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Studies on Cardiovascular Physiological Actions of *Rubus coreanum*

-Influence on Catecholamine Secretion, Blood Pressure and Isolated Thoracic Aortic Strips-

조선대학교 대학원

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복분자의 심혈관계 생리작용에 관한 연구

-카테콜아민 분비, 혈압 및 적출 가슴대동맥편에 대한 영향-

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지도교수 서 영 환

이 논문을 이학박사 학위신청논문으로 제출함.

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최 미 성

(지도교수 : **서 영 환**)

조선대학교 대학원 체육학과

예비실험에서 복분자 *(Rubus coreanum* MIQUEL, 覆盆子)로 양조한 복분자주에서 추출한 CH_2Cl_2분획, ethylacetate (EtOAc)분획, n-butanol (BuOH)분획 및 H_2O분획 (60 μg/ml)을 각각 흰쥐부신정맥 내로 투여 시 CH_2Cl_2분획이 ACh (5.32 mM)의 카테콜아민 (catecholamines, CA)분비반응에 대한 가장 강력한 억제작용을 나타내었다. 따라서, 본 연구의 목적은 복분자주에서 추출한 CH_2Cl_2분획이 흰쥐 적출부신의 관류모델에서 CA 유리작용에 미치는 영향, 흰쥐 혈압반응 및 적출 가슴대동맥에 대한 영향을 검색하여 그 작용기전을 규명코자 본 연구를 시행하여 다음과 같은 결과를 얻었다.

가. 흰쥐 관류부신에서 카테콜아민 분비에 대한 영향:

복분자 CH_2Cl_2분획(20~180 μg/ml)을 각각 부신정맥 내로 90분간 관류 시 ACh (5.32 mM), 고칼륨 (56 mM, 막탈분극제), DMPP (100 μM, 선택성

니코틴수용체 작용제), 및 McN-A-343 (100 μM, 선택성 무스카린 M_1-수용체 작용제)에 의한 CA 분비반응을 억제하였다. 그러나, CH_2Cl_2분획 자체는 기초 CA 분비량에 영향을 미치지 않았다. 또한, CH_2Cl_2분획(60 μg/ml) 존재 하에서, 선택성 나트륨통로 활성화제인 veratridine (100 μM), L형 칼슘통로 활성화제인 Bay-K-8644 (10 μM) 및 세포질내 내형질세망막에서 Ca^{2+}-ATPase 억제제인 cyclopiazonic acid (10 μM)에 의한 CA 분비반응이 유의하게 억제되었다. 흥미롭게도, CH_2Cl_2분획(60 μg/ml) 과 L-NAME (NO Synthase 억제제, 30 μM)과 함께 90분간 동시 처치하였을 때 ACh, 고농도의 K^+, DMPP, McN-A-343, veratridine, Bay-K-8644 및 cyclopiazonic acid의 CA 분비효과가 CH_2Cl_2분획 단독처치 시 나타나는 억제효과에 비교하여 상응하는 대조치의 수준까지 회복되었다. 또한 실제로 CH_2Cl_2분획을 처치한 후에 NO 유리량이 기초 유리량에 비해 현저하게 증가하였다.

나. 정상혈압흰쥐 및 자연발증고혈압쥐의 적출 가슴대동맥편에서 혈관수축 작용에 대한 영향:

정상혈압 흰쥐에서 적출 분리한 가슴대동맥편에서 phenylephrine (α_1-아드레날린 수용체 작용제)과 고칼륨(막탈분극제)은 각각 현저한 수축반응을 일으켰다. Phenylephrine (10^{-5} M)에 의한 수축반응은 CH_2Cl_2분획 (200~800 μg/ml)의 존재 하에서 용량의존적으로 현저하게 억제되었다. 또한 고칼륨에 의한 수축반응도 CH_2Cl_2분획 (200~800 μg/ml)의 존재 하에서는 용량의존적으로 뚜렷한 억제작용을 나타내었다. 그러나 NO합성효소 억제제인 L-NAME 존재 하에서 페닐에프린 및 고칼륨의 수축반응은 CH_2Cl_2분획의 단독처치 시에 비교하여 대조치의 상당한 수준으로 회복되었다. 혈관내피를 제거하기 위해 CHAPS처치한 가슴대동맥편에서 페닐에프린 및 고칼륨의

수축반응에 대한 CH_2Cl_2분획의 억제작용은 CH_2Cl_2분획의 단독처치 시에 비교하여 유의하게 약화되었다. 또한 자연발증 고혈압쥐의 적출대동맥편에서 phenylephrine (10^{-5} M)에 의한 수축반응은 복분자주 CH_2Cl_2분획(400 μg/ml)의 존재 하에서 현저하게 억제되었다. 또한 고칼륨에 의한 수축반응도 CH_2Cl_2분획 (400 μg/ml)의 의 존재 하에서 뚜렷한 억제작용을 나타내었다.

다. 정상혈압흰쥐 및 자연발증고혈압쥐의 혈압에 대한 영향:

CH_2Cl_2분획 (0.3~3.0 mg/kg)은 정상혈압흰쥐의 대퇴정맥 내로 주사 시 강력한 용량의존성 혈압하강 작용을 나타내었다. CH_2Cl_2분획 (1.0 mg/kg)의 혈압하강작용은 phentolamine (교감신경 α-아드레날린 수용체차단제, 1mg/kg, i.v.), chlorisondamine (자율신경절 차단제, 1mg/kg, i.v.), L-NAME (NO Synthase 억제제, 3mg/kg/30min), 그리고 sodium nitroprusside (NO유리-직접혈관확장제, 30μg/kg/30min) 등의 전처치에 의해서 현저하게 억제되었다. 정상혈압 흰쥐에서 CH_2Cl_2분획 (1, 3 및 10 mg/kg/30min, i.v.)은 norepinephrine의 혈압상승효과를 비교적 용량 의존적으로 뚜렷이 억제하였다. 자연발증 고혈압쥐에서도 CH_2Cl_2분획 (1.0~10.0 mg/kg)은 대퇴정맥 내로 주사 시 용량의존적으로 뚜렷한 혈압하강 작용을 나타내었다.

이와 같은 연구결과를 종합하여 보면, 여러 복분자 분획 중에서 본 연구에서 사용한 용량을 기초로 볼 때 정상혈압 흰쥐의 적출 관류 부신수질에서 ACh에 의한 카테콜아민 분비 억제작용 및 흰쥐 적출 대동맥편에서 PE 및 고칼륨에 의한 수축반응에 대한 이완작용이 CH_2Cl_2분획이 가장 강력한 활성을 나타내었다. 따라서 CH_2Cl_2 분획은 흰쥐 관류 부신수질에서 콜린성(니코틴 및 무스카린 수용체)흥분작용 및 막탈분극에 의한 CA 분비작용에 대하여 억제작용을 나타내었다. 이러한 CH_2Cl_2 분획의 CA분비억제작용은 흰쥐 적출 부신수질에서 L-형 칼슘통로 및

나트륨 통로를 통한 크롬친화세포내로 칼슘 및 나트륨의 유입과 세포내 칼슘저장고로부터 칼슘유리를 억제하며, 이는 적어도 NO Synthase의 활성화에 의한 NO생성증가에 기인되는 것으로 생각된다. 또한 본 연구에서 CH_2Cl_2분획은 자연발증고혈압쥐 및 정상혈압 흰쥐로부터 적출 분리한 대동맥편에서 뚜렷한 혈관 이완작용을 나타내며, 이러한 CH_2Cl_2분획의 혈관이완작용은 적어도 산화질소 합성효소 (Nitric Oxide Synthase)를 활성화하여 NO생산을 증가시킴으로써 나타나는 것으로 사료된다. 나아가 CH_2Cl_2분획은 자연발증고혈압쥐 및 정상혈압 흰쥐의 대퇴정맥내에 투여시 현저한 혈압하강작용을 나타내며, 또한 norepinephrine의 승압반응을 뚜렷하게 억제하였다. 이러한 CH_2Cl_2분획의 혈압하강작용은 아드레날린 α_1 수용체차단작용과 NO유리작용에 기인되는 것으로 사료된다.

