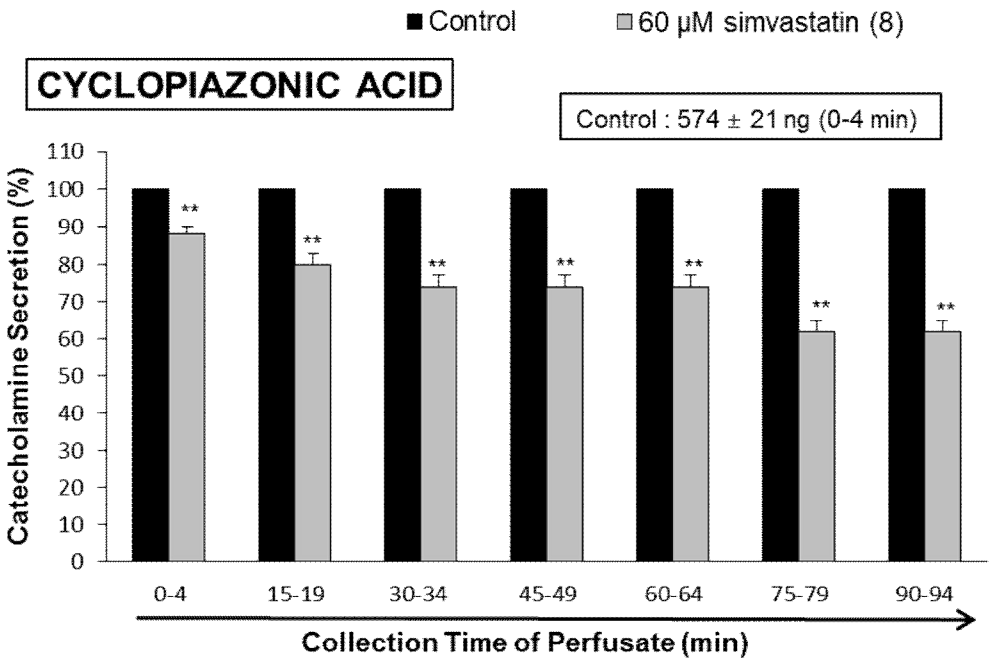

Fig. 8. Time-course effects of simvastatin on cyclopiazonic acid-evoked CA release in the perfused rat adrenal medulla. Cyclopiazonic acid (10 μM) was perfused into an adrenal vein for 4 min at 15 min intervals during perfusion of 60 μM simvastatin for 90 min. Cyclopiazonic acid-evoked perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

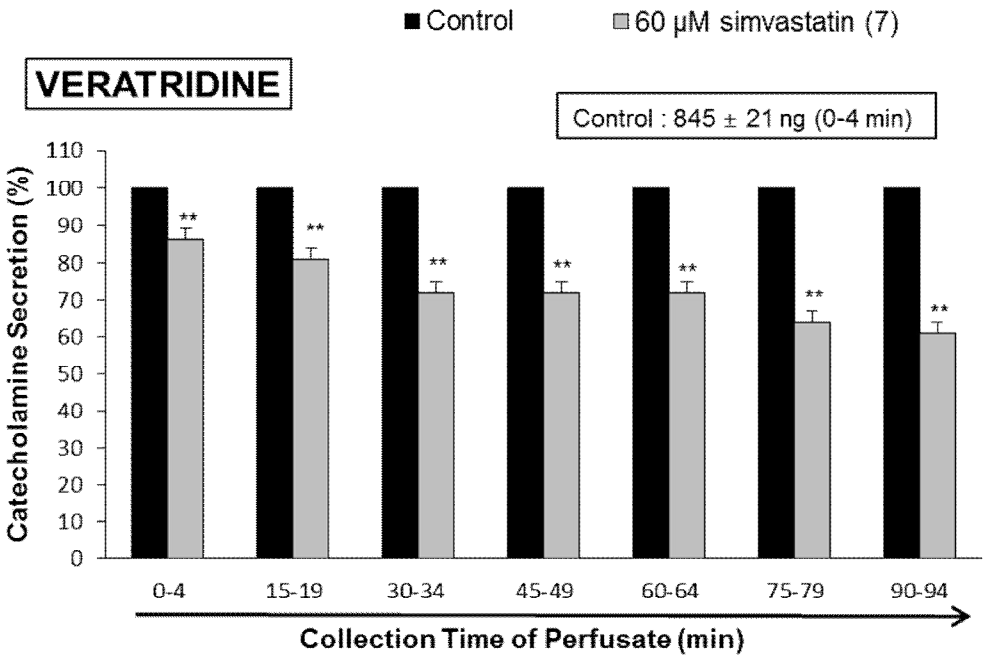

Fig. 9. Time-course effects of simvastatin on veratridine-evoked CA release in the perfused rat adrenal medulla. Veratridine (50 μM) was given into an adrenal vein for 4 min at 15 min intervals during perfusion of 60 μM simvastatin for 90 min. Veratridine-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$.

Influence of simvastatin plus L-NAME on CA release evoked by ACh, DMPP, McN-A-343, angiotensin II, high K^+, Bay-K-8644, cyclopiazonic acid and veratridine from the perfused rat adrenal medulla

It has also been found that, in the present study, simvastatin greatly inhibited the CA release evoked by activation of angiotensin II receptors as well as cholinergic receptors in the perfused rat adrenal medulla. Therefore, in order to determine the relationship between NO and simvastatin-induced inhibitory action on the CA release in the perfused rat adrenal medulla, the influence of L-NAME on simvastatin-induced inhibitory responses of CA release evoked by DMPP, ACh, McN-A-343, Ang II, Bay-K-8644, high K^+, cyclopiazonic acid and veratridine was investigated.

In the present study, during the simultaneous loading with L-NAME (30 μM) and simvastatin (60 μM) for 90 min, in 8 rat adrenal medullae, ACh (5.32 mM)-evoked CA secretion was mostly recovered to 100~92% of the control secretion (1330±43 ng for 0-4 min) compared to that of simvastatin (60 μM)-treated alone, as shown in Fig. 10.

Also, during simultaneous perfusion of L-NAME (30 μM) and simvastatin (60 μM) for 90 min, DMPP- and McN-A-343-evoked CA secretion was recovered mostly to their control levels (100~92%) compared to that of the simvastatin-treatment alone (Fig. 11 and 12).

Moreover, during simultaneous loading of L-NAME (30 μM) and simvastatin (60 μM) for 90 min from 8 rat adrenal medullae, the Ang II (100 nM)-evoked CA secretion was also restored to 100~86% of the control secretion (604±30 ng for 0-4 min), in comparison to the inhibitory effect of simvastatin-treatment alone on Ang II-induced CA secretion, as in Fig. 13.

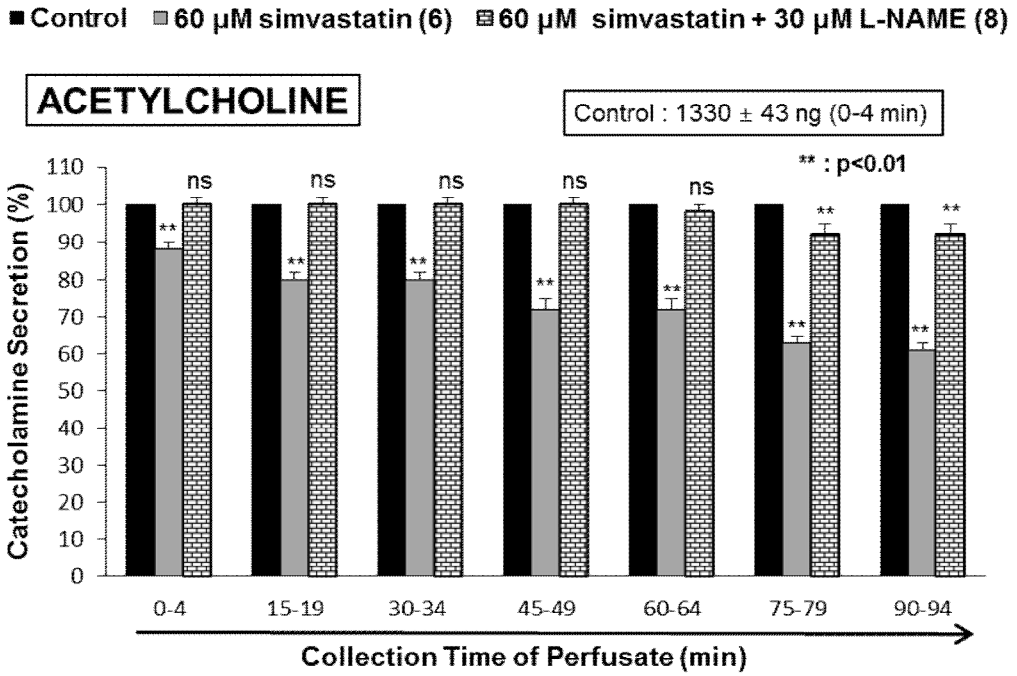

Fig. 10. Effects of simvastatin plus L-NAME on acetylcholine-evoked CA secretion in the perfused rat adrenal medulla. The CA release by a single injection of ACh (5.32 mM) in a volume of 50 μL was made at 15 min intervals during simultaneous perfusion of 60 μM simvastatin plus 30μM L-NAME for 90 min. Statistical difference was analyzed by comparing the control with simvastatin-treated alone group or group treated with simvastatin+L-NAME. Acetylcholine-evoked perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