이와 같은 연구결과를 기반으로 보면 복분자로부터 추출한 CH_2Cl_2분획이 심혈관계 질환치료에 유효한 성분을 함유하고 있는 것으로 사료된다. 나아가 이 같은 사실은 복분자 음용이 생활습관병의 하나인 고혈압을 비롯한 심혈관계 질환의 예방 및 치료에 유익한 것으로 생각된다. 이외에도 본 연구결과로 보아, 복분자로부터 추출한 유효성분을 함유한 스포츠 음료섭취가 생리학적으로 운동선수의 심혈관계를 안정화시키는데 도움이 되며, 경기력 향상에도 유익할 것으로 기대된다.

I. INTRODUCTION

Rubus coreanum MIQUEL (覆盆子) has been presently used in treating the disease of the aged, spermatorrhea and impotence in oriental medicine. It is also the principal products of Gochang county, Chonbuk province, Korea, where is famous for wine brewed from *Rubus coreanum* MIQUEL (Bokboonja liquor, 복분자주). So far *Rubus coreanum* has been found to possess several polyphenolic compounds, such as (-)-epicatechin, (+)-catechin, proanthocyanidin, etc. Ethanol extract of *Rubus coreanum* showed the antioxidative activity with inhibitory effects on linoleic acid oxidation and LDL oxidation (Lee and Do, 2000). Cho (2005) found that total phenol content of extract from *Rubus coreanum* M. was contained highly in hot-water extract than other extracts. These extracts elicited antioxidant protection as well as inhibitory activities on xanthine oxidase, pancreatin, α-amylase, and angiotensin converting enzyme (Cho, 2005).

Recently, it has been demonstrated that polyphenol compounds (PCRC), isolated from Bokboonja liquor, inhibits the CA secretory responses evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused adrenal gland of the normotensive rats (Kee and Lim, 2007) and spontaneously hypertensive rats (Yu et al., 2009). It seems that this inhibitory effect of PCRC is exerted by inhibiting both the Ca^{2+} influx into the rat adrenal medullary chromaffin cells and the uptake of Ca^{2+} into the cytoplasmic calcium store partly through the increased NO

production due to the activation of nitric oxide synthase (Kee and Lim, 2007; Yu et al., 2009*).*

Generally, the presence of polyphenolic compounds is widespread among plants and plant products (Formica and Regelson 1995; Zenebe and Pecháňová 2002). Several epidemiological studies have shown that consumption of foods rich in polyphenolic compounds is associated with lower incidence of cardiovascular disease. It was hypothesized that the cardioprotective effect of polyphenols results from their ability to protect low-density lipoprotein from oxidation, to prevent platelet aggregation and leukocyte adhesion, and to promote relaxation of vascular smooth muscle (Keli et al., 1996; Hertog et al., 1997). Polyphenols also act on other targets involved in the metabolism of mammalian cells, including nitric oxide (NO), which by itself regulates hemostasis (Palmer et al., 1987), thrombus development (Radomski et al., 1987) and vascular tone (Moncada et al., 1991; Zenebe et al., 2003). The beneficial properties of NO may therefore explain, at least in part, the beneficial effects of plant polyphenols. Several authors have reported that extracts from grapes and wine induce endothelium-dependent relaxation via enhanced generation and/or increased biological activity of NO leading to the elevation of cGMP levels (Fitzpatrick et al., 1993; Flesch et al., 1998). The critical step for the activation of NO synthase in endothelial cells is the increase in Ca^{2+} concentration leading to the production of NO and the subsequent endothelium-dependent vasorelaxation (Andriambeloson et al., 1999). The biological activity of NO can be effectively

increased by the scavengers of oxygen-free radicals (Bouloumié et al., 1997).

As aforementioned, there are many reports about the effects of red wine on cardiovascular system. Despite of these studies, there are so far few reports on *in vitro* functional effects of fractions isolated from Bokboonja wine on the cardiovascular system. Therefore, the aim of the present study was to investigate the ability of some fractions isolated from Bokboonja wine on the blood pressure, CA secretion in the perfused model of the adrenal gland, and the contractility of the thoracic aorta isolated from normotensive and spontaneously hypertensive rats, and to clarify its mechanism of action.

Ⅱ. MATERIALS AND METHODS

Experimental procedure

Mature male Sprague-Dowley rats and spontaneously hypertensive rats (purchased from DAMOOL SCIENCE, International Customer Service, Seoul, Korea), weighing 200 to 300 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 (50 mg/kg) intraperitoneally, and tied in supine position on fixing panel.

1. Catecholamine Secretion

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 (Fig. 1).

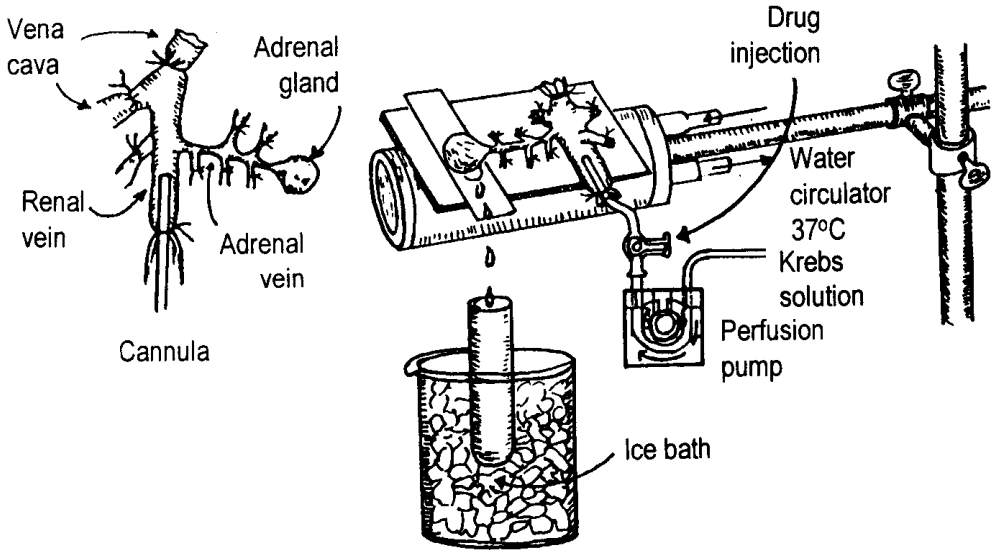

Fig. 1. Schematic drawing of the preparation used to study the CA secretion in the isolated perfused rat adrenal gland.

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 x 10^{-3} M) and KCl (5.6 x 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), veratridine (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, veratridine, 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 perfusate 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 several fractions extracted from *Rubus coreanum* on the spontaneous and evoked secretion, the adrenal gland was perfused with normal

Krebs solution for 90 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 fractions of *Rubus coreanum*, and the perfusates were collected for the same period as that for the background sample. The perfusate of adrenal gland was collected in chilled tubes.

Measurement of catecholamines: The content of CA in 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 fluorospectrophotometer (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 CA content in the perfusate was expressed in terms of norepinephrine (base) equivalents.

Measurement of NO release: NO release was measured using a NO-selective microelectrode (ami700, Innovative Instruments Inc) and an amplifier (inNo meter, Innovative Instruments Inc). Adrenomedullary NO production was quantified as the integrated signal detected by the microelectrode after perfusion of extracts of *Rubus coreanum* into rat adrenal medulla, as previously described (McVeigh et al., 2002). The electrode was calibrated by

producing standardized concentrations of NO in 0.5% (wt/vol) KI in 0.1 mol/LH2SO4 from $NaNO_2$ standards. NO release was quantitated as the current detected at the electrode after loading extracts of *Rubus coreanum* into adrenal medulla. NO release was calculated as picomoles.

2. Vasorelaxation

Isolation of Thoracic Aortic Strips: The thorax was opened by a midline incision, and the heart and surrounding area were exposed by placing three hook retractors. The heart and portion of the lung were not removed, but pushed over to the right side and covered by saline-soaked gauge pads in order to obtain enough working space for isolating thoracic aortic vessel. The aorta was isolated from the proximal part of the heart to the vicinity of liver and immediately immersed in cold Krebs solution. The blood within the aorta was rapidly removed. The aorta was cut into the ring of 4-5 mm length (Fig. 2).

Recording of Mechanical Activity: The ring segment of aorta was mounted in a muscle bath by sliding the ring over two parallel stainless-steel hooks (0.15 mm in diameter). The lower hook was fixed on bottom of the bath and the upper was connected to isometric transducer (Grass FT. 03). The signal from the transducer was displayed on a polygraph (Grass Instruments Model 79). The volume of bath was 25 ml and the bath solution was saturated with 95% O_2 and

5% CO_2 at 37^0C. The composition (mM) of Krebs was: 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 final pH of the solution was maintained at 7.4 - 7.5. During equilibration period of 2 hours, the resting tension was adjusted to 0.5 g. After the equilibration period, the ring was challenged with 35 mM KCl two times, and if it responded with contraction, the proper experiment was started. Vasoconstrictors were administered into the bath in order to obtain dose-response curves. In the subsequent experiments, under the presence of extracts of *Rubus coreanum*, some vasoconstrictors were administered, respectively. The data were expressed as % of the control tension.