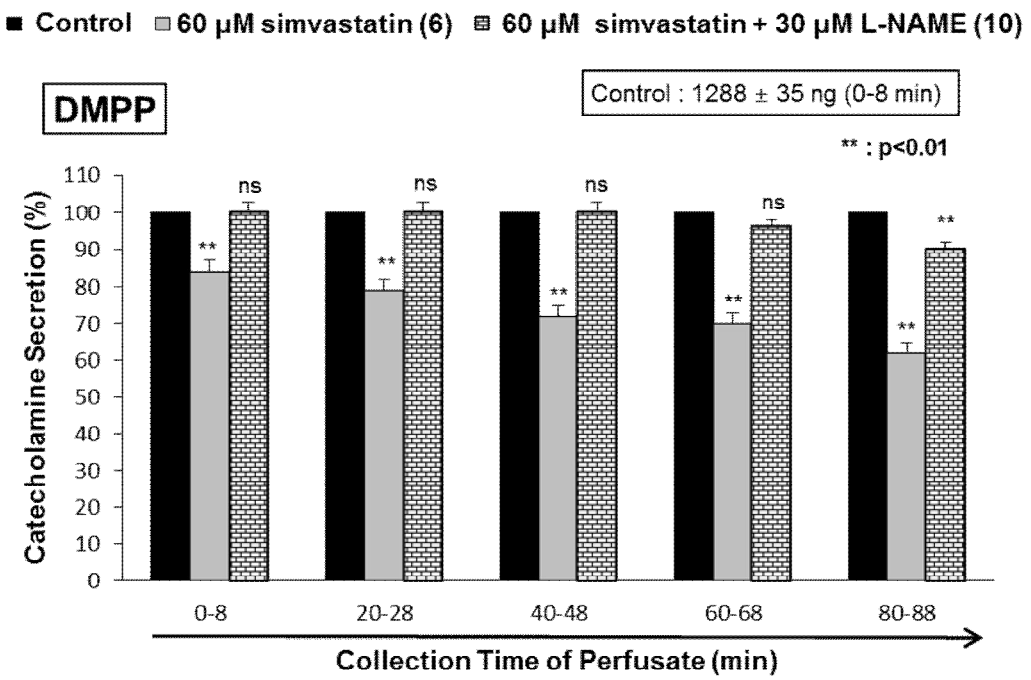

Fig. 11. Effects of simvastatin plus L-NAME on DMPP-evoked CA release in the perfused rat adrenal medulla. The CA release by perfusion of DMPP (100 μM) for 2 min was made at 20 min intervals during simultaneous perfusion of 60 μM simvastatin plus 30μM L-NAME for 90 min. DMPP-induced perfusate was collected for 8 minutes. Other legends are the same as in Fig. 2. **: p<0.01. ns: Not statistically significant.

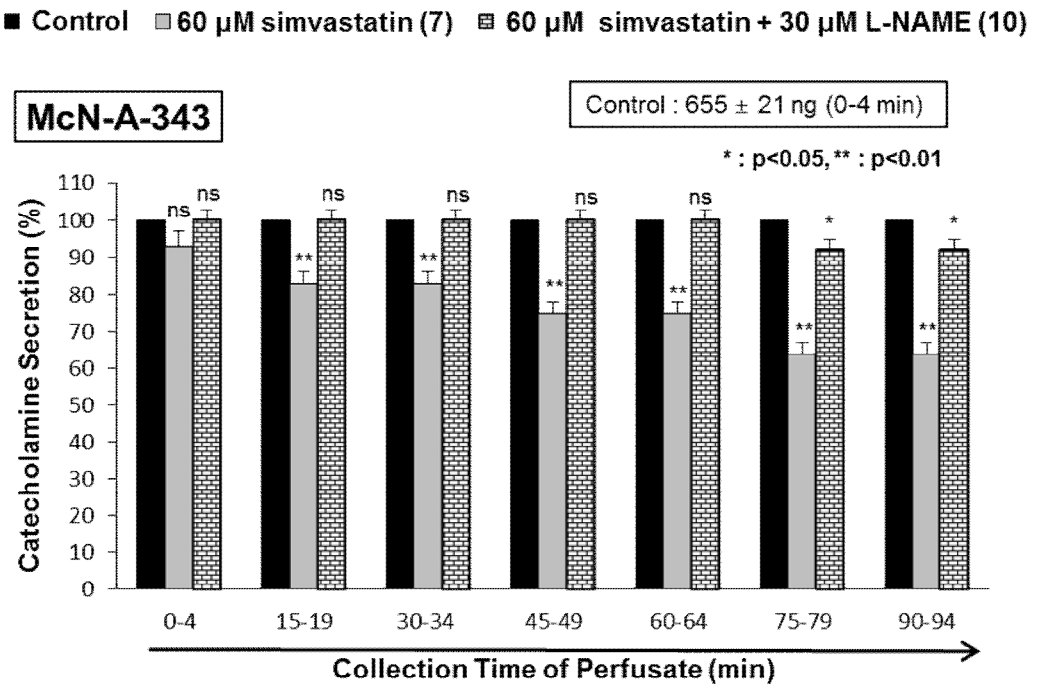

Fig. 12. Effects of simvastatin plus L-NAME on McN-A-343-evoked CA release in the perfused rat adrenal medulla. The CA release by perfusion of McN-A-343 (100 μM) for 4 min was produced at 15 min intervals during simultaneous perfusion of 60 μM simvastatin plus 30μM L-NAME for 90 min. McN-A-343-induced perfusate was collected for 8 minutes. Other legends are the same as in Fig. 2. *: p<0.05, **: p<0.01. ns: Not statistically significant.

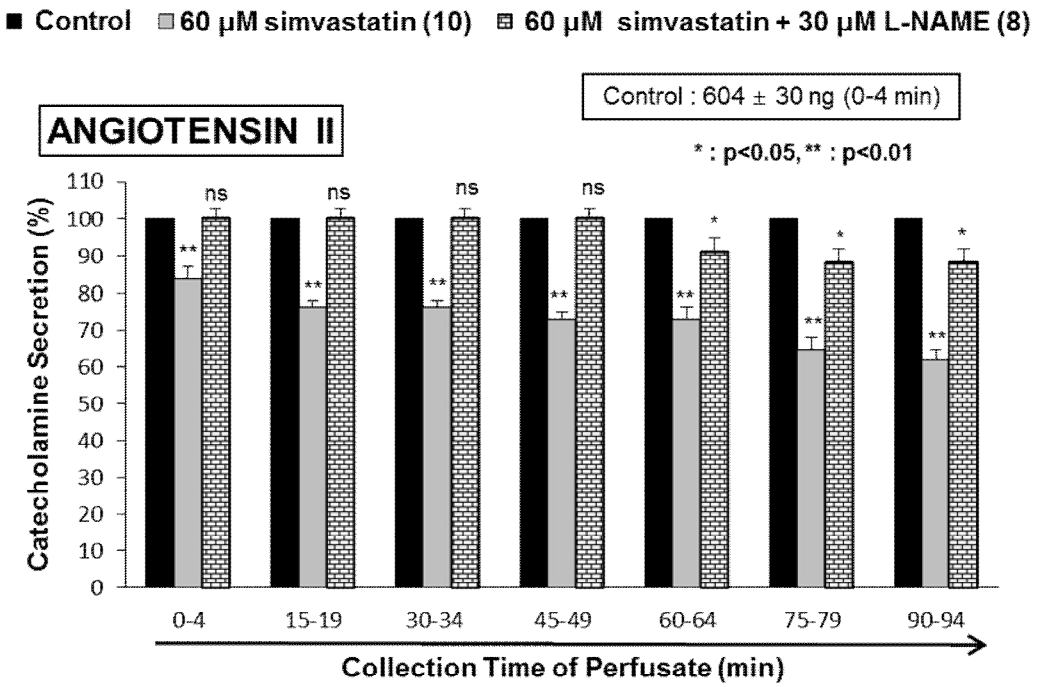

Fig. 13. Effects of simvastatin plus L-NAME on angiotensin II-evoked CA release in the perfused rat adrenal medulla. The CA release by perfusion of angiotensin II (100 nM) for 2 min was measured at 15 min intervals during simultaneous loading of 60 μM simvastatin plus 30μM L-NAME for 90 min. Angiotensin II-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: p<0.01. ns: Not statistically significant.

The simultaneous perfusion of simvastatin (60 μM) and L-NAME (30 μM) for 90 min, high K^+ (56 mM)-evoked CA secretion was also recovered to 100~88% of the control release (779±36 ng for 0-4 min), in which there was a significant difference compared to the inhibitory effect of simvastatin-treatment alone on high K^+ (56 mM)-evoked CA secretion (Fig. 14).

The coexistence of simvastatin (60 μM) and L-NAME (30 μM) for 90 min restore the CA release evoked by Bay-K-8644 (10 μM) and cyclopiazonic acid (10 μM) mostly to 100~88% (Bay-K-8644) and 100~86% (cyclopiazonic acid) of their control secretory responses (627±22 ng/0-4 min for Bay-K-8644; 575±25 ng/0-4 min for cyclopiazonic acid), respectively, compared to the inhibitory effect of simvastatin-treatment alone, as shown in Fig. 15 and 16.