Removal of Endothelium: A solution containing 0.4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) was perfused for 30 s to remove the endothelium (Moore et al., 1990), followed by washout with the drug-free solution. The effect of CHAPS was confirmed by the absence of a flow increase due to 10^{-6} M acetylcholine and the presence of a response to 10^{-6} M sodium nitroprusside before the experiments were started. The vasoconstrictor-induced response of non-treated (control) and CHAPS-treated preparations was compared in parallel.

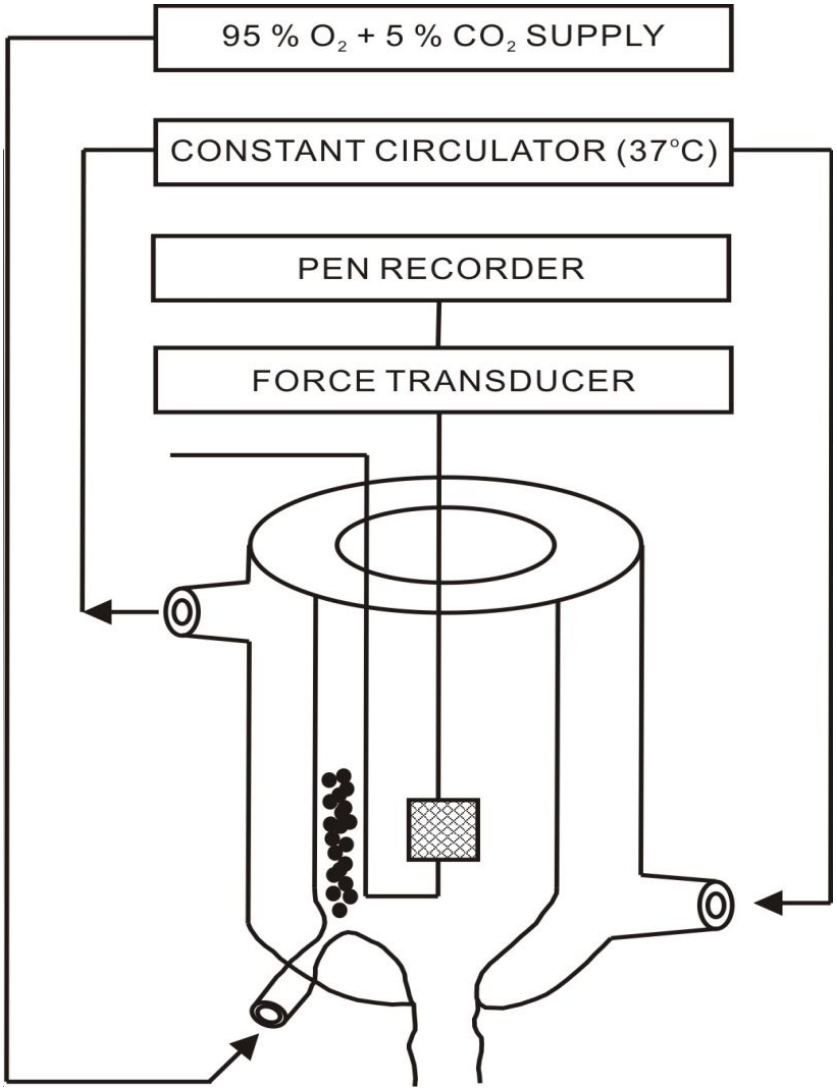

Fig. 2. A schematic representation of the isometric contraction recording system with a vertical chamber. The chamber (25 ml) was maintained at 37 ℃ with temperature-regulated circulator and aerated with 95 % O_2 and 5 % CO_2.

3. Blood Pressure

Preparation for measurement of Arterial pressure: The animal was tied in supine position on fixing panel to insert a T- formed cannula into the tachea for securing free air passage. The rectal temperature was maintained at 37-38^{o}C by a thermostatically controlling blanket and heating lamp throughout the course of the experiment.

Measurement of Blood Pressure: In order to observe the change of arterial pressure, one of the common carotid arteries or of the femoral arteries was catheterized with polyethylene tubing [outside diameter (o.d): 0.5mm]. The tubing was connected to a pressure transducer (Gould Co., U.S.A.) and pulse of mean arterial blood pressure was recorded on a biological polygraph (Grass Co., U.S.A.) continuously. The chart speed was adjusted to 2 cm per minute. The artery tubing was filled with heparin solution (400 I.U.) to prevent the blood coagulation during the experiment. Another cannulation with polyethylene tubing (o.d.: 0.3mm) was made into a femoral vein for the administration of drugs and supplemental anesthetic agents as needed to maintain light surgical anesthesia. Each rat was left undisturbed for at least 30 minutes after completion of the operative procedures to permit cardiovascular parameters to be stabilized and drugs under investigation were administered at intervals of 60 minutes.

4. Fractionation of Rubus coreanum

Fractionation of *Rubus coreanum* extract was made from a 1-year old wine brewed from *Rubus coreanum* Miquel (覆盆子) at the Research Institute of Bokboonja, Gochang County, Cheollabukdo Province, Korea as shown in Fig. 3: wine of *Rubus coreanum* was concentrated in a vacuum. And then it was extracted with methylene chloride (CH_2Cl_2) followed by extraction with ethylacetate (EtOAc) and n-butanol. These fractions were concentrated by vacuum, evaporation and atomized, lyophilized by freezing dryer (Coldvac -80, Hanil R & D, Korea). Extract of 2.095 g CH_2Cl_2, 10.968 g EtAc and 9.057 g n-butanol was obtained from 6 L Bokboonja wine, respectively. The working solution of these extracts was prepared by dissolving in 0.9% NaCl solution or DMSO on the day of each experiment and filtered before administration and diluted appropriately with Krebs-bicarbonate solution (final concentration of alcohol was less than 0.1 %).

Statistical analysis

The statistical difference between the control and the 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).

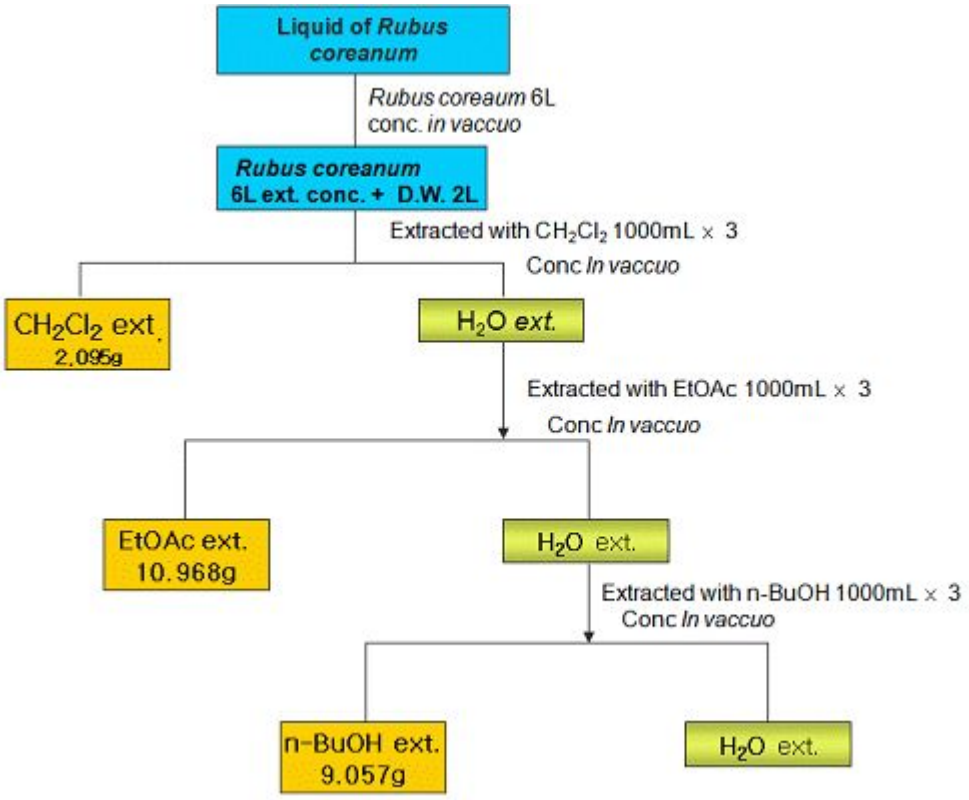

Fig. 3. Fractionation procedure of *Rubus coreanum*

Drugs and their sources

The following drugs were used: phenylephrine hydrochloride, potassium chloride, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), 1.1-dimethyl-4 -phenyl piperazinium iodide (DMPP), acetylcholine chloride, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), norepinephrine bitartrate, veratridine chloride, cyclopiazonic acid, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-car boxylate (BAY-K-8644), (Sigma Chemical Co., U.S.A.), chlorisondamine chloride,

phentolamine mesylate (CIBA Co., U.S.A.), thiopental sodium and heparin sodium (Daehan Choongwae Pharm. Co., Korea), and 3-(m-cholro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium 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 and CH_2Cl_2 fraction, which were 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 except CH_2Cl_2 fraction used were expressed in terms of molar base.