During simultaneous perfusion of simvastatin (60 μM) and L-NAME (30 μM) for 90 min, there was also vastly a full recovery (100~91%) of the control secretion (845±21 ng for 0-4 min) in veratridine (50 μM)-evoked CA release compared to that of the inhibitory effect of simvastatin-treatment alone (Fig. 17).

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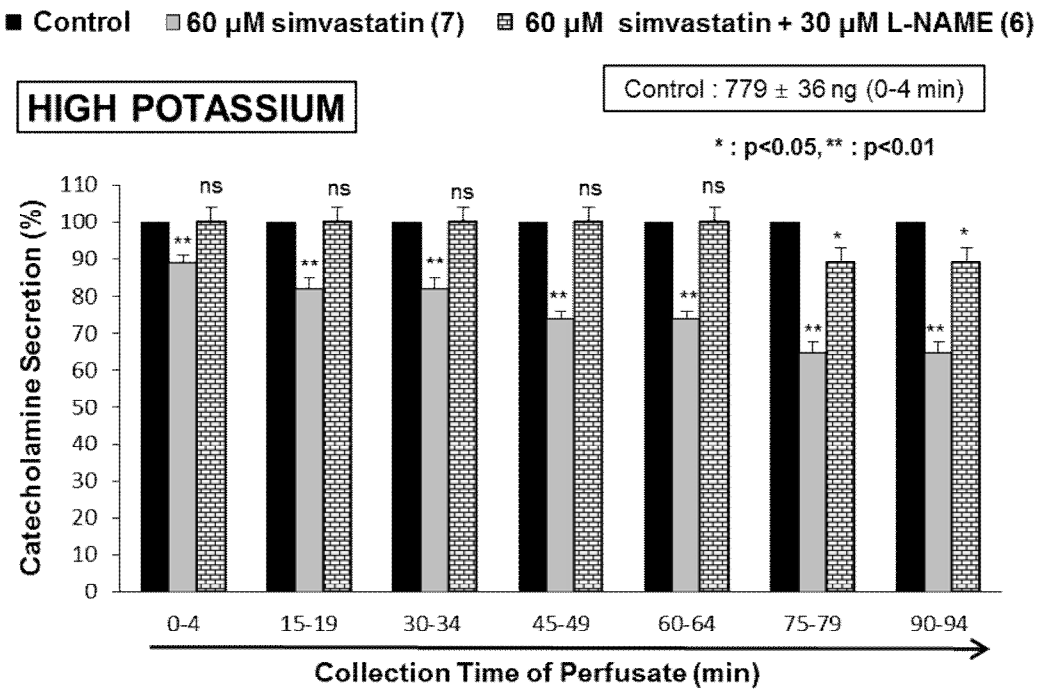

Fig. 14. Influence of simvastatin plus L-NAME on high potassium-evoked CA secretion in the perfused rat adrenal medulla. The CA release by a single injection of high potassium (56 mM) in a volume of 50 μL was made at 15 intervals during simultaneous perfusion of 60 μM simvastatin plus 30μM L-NAME for 90 min. Statistical difference was analyzed by comparing the control with group of simvastatin-treated alone or group treated with simvastatin+L-NAME. High potassium-induced perfusates were collected for 4 minutes. Other legends are the same as in Fig. 2., **: P < 0.01. ns: Not statistically significant.

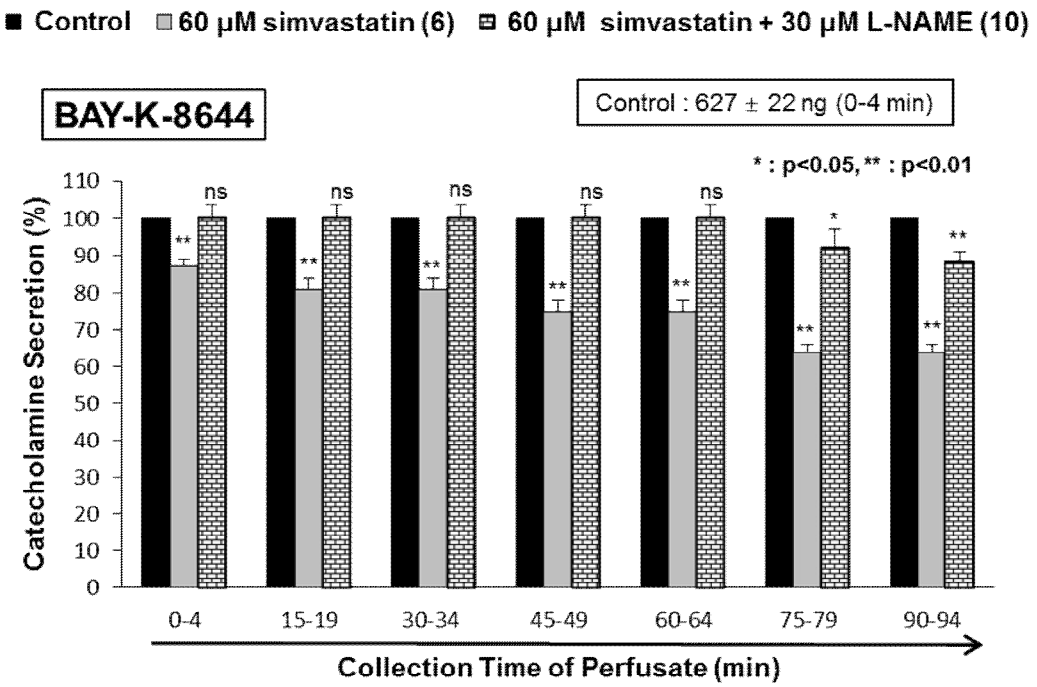

Fig. 15. Effects of simvastatin plus L-NAME on Bay-K-8644-evoked CA release in the perfused rat adrenal medulla. The CA release by perfusion of Bay-K-8644 (10 μM) for 4 min was made at 15 min intervals during simultaneous loading of 60 μM simvastatin plus 30μM L-NAME for 90 min. Bay-K-8644-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: p<0.01. ns: Not statistically significant.

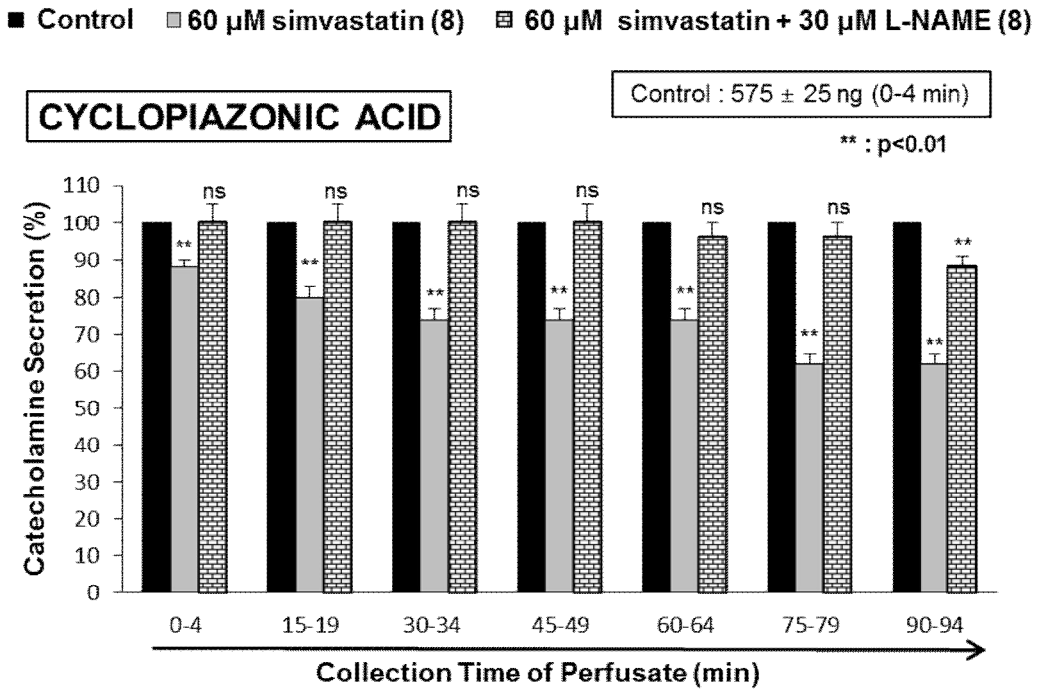

Fig. 16. Effects of simvastatin plus L-NAME on cyclopiazonic acid-induced CA secretion in the perfused rat adrenal medulla. The CA release by perfusion of cyclopiazonic acid (10 μM) for 4 min was produced at 15 min intervals during simultaneous perfusion of 60 μM simvastatin plus 30μM L-NAME for 90 min. Cyclopiazonic acid -induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: p<0.01. ns: Not statistically significant.