III. RESULTS

A. Influence of CH_2Cl_2 fraction on the CA secretion from the perfused rat adrenal glands

Influence of four fractions extracted from Rubus coreanum on ACh-evoked CA secretion from the perfused rat adrenal glands

In order to stabilize the secretory function of adrenal medulla, it was perfused with oxygenated Krebs-bicarbonate solution for 1 hr and basal CA release from the adrenal glands amounted to 21±2 ng for 2 min (n=9). Previously, it has been found that PCRC exhibit inhibitory effect on the CA secretion from the perfused adrenal medulla of SHRs (Yu et al., 2009). Therefore, it was attempted to examine effects of four fractions (ethylacetate [EtOAc], methylene chloride [CH_2Cl_2], n-butanol [BuOH], and water [H_2O]) isolated from *Rubus coreanum* on ACh-evoked CA secretion from the isolated perfused rat adrenal medulla. When each fraction was perfused into adrenal vein for 90 min, CH_2Cl_2 (60 μg/mL), EtOAc (60 μg/mL), BuOH (60 μg/mL), and H_2O (60 μg/mL) reduced ACh-evoked CA secretion maximally to 77%, 79%, 89% and 90% of the control response, respectively (Fig. 4). Based on these results, for the ACh (5.32 mM)-evoked CA release, the following rank order of inhibitory potency to these fractions was obtained: CH_2Cl_2 **>**EtOAc**>>**BuOH**>**H_2O. Because of this finding, therefore, CH_2Cl_2 fraction only among four fractions was used in the present study.

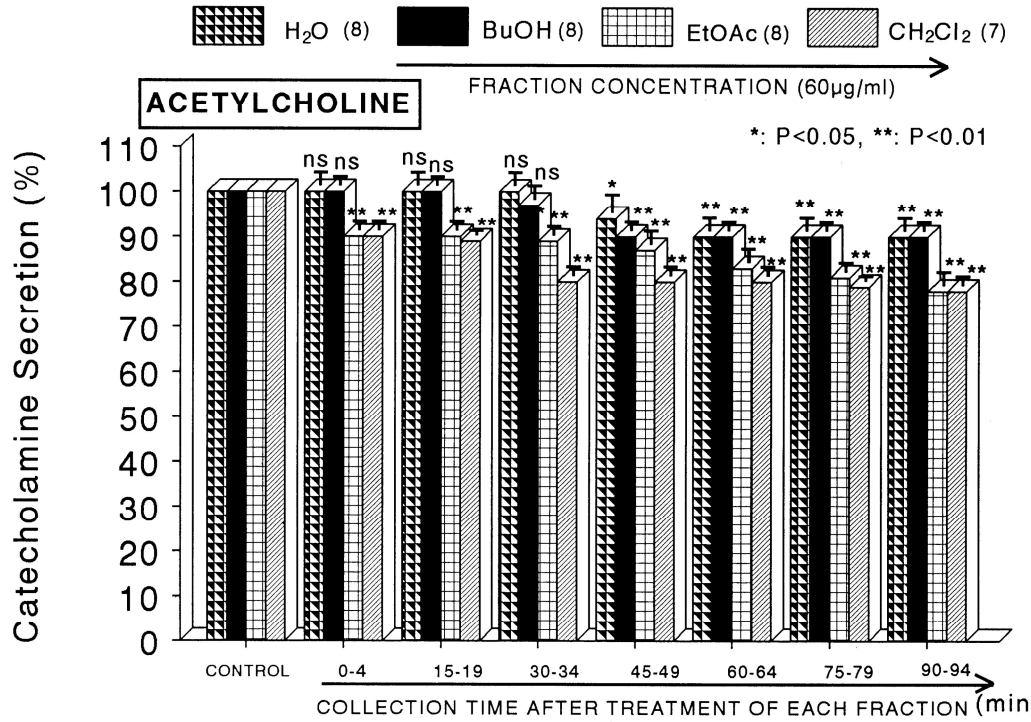

Fig. 4. Comparative effects of four fractions (water [H_2O], butanol [BuOH], ethylacetate [EtOAc], and methylene chloride [CH_2Cl_2]) extracted from *Rubus coreanum* on the secretion of catecholamines (CA) evoked by acetylcholine (ACh) from the isolated perfused rat adrenal glands. The CA secretion by a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 ml was evoked at 15 min intervals after preloading with 60 μg/mL of each fraction for 90 min as indicated at an arrow mark. Numbers in the parenthesis indicate number of rat adrenal glands. Vertical bars on the columns represent the standard error of the mean (S.E.M.). Ordinate: the amounts of CA secreted from the adrenal gland (% of control). Abscissa: collection time of perfusate (min). Statistical difference was obtained by comparing the corresponding control (CONTROL) with 60 μg/mL concentration-pretreated group of each fraction. ACh-induced perfusate was collected for 4 minutes. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

Effects of CH_2Cl_2 fraction on the CA secretion evoked by ACh, high K^+, DMPP and McN-A-343 from the perfused rat adrenal glands

As mentioned above, CH_2Cl_2 fraction among four fractions exhibited the most powerful inhibitory effect on the ACh-evoked CA secretion from the perfused rat adrenal medulla. Thus, it was attempted initially to examine the effects of CH_2Cl_2 fraction itself on CA secretion from the perfused model of the rat adrenal glands. However, in the present study, CH_2Cl_2 fraction (20 ~ 180 μg/ml) itself did not affect the basal CA output from the adrenal medulla (data not shown). Therefore, it was decided to investigate the effects of CH_2Cl_2 fraction on cholinergic receptor stimulation- as well as membrane depolarization-evoked CA secretion. Secretagogues were given at 15 to 20 min-intervals. CH_2Cl_2 fraction was present for 90 minutes after the establishment of the control release.

When ACh (5.32×10^{-3} M) in a volume of 0.05 ml was injected into the perfusion stream, the amount of CA secreted was 1294±24 ng for 4 min. However, during the perfusion with CH_2Cl_2 fraction in the range of 20 ~ 180 μg/ml for 90 min ACh-stimulated CA secretion was relatively concentration- and time-dependently inhibited. As shown in Fig. 5, in the presence of CH_2Cl_2 fraction, the CA releasing response of ACh was maximally inhibited to 60% of the corresponding control release (100%).