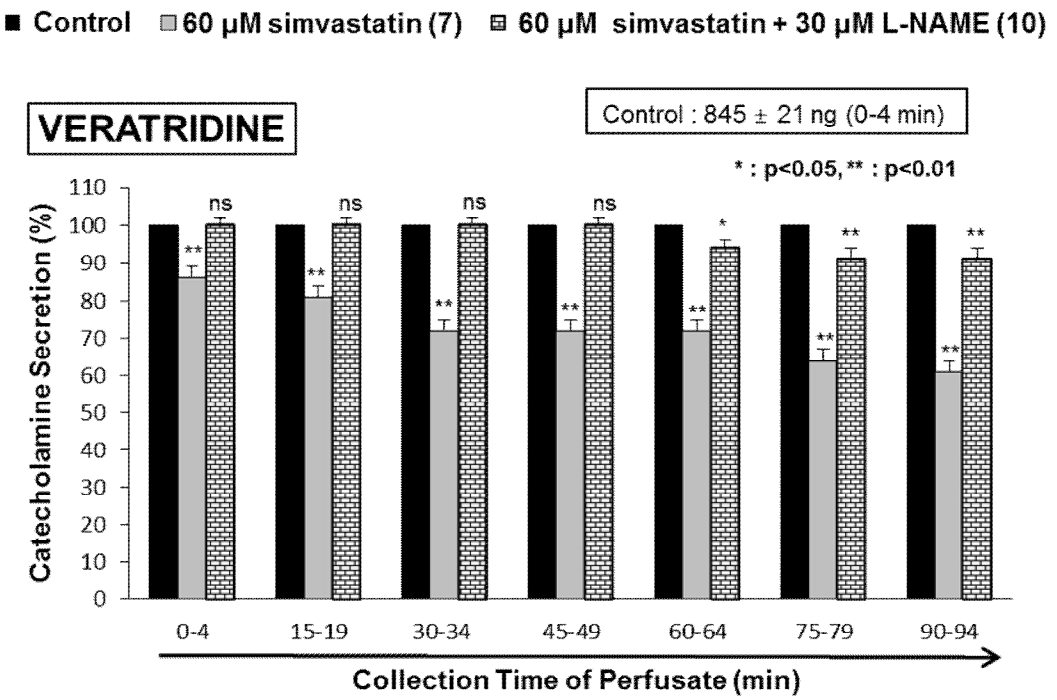

Fig. 17. Effects of simvastatin plus L-NAME on veratridine-evoked CA secretion in the perfused rat adrenal medulla. The CA release by perfusion of veratridine (100 μM) for 4 min was produced at 15 min intervals during simultaneous perfusion of 60 μM simvastatin plus 30μM L-NAME for 90 min. Veratridine-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. *: $p<0.05$, **: $p<0.01$. ns: Not statistically significant.

Influence of simvastatin on the level of nitric oxide in the perfused rat adrenal medulla

As shown in Fig. 10~17, the CA release evoked by DMPP, ACh, McN-A-343, high K^+, Ang II, cyclopiazonic acid, Bay-K-8644, and veratridine were markedly recovered to the control level during simultaneous perfusion of simvastatin and L-NAME. Thus, it was tried directly to assay the level of NO released from rat adrenal medulla after the perfusion of simvastatin. Moreover, it has been found that simvastatin induced vascular relaxation in the rat aorta and inferior mesenteric artery (De Sotomayor et al., 2000) as well as in the rat superior mesenteric arterial rings (Chen et al., 2016), through an endothelium-dependent pathway. Moreover, simvastatin protected the vascular endothelium against damage induced by LDL or oxidized LDL, and relaxed the thoracic aorta in rats (Jiang et al., 2004).

In 5 adrenal medullae, the basal release of NO from the adrenal medulla before perfusion of simvastatin was 35.9±3 picomoles. However, 8 min after perfusion with 60 μM simvastatin it was significantly elevated to 53.9±8picomoles, which was 150% of the basal release, as shown in Fig. 18.

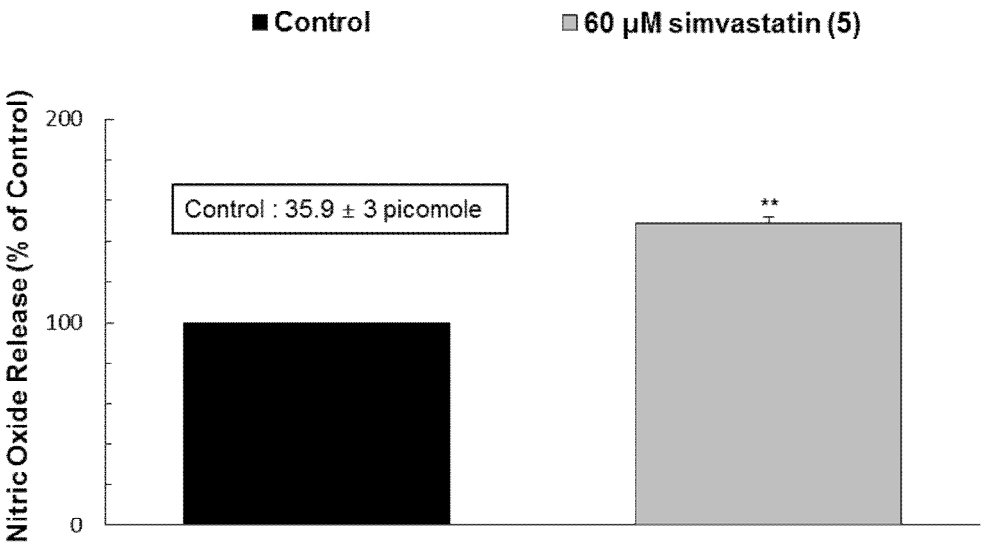

Fig. 18. Effects of simvastatin on level of nitric oxide release in the perfused rat adrenal medulla. Perfusate sample was collected for 8 min after the perfusion of simvastatin (60 μM) at a rate of 0.31 mL/min. Ordinate: the amounts of nitric oxide released from the adrenal medulla (% of control). Abscissa: Treatment (before and after simvastatin). Statistical difference was analyzed by comparing the control group with simvastatin-treated group. **: $p<0.01$.

Effects of mevalonolactone, pravastatin and atrorvastatin on the ACh-induced CA secretion from the perfused rat adrenal medulla

It was investigated whether the presence of mevalonolactone, which is nonenzymatically converted to mevalonate, the first metabolite of HMG-CoA, affects simvastatin-induced inhibitory effect on ACh-evoked CA secretion. However, ACh-induced CA secretory response in the presence of 200 μM mevalonolactone for 90 min was not influenced in comparison of that of its control secretory response, as shown in Fig. 19. Furthermore, simvastatin (60 μM) significantly diminished ACh-induced CA secretory response in the absence as well as the presence of 200 μM mevalonolactone (Fig. 20).

Interestingly, pravastatin (60 μM), another statin of HMG-CoA reductase inhibitors, never affected ACh-induced CA secretory response in the perfused rat adrenal medulla as shown in Fig. 21. However, atorvastatin (60 μM), one of statins, significantly depressed ACh-induced CA secretory response to 82% of its control response, although it was meek compared to that of simvastatin (Fig. 22).

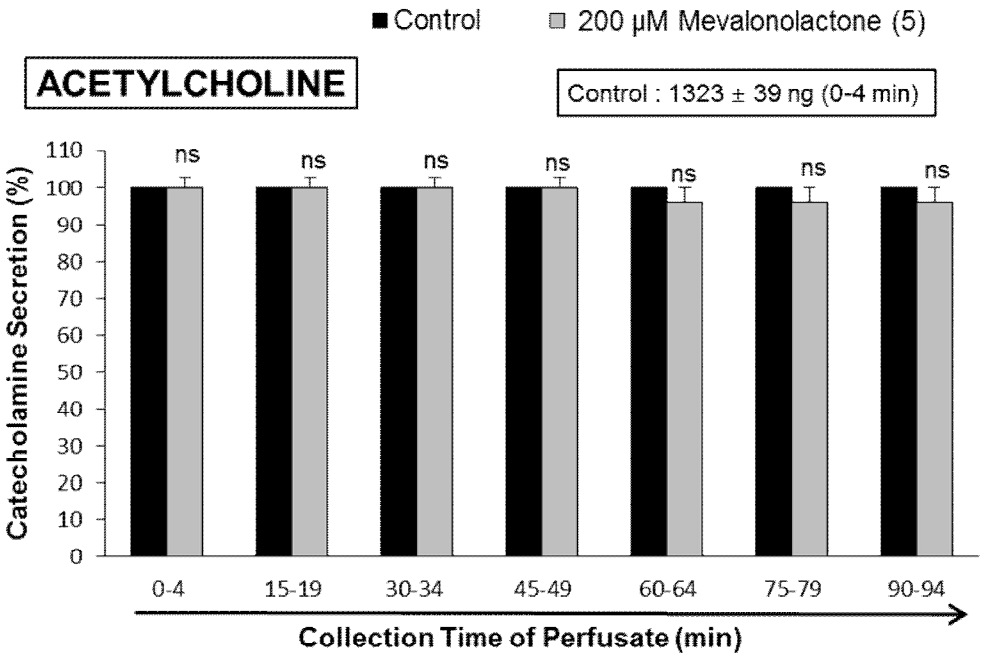

Fig. 19. Influence of mevalonolactone on acetylcholine-evoked CA release in the perfused rat adrenal medulla. The CA release by a single injection of ACh (5.32 mM) in a volume of 50 μL was made at 15 min intervals during loading with 60 μM mevalonolactone for 90 min as denoted by the arrow marks. Acetylcholine-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

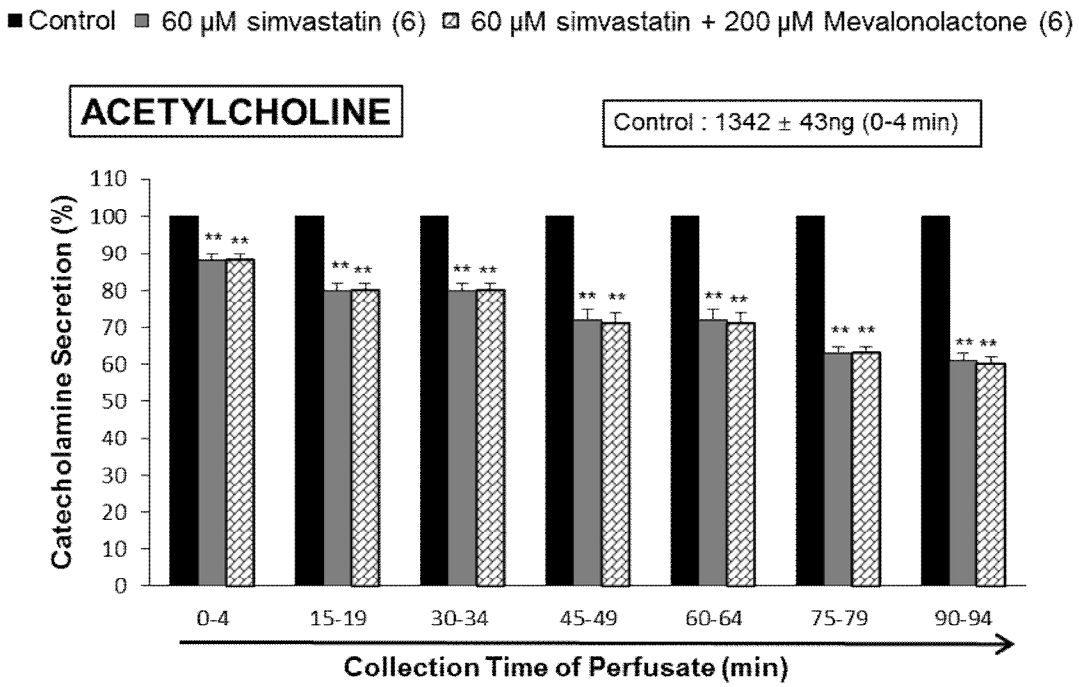

FIG. 20. Comparative time-course effects of simvastatin and mevalonolactone plus simvastatin on acetylcholine-evoked CA release in the perfused rat adrenal glands. The CA release by a single injection of ACh (5.32 mM) in a volume of 50 μL was made at 15 min intervals during perfusion of simvastatin (60 μM) or mevalonolactone (200 μM) plus simvastatin (60 μM) for 90 min, respectively, as denoted by the arrow mark. Acetylcholine-induced perfusates were collected for 4 minutes. Other legends are the same as in Fig. 2. *: $P < 0.05$ or **: $P < 0.01$ control group vs simvastatin (60 μM)-treated group or mevalonolactone (200 μM) + simvastatin (60 μM)-treated group.

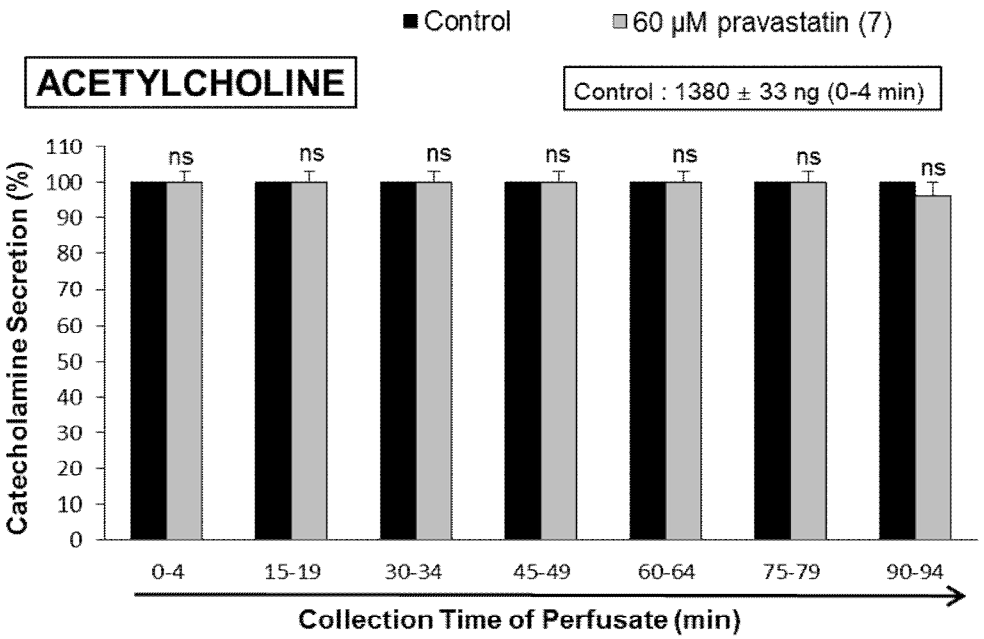

Fig. 21. Influence of pravastatin on acetylcholine-evoked CA release in the perfused rat adrenal medulla. The CA release by a single injection of ACh (5.32 mM) in a volume of 50 μL was made at 15 min intervals during loading with 60 μM pravastatin for 90 min as denoted by the arrow marks. Acetylcholine-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

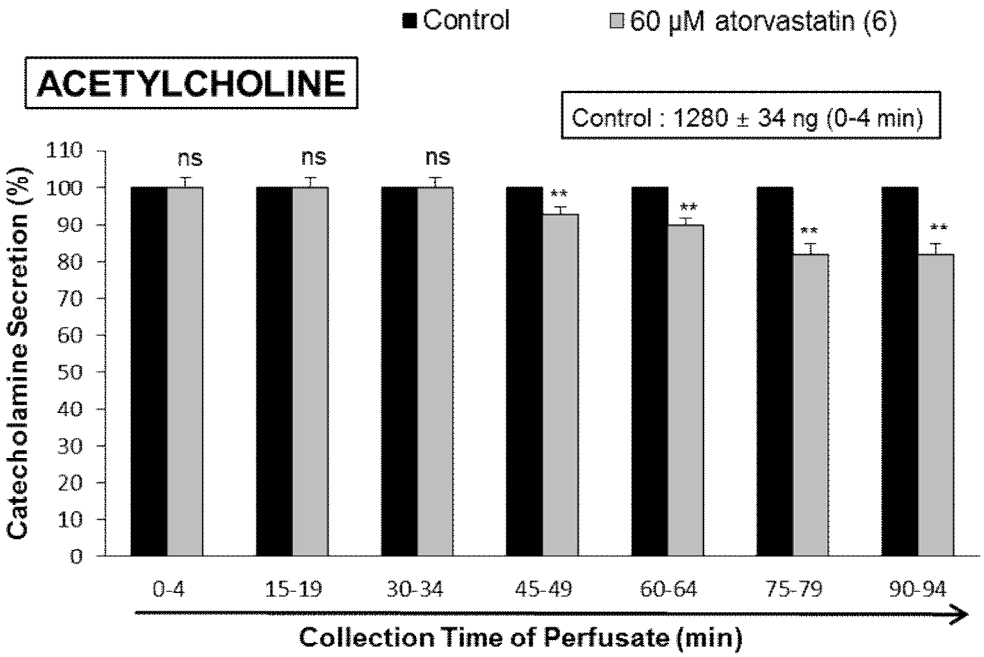

Fig. 22. Influence of atorvastatin on acetylcholine-evoked CA release in the perfused rat adrenal medulla. The CA release by a single injection of ACh (5.32 mM) in a volume of 50 μL was made at 15 min intervals during loading with 60 μM atorvastatin for 90 min as denoted by the arrow marks. Acetylcholine-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. **: $p<0.01$. ns: Not statistically significant.

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

In the present study, simvastatin or fimasartan (an angiotensin II type 1 (AT_1) receptor-selective antagonist (Kim et al., 2012) caused inhibitory effects on the CA secretion by activation of Ang II AT_1 receptors as well as cholinergic receptors in the perfused rat adrenal medulla. Thus, in order to investigate the combined effects of simvastatin and fimasartan on ACh-evoked CA release, it was attempted to determine inhibitory effects of simvastatin plus fimasartan on ACh-induced CA secretion.

In the simultaneous presence of fimasartan (15 μM) and simvastatin (60 μM) for 90 min, ACh (5.32 mM)-evoked CA secretion was more markedly depressed to ~54% of the control release (1189±33 ng for 0-4 min), in comparison to the inhibitory effect evoked by fimasartan-treatment alone, as shown in Fig. 23. Also, there was statistically difference in inhibitory effect between fimasartan versus simvastatin plus fimasartan on ACh-evoked CA release (Fig. 23).