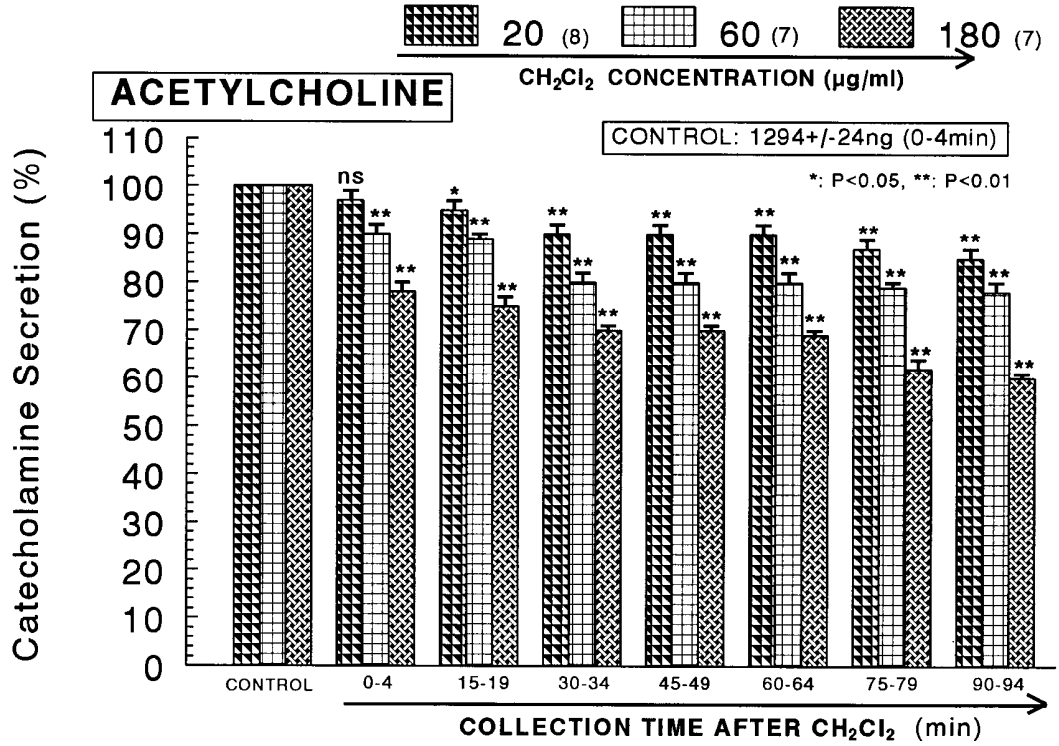

Fig. 5. Dose-dependent effect of CH_2Cl_2 fraction on the acetylcholine-evoked CA secretory responses from the isolated perfused rat adrenal glands. The CA secretion by a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 ml was evoked at 15 min intervals after preloading with 20, 60, 180 μg/mLof CH_2Cl_2 fraction for 90 min as indicated at an arrow mark. Other legengds are the same as in Fig. 4. Statistical difference was obtained by comparing the corresponding control (CONT) with each concentration-pretreated group of CH_2Cl_2. ACh-induced perfusate was collected for 4 minutes. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

Also, it has been found that depolarizing agent like KCl stimulates markedly CA secretion (691±21 ng for 0-4 min). High K^+ (5.6 x 10^{-2} M)-stimulated CA secretion during loading with 20 μg/ml CH_2Cl_2 fraction was not affected for the first 30 min period as compared with its corresponding control secretion (Fig. 6). However, following the perfusion with higher concentrations of CH_2Cl_2 fraction (60 ~ 180 μg/ml), high K^+-stimulated CA secretion was inhibited maximally to 55% of the control for the last period (90 min), although it was not initially affected at 60 μg/ml of CH_2Cl_2 fraction. DMPP (10^{-4} M), which is a selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion (1296±24 ng for 0-8 min). However, as shown in Fig. 7, DMPP-stimulated CA secretion during the perfusion with CH_2Cl_2 fraction was greatly reduced to 62% of the control release. McN-A-343 (10^{-4} M), which is a selective muscarinic M_1-agonist (Hammer and Giachetti, 1982), perfused into an adrenal gland for 4 min caused an increased CA secretion (576±20 ng for 0-4 min). However, as shown in Fig. 8, McN-A-343-stimulated CA secretion in the presence of CH_2Cl_2 fraction was depressed markedly to 57% of the corresponding control secretion (100%).

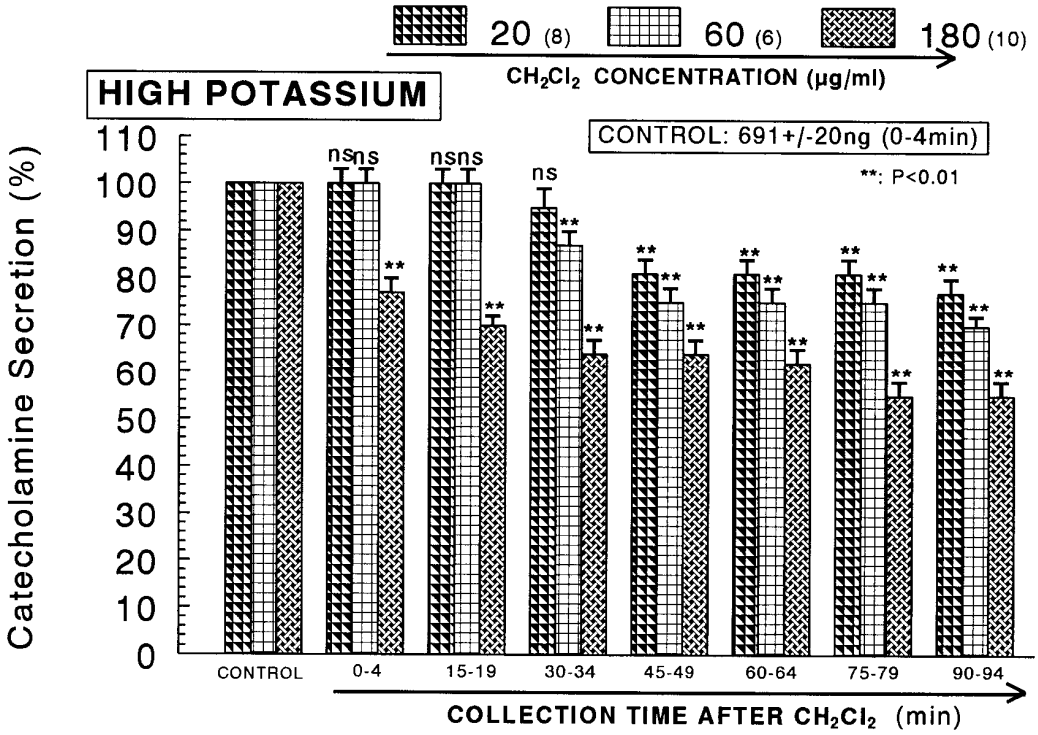

Fig. 6. Dose-dependent effect of CH_2Cl_2 fraction on the high K^+–evoked CA secretory responses from the isolated perfused rat adrenal glands. CA secretion by a single injection of K^+ (5.6×10^{-2} M) was injected in a volume of 0.1 ml at 15 min intervals after preloading with 20, 60, 180 μg/mLof CH_2Cl_2 for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT) with each concentration-pretreated group of CH_2Cl_2. K^+-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 4. **: $P < 0.01$. ns: Statistically not significant.

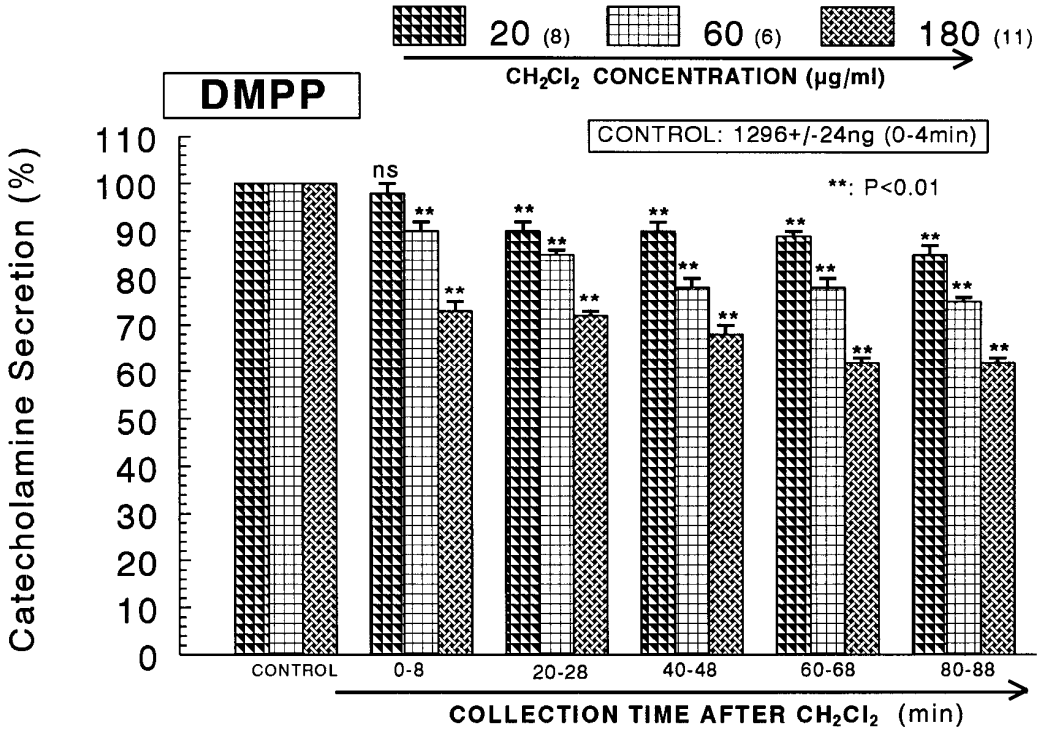

Fig. 7. Dose-dependent effect of CH_2Cl_2 fraction on the DMPP-evoked CA secretory responses from the isolated perfused rat adrenal glands. CA secretion by the perfusion of DPPP (10^{-4} M) was infused for 2 min at 20 min intervals after preloading with 20, 60, 180 μg/mLof CH_2Cl_2 for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONTROL, 1296±24 ng for 8 min) with each concentration-pretreated group of CH_2Cl_2. DMPP-induced perfusate was collected for 8 minutes. Other legends are the same as in Fig. 4. **: $P < 0.01$. ns: Statistically not significant.