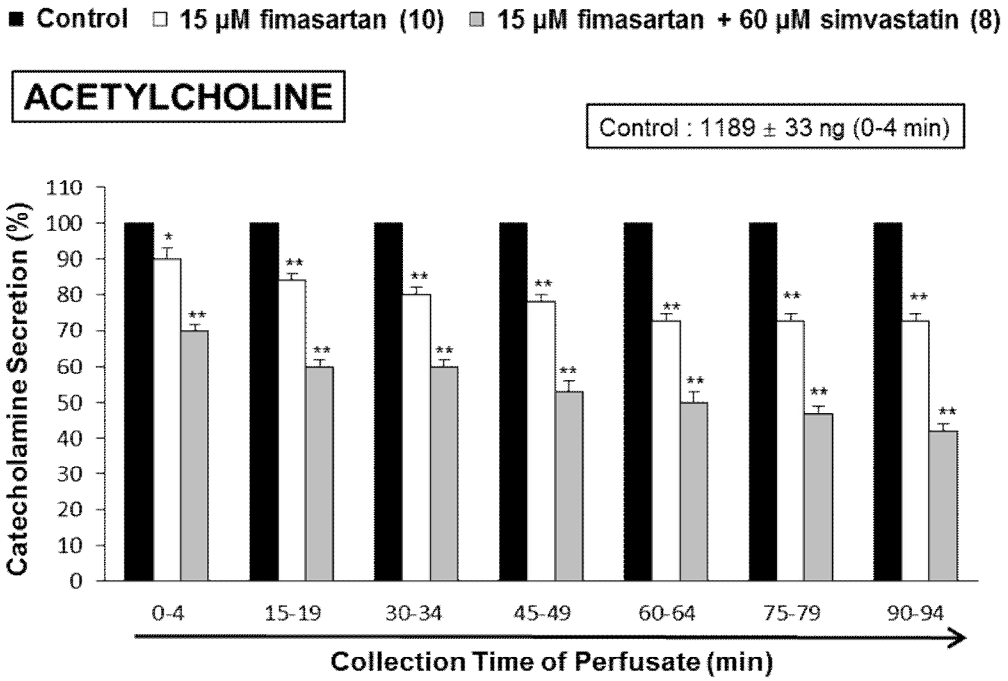

Fig. 23. Comparative time-course effects of fimasartan and simvastatin plus fimasartan on acetylcholine-induced CA release in the perfused rat adrenal glands. The CA release by a single injection of ACh (5.32 mM) in a volume of 50 μL was evoked at 15 min intervals during loading of fimasartan (15 μM) or fimasartan (15 μM) plus simvastatin (60 μM) for 90 min, respectively, as denoted by the arrow mark. Acetylcholine-induced perfusates were collected for 4 minutes. Other legends are the same as in Fig. 2. *: $P < 0.05$ or **: $P < 0.01$ control group vs fimasartan (15 μM)-treated group or fimasartan (15 μM) + simvastatin (60 μM)-treated group.

IV. DISCUSSION

The present data are the first evidence showing that simvastatin markedly diminishes the CA secretion induced by stimulation of AT_1 receptors as well as cholinergic nicotinic receptors from the perfused model of the rat adrenal medulla. It appears that this simvastatin-induced inhibitory action is derived from inhibition of both Na^+ and Ca^{2+} influx via their ion channels into the rat adrenal chromaffin cells and also by repression of Ca^{2+} mobilization from the cytoplasmic Ca^{2+} pool at least due to increased NO release through activation of neuronal NO synthase, which is in relation to the antagonism of neuronal nicotinic receptors and AT_1 receptors.

In the present work, under the coexistence of simvastatin and L-NAME (an NO synthase inhibitor), the CA secretory responses evoked by ACh, Ang II, DMPP, McN-A-343, high K^+, veratridine, Bay-K-8644, and cyclopiazonic acid were recovered virtually to the extent of the control level compared to those of simvastatin-treatment alone. Moreover, substantially, after loading of simvastatin into adrenal medulla, NO production was significantly increased as shown in Fig. 18. Taking account of these results, in the present study, it is considered that simvastatin depresses the CA secretory response evoked by several secretagogues through elevation of NO production in rat adrenomedullary chromaffin cells.

In support of this idea, in a series of studies, the demonstration that statins are able to enhance local NO generation in endothelial cells, by increasing the half-life of eNOS (endothelial NO synthase) mRNA, was fundamental to the acceptance of the emerging evidence for lipid-independent effects (Laufs et al., 1997). Statins retain their ability to increase eNOS in the presence of oxidized LDL and under hypoxic conditions (Laufs et al., 1998; Laufs et al., 1997). In addition, statins exert further beneficial effects on the endothelium through their inhibition of the expression of the potent vasoconstrictor endothelin-1 (Hernandez-Perera et al., 2000). These actions have now been demonstrated for a number of different statins, including simvastatin, lovastatin, atorvastatin, pravastatin and fluvastatin in *in vivo* and *in vitro* studies (Lefer et al., 2001).

Also, it has been reported that the NOS inhibitor, L-NAME enhances K^+-stimulated CA secretion

in cultured bovine chromaffin cells (Torres et al., 1994) and also that sodium nitroprusside (SNP) inhibits ACh-induced CA secretion in bovine chromaffin cells (Uchiyama et al., 1994). Results of these studies indicate that NO may play an inhibitory role in the regulation of the CA secretion. Moreover, the presence of endothelial cells has been reported to reduce the K^+-induced or the nicotinic receptor agonist DMPP-induced CA secretion in cultured bovine chromaffin cells (Torres et al., 1994), suggesting that not only nNOS but also eNOS may play roles in modulating adrenal CA secretion. In view of previous reports, the present results strongly suggest that simvastatin can activate nNOS in the rat adrenomedullary chromaffin cells, inhibiting the CA secretion through elevation of NO production, in addition to the direct inhibitory action on the CA secretion. In supporting of this finding, in aorta isolated from hypertensive rats, endothelium-dependent ACh-induced relaxations are decreased by different mechanisms, involving not only enhanced production of PGH_2 but also impaired formation of nitric oxide, although the response of vascular smooth muscle to nitric oxide does not differ significantly with hypertension (Küng and Lüsher, 1995). When nitric oxide synthase was blocked by incubation with L-NOARG, relaxation due to ACh was modified in all groups of animals: simvastatin (1 and 2 mg/kg) and untreated rats, independent of whether they were SHR or WKY (Alvarez de Sotomayor et al., 1999). This fact made conclusion that nitric oxide was the main factor involved in improvement of the endothelial function of simvastatin-treated SHR rats. This effect may be due to activation of nitric oxide synthase, an increase in the release of nitric oxide or an increase of vascular smooth muscle sensitivity to nitric oxide (Alvarez de Sotomayor et al., 1999). This finding is in accordance with other studies carried out in human endothelial cells in the presence of HMGCoA reductase inhibitors (Laufs et al., 1997) and in a vascular bed in subjects receiving lipid-lowering therapy (Stroes et al., 1995; O'Driscoll et al., 1997). It has been shown that simvastatin completely normalized elevated systolic blood pressure and improved impaired endothelium-dependent relaxation of the aorta of hereditary hypertriglyceridemic (hHTG) rats (Török et al., 2007). Beneficial effect of simvastatin against endothelial dysfunction in the aorta of hHTG rats may have several plausible explanations. Statins act beneficially on vascular smooth muscle. Alvarez de Sotomayor *et al.* (1999) have shown that simvastatin improved both endothelium-dependent and

–independent phenylephrine-recontracted arteries from SHRs independently of the presence of endothelium.

Furthermore, statins may protect the vessels by reducing oxidative stress. Simvastatin attenuated superoxide anion formation in the endothelium along with the increase of NO synthase activity and restoration of endothelial NO-induced vasorelaxation in large arteries following myocardial infarction (Wagner et al., 2000, Bates et al., 2002). Finally, statins may improve endothelial function through enhancing eNOS expression and activity. Török et al (2007) have reported that simvastatin, along with blood pressure reduction, improved ACh-induced relaxation of the aorta. Simultaneously, simvastatin increased the expression of endothelial NO synthase protein, which could participate in improved NO production and represent a mechanism underlying the improvement of endothelium-dependent vascular relaxation (Török et al., 2007). Vascular endothelium located between vascular smooth muscle and circulating blood is known to be important in regulation of vascular tone. Vasorelaxation is mediated by vasorelaxant substances synthesized and released into the endothelium (Rubanyi, 1993). In the recent study, the relaxant effect induced by simvastatin was attenuated in the rat superior mesenteric artery rings without endothelium, suggesting that simvastatin also relaxes arteries through an endothelium-dependent pathway (Chen et al., 2016). Furthermore, the eNOS inhibitor L-NAME significantly reduced the vasorelaxation induced by simvastatin. In view of these previous results, in the present work, it is concluded that simvastatin can reduce the CA secretion evoked by activation of AT_1 receptors as well as cholinergic nicotinic receptors at least through elevation of NO production due to activation of neuronal NO synthase in the rat adrenal medulla.

In contrast, it has been reported that L-NAME inhibits ACh-induced CA secretion in bovine chromaffin cells (Uchiyama et al., 1994), and also that the NO donor SNP enhances nicotine-induced CA secretion in cultured bovine chromaffin cells (O'Sullivan and Burgoyne, 1990). These findings indicate that NO may enhance cholinergic agonist-induced CA secretion. On the other hand, a few in vivo studies have suggested that NO does not play a role in regulation of adrenal CA secretion (Breslow et al., 1992; 1993).

Generally, the adrenal medulla has been utilized as a good model system to investigate many

cellular activities including not only noradrenergic nerve cells but also many neurons. During neurogenic stimulation of the adrenal medulla, ACh is liberated from splanchnic nerve endings and stimulates cholinergic receptors on the chromaffin cell membrane (Viveros, 1975). This stimulation starts a series of events as stimulus-secretion coupling, resulting in the exocytotic CA secretion and other constituents of the releasing vesicles into the extracellular gap. Mostly, two mechanisms are included in the release of adrenal medulla hormones. Upon activation of splanchnic nerves, ACh is liberated from the nerve terminals, and then stimulates nicotinic receptors and the CA release. Based on this finding, the present results demonstrated that simvastatin inhibits the CA release evoked by activation of AT_1 receptors as well as cholinergic (nicotinic and muscarinic) receptors in the adrenal medulla. These experimental results indicate that simvastatin-induced inhibitory effects may contribute partly to its hypotensive mechanism. ACh, the physiological presynaptic transmitter at the adrenal medulla, which is released by depolarizing splanchnic nerve terminals and then stimulates nicotinic receptors, secrets the CA, and induces dopamine β-hydroxylase by calcium-dependent secretory process (Dixon et al., 1975; Viveros et al., 1968). In view of this finding, the present results suggest that simvastatin may inhibit CA release evoked by nicotinic stimulation from the splanchnic nerve terminal via the blockade of neuronal nicotinic receptors.

Generally, it has been known that the CA release from the adrenomedullary cells in response to splanchnic nerve stimulation or nicotinic agonist is exerted by stimulation of nicotinic receptors located on the chromaffin cells. The exocytotic CA release in the adrenomedullary chromaffin cells seems to be essentially similar to that occurring in noradrenergic axons (Douglas, 1968; Sorimachi and Yoshida, 1979). ACh-induced CA secretion has shown to be caused through stimulation of both nicotinic and muscarinic receptors in guinea-pig adrenal gland (Nakazato et al., 1988) as well as in the perfused rat adrenal glands (Lim and Hwang, 1991).

In the present study, simvastatin also time-dependently suppressed the CA secretory response induced by Bay-K-8644, which is known to activate L-type voltage-dependent Ca^{2+} channels (Garcia et al., 1984; Schramm et al., 1983), as well as by high K+, a direct membrane depolarizer. This finding indicates that simvastatin may depress Ca^{2+} influx via voltage-sensitive Ca^{2+} channels to the rat adrenal medullary cells. In support of this idea, in cultured bovine adrenal medullary cells,

nicotinic (but not muscarinic) receptors mediate the Ca^{2+}-dependent CA secretion (Fisher et al., 1981; Yanagihara et al., 1979). It has also been known that the stimulation of nicotinic receptors facilitates the CA secretion by increasing Ca^{2+} entry through receptor-linked and/or voltage-dependent Ca^{2+} channels in both perfused rat adrenal glands (Lim and Hwang, 1991; Wakade and Wakade, 1983) and isolated bovine adrenal chromaffin cells (Wakade and Wakade, 1983; Kilpatrick et al., 1981; 1982; Knight and Kesteven, 1983). It has been reported that the adrenomedullary chromaffin cells have (i) nicotinic receptor-operated ionic channels, responsible for carbachol-induced Na^{+} influx, (ii) voltage-dependent Na^{+} channels, responsible for veratridine-induced Na^{+} influx and (iii) voltage-dependent Ca^{2+} channels (VDCC), suggesting that the influx of Na^{+} caused either by carbachol or by veratridine leads to activate voltage-dependent Ca^{2+} channels by altering membrane potentials, whereas high K^{+} directly activates voltage-dependent Ca^{2+} channels without increasing Na^{+} influx (Wada et al., 1985b). In the present study, the CA secretion evoked by high K^{+} as well as Bay-k-8644 was markedly diminished in the presence of simvastatin. These results indicate that this simvastatin-induced inhibitory effect on the CA secretion is mediated by the direct suppression of Ca^{2+} influx via VDCC into the adrenomedullary chromaffin cells. Furthermore, slight elevation in the extracellular K^{+} concentration increases both the frequency of spontaneous action potentials and the CA secretion (Kidokoro and Ritchie, 1980), suggesting that the influx of Ca^{2+} that occurs during action potentials is directly linked to the rate of secretion. The present findings that simvastatin reduced the CA release evoked by Bay-K-8644 as well as high K^{+} suggest that simvastatin can inhibit directly 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 simvastatin on the CA secretory responses induced by ACh, DMPP, veratridine, Bay-K-8644 and high K^{+} may be mediated by inhibiting Ca^{2+} influx via voltage-dependent Ca^{2+} channels by activation of nicotinic receptor-operated ionic channels, responsible for carbachol-evoked Na^{+} influx, as well as of voltage-sensitive Na^{+} channels, responsible for veratridine-evoked Na^{+} influx, and VDCC.

In support of these findings, it has been shown that, in cultured bovine adrenal chromaffin cells, simvastatin attenuates $^{22}Na^{+}$ and/or $^{45}Ca^{2+}$ influx and CA secretion induced by ACh, veratridine and 56 mM K^{+}, which activate receptor-operated Na^{+} channels, voltage-dependent Na^{+} channels and Ca^{2+} channels, respectively (Matsuda et al., 2008). This simvastatin-induced voltage-dependent Ca^{2+} channels is compatible with previous reports, showing that simvastatin reduced the L-type Ca^{2+} current in rat pancreatic β-cells (Yada et al., 1999) and in brain cerebral cortex of rats (Bergdahl et al., 2003). Therefore, it seems that simvastatin inhibits CA secretion evoked by ACh, veratridine, Bay-K-8644, and high K^{+} through nicotinic ACh receptor-operated ion channels, voltage-dependent Na^{+} channels, and voltage-dependent Ca^{2+} channels, respectively. To the best of my knowledge, this is the first evidence showing the inhibitory effects of simvastatin on the CA secretion, Na^{+} and Ca^{2+} ion channels in the perfused model of isolated rat adrenal medulla.

The present study has also shown that simvastatin 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 simvastatin-evoked inhibitory effect on cyclopiazonic acid-evoked CA release may also be associated to the mobilization of intracellular Ca^{2+} from the cytoplasmic Ca^{2+} pool. This is consistent with the findings obtained in skinned smooth muscle fibers of the longitudinal layer of the guinea-pig ileum, where Ca^{2+}-uptake was also inhibited by cyclopiazonic acid (Uyama et al., 1992). It has been found that cyclopiazonic acid easily penetrates into the cytoplasm through the plasma membrane and reduces Ca^{2+}-ATPase activity in sarcoplasmic/endoplasmic reticulum, resulting in an increase in the subsequent Ca^{2+} release from those storage sites (Suzuki et al., 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, in the present study, it can be speculated that simvastatin-induced inhibitory effect on the CA release of McN-A-343 may be related to the

mobilization of intracellular Ca^{2+} from the cytoplasmic Ca^{2+} pool. This indicates that this simvastatin has an inhibitory activity on the Ca^{2+} release from the intracellular store evoked by stimulation of muscarinic ACh receptors, which is some responsible for the CA release. The present results suggest that simvastatin-induced suppression of the CA release evoked by McN-A-343 and cyclopiazonic acid may be exerted by the reduction of Ca^{2+} release produced by stimulation of ACh muscarinic receptors from the intracellular pools. However, in the present study, it is uncertain whether simvastatin-evoked inhibitory action on Ca^{2+} movement from intracellular store is due to the indirect action on the PI pathway or its direct action. To clarify this nature of simvastatin, more detailed investigation is required in the next study.

It is generally well-known that statins block the conversion of HMG-CoA to mevalonate and subsequently inhibit the biosynthesis of cholesterols, which shows the main effect of statins. A previous study showed that, in neonatal rat cardiac myocytes, atorvastatin suppressed the stimulatory effect of isoprenaline on cyclic AMP accumulation, which was abolished by mevalonate, suggesting an HMG-CoA reductase-dependent pathway (Mühlhaüser et al., 2006). However, in the present study, it has been found that mevalonolactone, which is nonenzymatically converted to mevalonate, the first metabolite of HMG-CoA, did not block the inhibitory effect of simvastatin on ACh-evoked CA secretion. Therefore, it appears that the inhibitory effect of simvastatin on ACh-evoked CA secretion is not responsible for its inhibition of mevalonate-derived isoprenoid biosynthesis. Although pravastatin, a hydrophilic statin, as well as simvastatin and atorvastatin, lipophilic statins, are reported to inhibit mevalonate synthesis (Endo, 1992), in the present study, pravastatin did not inhibit ACh-evoked CA secretion in the perfused model of the isolated rat adrenal glands. In support of this idea, several previous studies have demonstrated that lovastatin, a lipophilic statin, inhibited the L-type Ca^{2+} current and reduced the intracellular free Ca^{2+} concentration and contraction in the rat cerebral artery, but that pravastatin did not (Bergdahl et al., 2003), and that simvastatin, but not paravastatin, inhibited glucose-induced cytosolic Ca^{2+} signaling and insulin secretion due to blockade of L-type Ca^{2+} channels in rat islet β-cells (Yada et al., 1999). Although atorvastatin and simvastatin reduced AF recurrence after electrical cardioversion (EC) (Siu et al., 2003), use of pravastatin before EC did not decrease AF recurrence