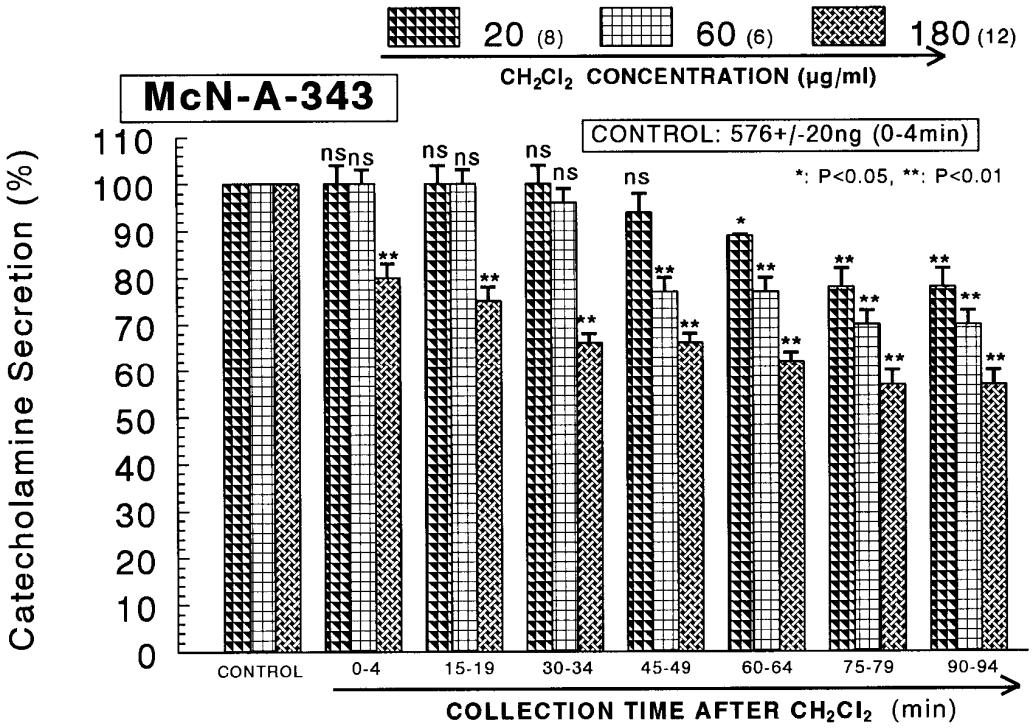

Fig. 8. Dose-dependent effect of CH_2Cl_2 fraction on the McN-A-343-evoked CA secretory responses from the isolated perfused rat adrenal glands. CA secretion by the perfusion of McN-A-343 (10^{-4} M) was infused for 4 min at 15 min intervals after preloading with 20, 60, 180 μg/mLof CH_2Cl_2 for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONTROL) with each concentration-pretreated group of CH_2Cl_2 fraction. McN-A-343-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 4. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

Effects of CH_2Cl_2 fraction on the CA secretion evoked by Bay-K-8644, cyclopiazonic acid and veratridine from the perfused rat adrenal glands

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 CH_2Cl_2 fraction on Bay-K-8644-stimulated CA secretion from the isolated perfused rat adrenal glands. Bay-K-8644 (10^{-5} M)-stimulated CA secretion in the presence of CH_2Cl_2 fraction was greatly blocked to 70% of the control except for the early 15 min period as compared to the corresponding control release (469±18 ng for 0-4 min) from 6 rat adrenal glands, as shown in Fig. 9. 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 CH_2Cl_2 fraction on cyclopiazonic acid-evoked CA secretory response was observed as shown in Fig. 10. However, in the presence of CH_2Cl_2 fraction in 6 rat adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was also inhibited to 73% of the control response (448±19 ng for 0-4 min). It has been known that veratridine-induced Na^+ influx mediated through Na^+ channels increased Ca^{2+} influx via activation of voltage-dependent Ca^{2+} channels and produced the exocytotic CA secretion in cultured bovine adrenal medullary cells (Wada et al., 1985a). As shown in Fig. 11, veratridine greatly produced CA secretion (1056±21 ng for 0-4 min). CH_2Cl_2 fraction (60 μg/ml) also attenuated veratridine-induced CA secretion by 58% of the corresponding control release in a time-dependent manner.

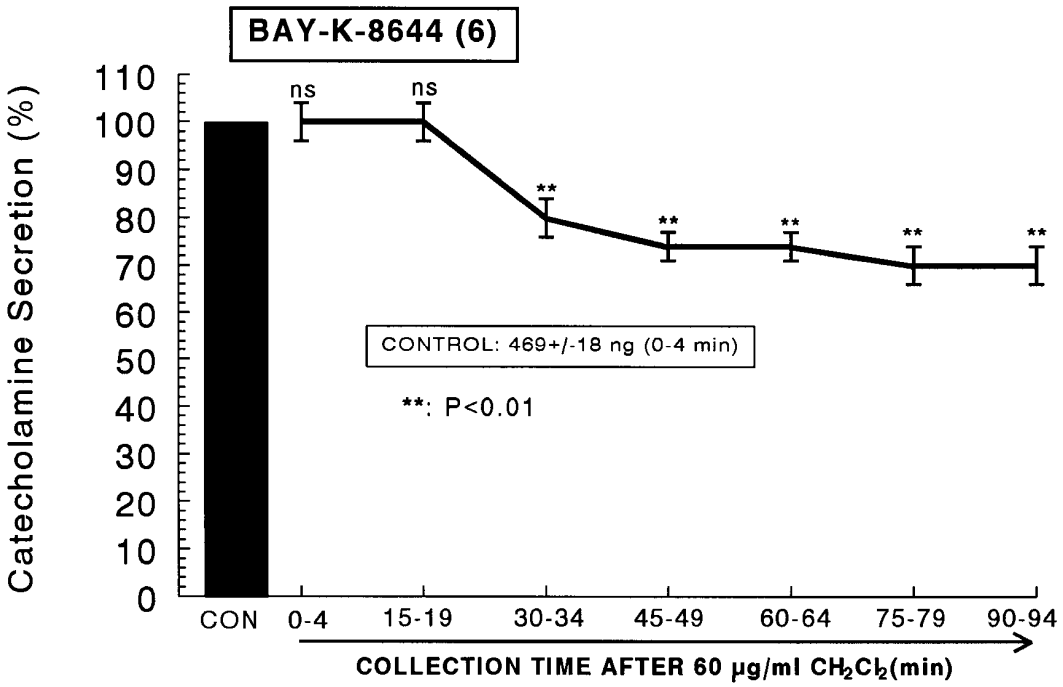

Fig. 9. Time-course effects of CH_2Cl_2 fraction on Bay-K-8644-evoked CA release in the perfused rat adrenal glands. Bay-K-8644 (10^{-5} M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with of CH_2Cl_2 fraction (60 µg/mL) for 90 min. Statistical difference was obtained by comparing the corresponding control (CON) with each period after pretreatment with CH_2Cl_2 fraction. Other legends are the same as in Fig. 4. **: $P < 0.01$. ns: Statistically not significant.

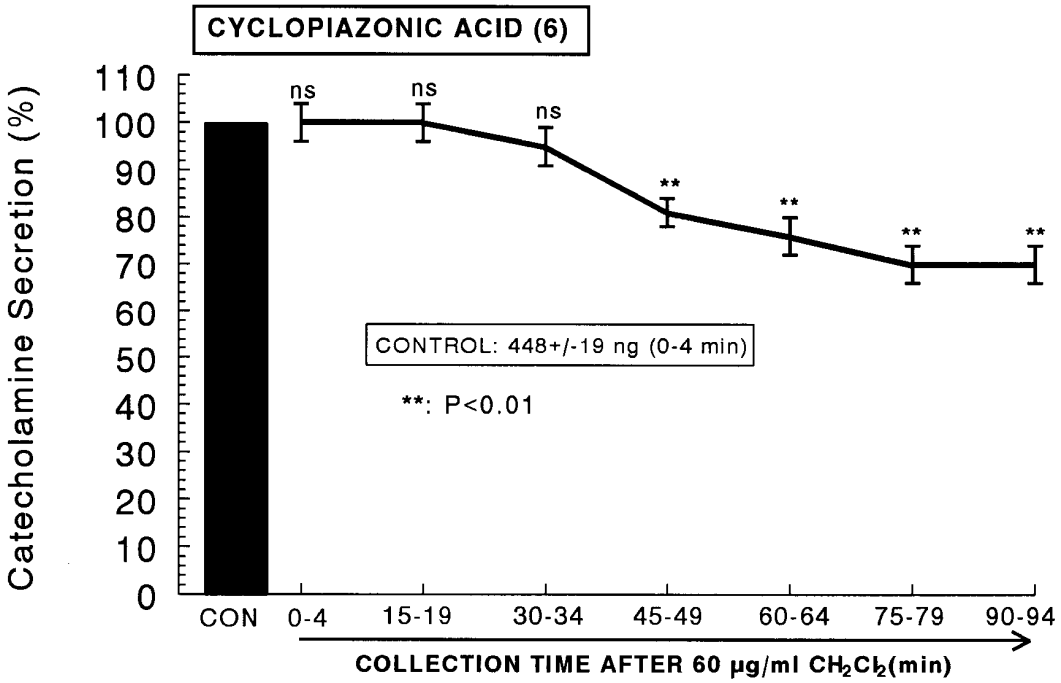

Fig. 10. Time-course effects of CH_2Cl_2 fraction on cyclopiazonic acid-evoked CA release in the perfused rat adrenal glands. Cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with of CH_2Cl_2 fraction (60 μg/mL) for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CON) with each period after pretreatment with CH_2Cl_2 fraction. Other legends are the same as in Fig. 4. **: $P < 0.01$. ns: Statistically not significant.

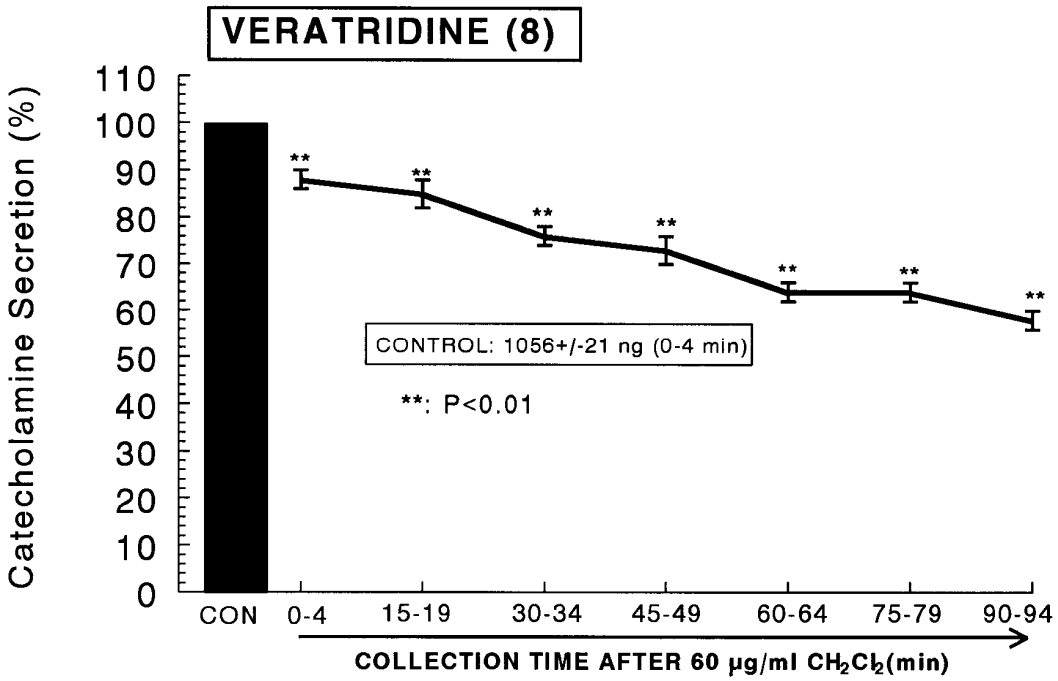

Fig. 11. Time-course effects of CH_2Cl_2 fraction on veratridine-evoked CA release in the perfused rat adrenal glands. Veratridine (10^{-4} M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with CH_2Cl_2 fraction (60 μg/ml) for 90 min. Statistical difference was obtained by comparing the corresponding control with each period after pretreatment with CH_2Cl_2 fraction. Other legends are the same as in Fig. 4. **: $P < 0.01$.

Effects of CH_2Cl_2 fraction plus L-NAME on the CA release evoked by ACh, high K^+, DMPP, McN-A-343, BAY-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

It has also been found that, in this study, CH_2Cl_2 fraction inhibited the CA secretory response evoked by cholinergic stimulation in the perfused rat adrenal glands. Therefore, to study the relationship between NO and CH_2Cl_2 fraction -induced inhibitory effects on the CA release from the rat adrenal glands, the effect of L-NAME on CH_2Cl_2 fraction-induced inhibitory responses of CA secretion evoked by cholinergic receptor-stimulation as well as membrane depolarization was examined. In the present study, in the simultaneous presence of CH_2Cl_2 fraction (60 μg/ml) and L-NAME (30 μM) for 90 min, ACh (5.32 mM)-evoked CA release was initially not affected at first 4 min, but later rather recovered to 89% of the corresponding control release at the period of 90-94 min compared to that of CH_2Cl_2 fraction (60 μg/ml)-treated group only, as illustrated in Fig. 12. High K^+ (56 mM)-evoked CA release in the presence of CH_2Cl_2 fraction (60 μg/ml) and L-NAME (30 μM) for 90 min was also not changed for 0-64 min, and then recovered to 84% of the corresponding control release at the last period of 90-94 min period in comparison to that of CH_2Cl_2 fraction (60 μg/ml)-treated group only (Fig. 13). As shown in Fig. 14, the simultaneous perfusion of CH_2Cl_2 fraction and L-NAME for 90 min no longer inhibited DMPP-evoked CA release for the period of 0-48 min while later rather recovered to 91% of the control release at the period of 60-88 min. Moreover, in the simultaneous presence of CH_2Cl_2 fraction and L-NAME for 90 min, McN-A-343-evoked CA secretory responses was also time-dependently recovered to 84% of the control secretion compared to that of

CH_2Cl_2 fraction (60 μg/ml)-treated group only from 8 glands as shown in Fig. 15, although they were not affected at period of 0-64 min.

As shown in Fig. 16, the simultaneous perfusion of CH_2Cl_2 fraction (60 μg/ml) and L-NAME (30 μM) for 90 min no longer inhibited the CA release evoked by Bay-K-8644 for the period of 0-64 min, and then also recovered to 83% of the control release at 75-94 min period in comparison to that of CH_2Cl_2 fraction (60 μg/ml)-treated group alone. In the presence of CH_2Cl_2 fraction (60 μg/ml) and L-NAME (30 μM) for 90 min, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was recovered to 82% of the control response (100%) at the period of 75-94 min in comparison to that of CH_2Cl_2 fraction (60 μg/ml)-treated group alone. the simultaneous perfusion of CH_2Cl_2 fraction (60 μg/ml) and L-NAME (30 μM) for 90 min did not inhibit the veratridine-evoked CA for the period of 0-34 min, finally recovered to 89% of the control release at 90-94 min period in comparison to that of CH_2Cl_2 fraction (60 μg/ml)-treated group alone (Fig. 18).