(Tveit et al., 2004) and rosuvastatin did not affect clinical outcome and AF occurrence (Maggioni et al., 2009; Tavazzi et al., 2008). Lipophilic statins improve cardiac sympathetic activity by reducing oxidative stress (Gomes et al., 2010; Tsutamoto et al., 2011), and an active metabolite of atorvastatin displays stronger antioxidant activity than rosuvastatin (Mason et al., 2006). Simvastatin but not pravastatin significantly reduces angiotensin II-induced calcium mobilization (Escobales et al., 1996), and simvastatin may exert direct anti-arrhythmic effect by suppressing events that trigger AF (Sicouri et al., 2011). Accordingly, it has been demonstrated that the inhibition of ACh-activated K^+ current (I_{KACh}) may represent another important anti-arrhythmic mechanism of simvastatin in mouse atrial cardiomyocytes (Cho et al, 2014). This inhibitory action on the (I_{KACh}) current was not reversed by addition of mevalonate, geranylgeranyl pyrophosphate, or farnesyl pyrophosphate, implying that simvastatin may suppress I_{KACh} independently from signaling proteins activated by isoprenylation (Cho et al, 2014). They observed the inhibition of I_{KACh} after administration of simvastatin, which suggested that inhibition of I_{KACh} does not involve metabolism of the drug but occurs through direct interaction of the drug with K^+ channels within the membrane (Cho et al, 2014). The highly lipophilic simvastatin has a strong affinity for the cell membrane (Sarr et al., 2008) and, consequently, it may has easy access to the intracellular space; this may explain the ability of simvastatin to effectively inhibit I_{KACh} in atrial myocytes (Cho et al, 2014). However, since hydrophilic pravastatin has limited access to the plasma membrane and intracellular space (Sarr et al., 2008), in the present study, this finding may explain the absence of effect on ACh-evoked CA secretion. In accordance with results of this study, it has been shown that the inhibitory effect of simvastatin on on ACh-evoked CA secretion does not involve its inhibition of mevalonate-derived isoprenoid synthesis, and that pravastatin does not inhibit on ACh-evoked CA secretion in cultured bovine adrenal medullary cells (Mastsuda et al., 2008). Based on these results, it is suggested that the inhibitory potency of statins on adrenal CA secretion as well as ion channels may correlate with their hydrophobicity.

In the present work, when both simvastatin and fimasartan (an AT_1 antagonist) were used in combination, their inhibition on ACh-evoked CA secretion was potentiated. In support of this idea, the losartan (an AT_1 antagonist)-enalapril (an angiotensin-converting enzyme inhibitor)

combination is more effective in decreasing blood pressure and increasing plasma active renin than doubling of the enalapril dose in normotensive male volunteers (Azizi et al., 1997). Based on the present results, the clinically combined administration of both simvastatin and fimasartan may contribute distinctly to the treatment of cardiovascular diseases such as angina pectoris, heart failure and hypertension.

In conclusion, as shown in Figure 24, the results of the present study have demonstrated that simvastatin reduces the CA release by activation of AT_1 receptors as well as cholinergic nicotinic receptors in the perfused model of the isolated rat adrenal medulla. It seems that this simvastatin-induced inhibitory effect on CA secretion is produced by suppressing influx of Na^+ and Ca^{2+} via their ionic channels into the adrenal chromaffin cells as well as by inhibiting the Ca^{2+} release from the cytoplasmic Ca^{2+} pool partly through the elevation of NO production due to the activation of NO synthase. Based on these results, the ingestion of simvastatin can be beneficial to improvement or prevent the cardiovascular diseases, through decreased CA secretion in adrenomedullary chromaffin cells and consequent reduced CA concentration in the circulation. The combined use of both simvastatin and fimasartan may dedicate clinically to the improvememt of cardiovascular diseases, including hypertension.

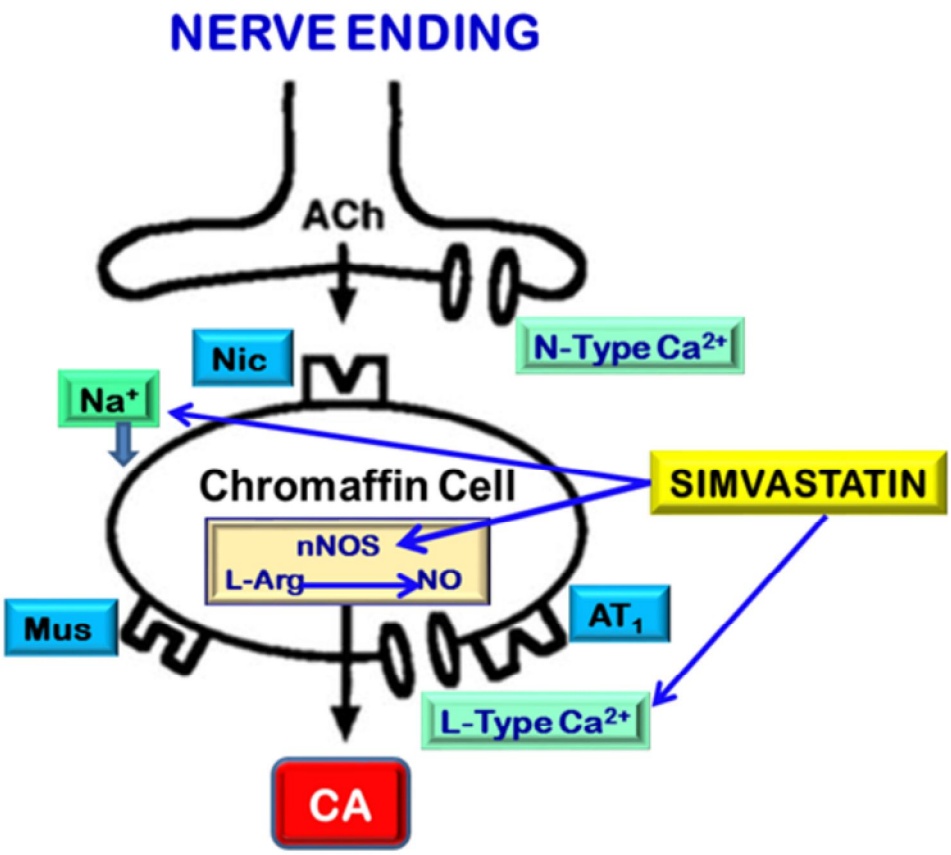

Fig. 24. Schematic diagram of possible action site of simvastatin in the rat adrenal medulla.

V. SUMMARY

It has been reported that the molecular mechanism of the action of statins, including simvastatin, is the inhibition of HMG-CoA reductase, which catalyzes the formation of mevalonate, a precursor of isoprenoids, and thereby of cholesterol (Goldstein and Brown, 1984), reducing deaths caused by myocardial infarction and stroke (Pedersen et al., 2000). Simvastatin has been reported to lower the plasma concentration of norepinephrine in rabbits associated with congestive heart failure (Pliquett et al., 2003). Plasma epinephrine concentration was slightly lower in patients treated with simvastatin therapy (Nette et al., 2005). Although an endogenous excess of catecholamines (CA) may be a risk factor for the development of atherosclerosis (Westfall and Westfall, 2005), it is not clear whether simvastatin affects the function of the adrenal medulla. The present study was aimed to investigate the characteristics of simvastatin, one of statins, on the CA release in the perfused model of the isolated rat adrenal gland, and also to clarify its mechanism of action. simvastatin (20~200 μM), given into an adrenal perfusion stream for 90 min, suppressed ACh (5.32 mM)-evoked CA release in a dose- and time-dependent fashion. Simvastatin (60 μM) also time-dependently inhibited the CA secretion evoked by DMPP (a selective neuronal nicotinic receptor agonist, 100 μM), McN-A-343 (a selective muscarinic M_1 receptor agonist, 100 μM), and angiotensin II (Ang II, 100 nM). Simvastatin itself did not alter spontaneous CA release (data not shown). Also, in the presence of simvastatin (60 μM), the secretory responses of CA evoked by high K^+ (56 mM, a direct membrane depolarizer), veratridine (a voltage-dependent Na^+ channel activator (50 μM), Bay-K-8644 (an L-type dihydropyridine Ca^{2+} channel activator, 10 μM), and cyclopiazonic acid (a cytoplasmic Ca^{2+}-ATPase inhibitor, 10 μM) were markedly inhibited, respectively. Interestingly, in the simultaneous presence of simvastatin (60 μM) and L-NAME (an inhibitor of NO synthase, 30 μM), the inhibitory responses of simvastatin on CA secretory response evoked by ACh, Ang II, DMPP, high K^+, McN-A-343, cyclopiazonic acid, Bay-K-8644, and veratridine were recovered similarly to the extent of the control release compared to the inhibitory activity of simvastatin-treatment alone, respectively. Actually, the amount of NO released from

adrenal medulla following perfusion of simvastatin (60 μM) was significantly inreased compared to the basal level. The reduction of CA secretion caused by simvastatin was not affected by treatment along with mevalonate, the first metabolite of HMG-CoA. In the presence of pravastatin (60 μM), ACh-evoked CA secretory responses were not influenced, while atorvastatin (60 μM) also significantly reduced them. In the simultaneous presence of simvastatin (60 μM) and fimasartan (15 μM), ACh-induced CA release was more markedly reduced compared to that of simvastatin (60 μM) or fimasartan (15 μM)-treated alone. These results, taken together, demonstrate that simvastatin reduces the CA secretion induced by cholinergic activation as well as by AT_1-receptor activation from the perfused model of the isolated rat adrenal medulla. It seems that this inhibitory effect of simvastatin is produced by reducing both the influx of Ca^{2+} and Na^{+} into the adrenal medullary chromaffin cells and also by repressing the movement of Ca^{2+} from the cytoplasmic calcium store, at least partly via the elevation of NO production by the activation of NO synthase, which is in releation to AT_1 receptors as well as neuronal nicotinic receptor blockade. Simvastatin-induced inhibitory effect of the CA secretion adrenal medulla is not thought to be directly associated with the inhibition of HMG-CoA reductase. When simvastatin and fimasartan were given in combination, their inhibitory activity were potentiated, which may also be clinically beneficial in improvement of cardiovascular diseases, including hypertension and heart failure.

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