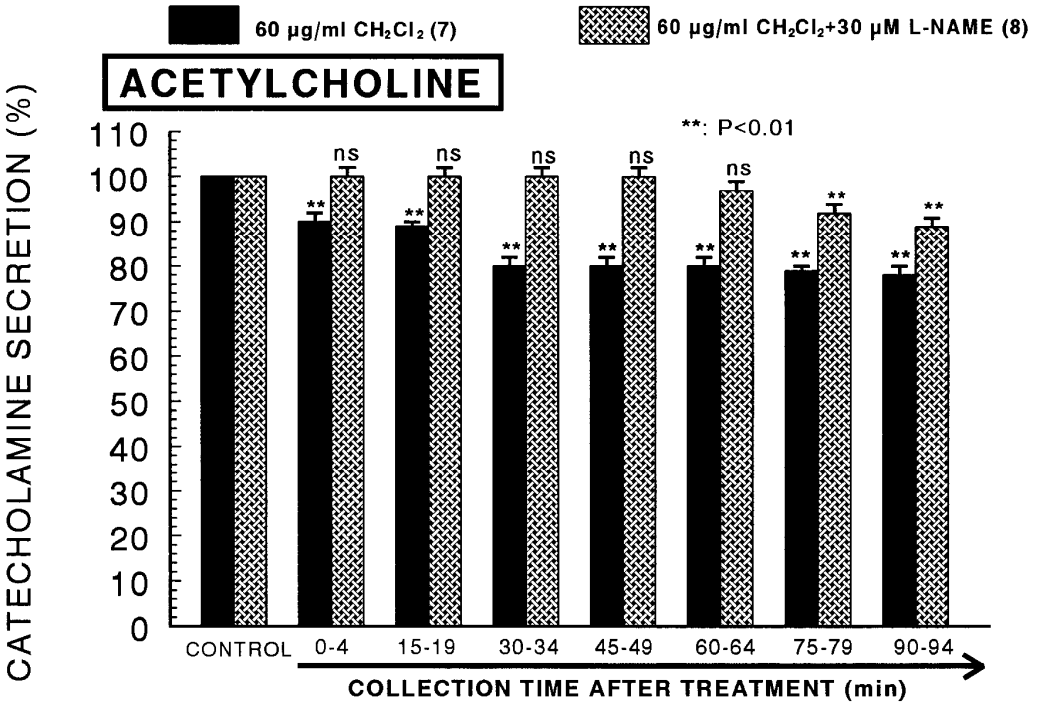

Fig. 12. Effects of CH_2Cl_2 fraction plus L-NAME on the ACh-evoked CA secretory responses from the isolated perfused rat adrenal glands. The CA secretion by a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 ml was induced before (CONTROL) and after preloading with CH_2Cl_2 fraction (60 μg/ml) plus L-NAME (30 μM) for 90 min. Perfusates were collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 4. **: $P < 0.01$. ns: Statistically not significant

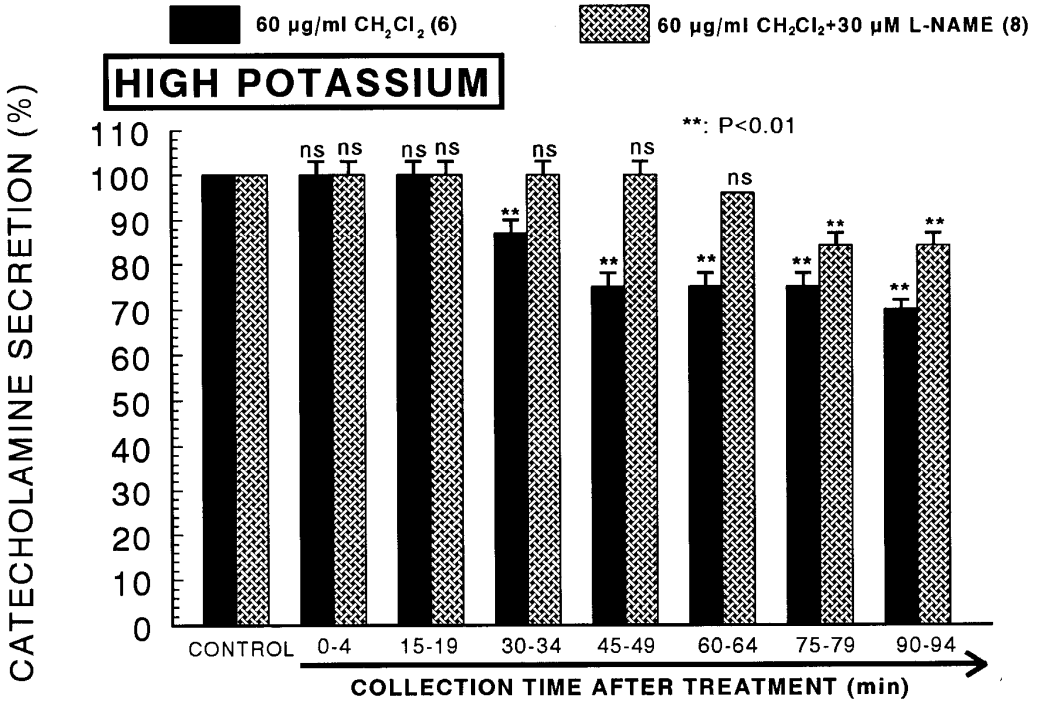

Fig. 13. Effects of CH_2Cl_2 fraction plus L-NAME on the high potassium-evoked CA secretory responses from the isolated perfused rat adrenal glands. The CA secretion by a single injection of high K^+ (5.6×10^{-2} M) in a volume of 0.05 ml was induced before (CONTROL) and after preloading with CH_2Cl_2 fraction (60 μg/ml) plus L-NAME (30 μM) for 90 min. Perfusates were collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 4 and 12. **: $P < 0.01$. ns: Statistically not significant.

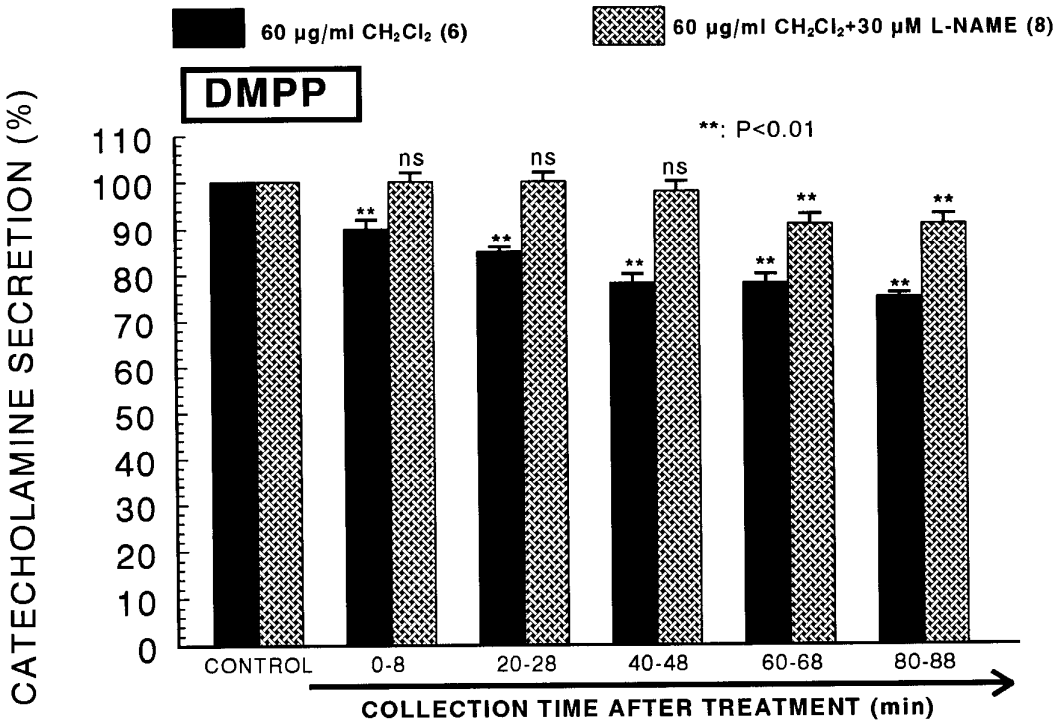

Fig. 14. Effects of CH_2Cl_2 fraction plus L-NAME on the DMPP-evoked CA secretory responses from the perfused rat adrenal glands. The CA secretion by perfusion of DMPP (10^{-4} M) for 2 min was induced before (CONTROL) and after preloading with CH_2Cl_2 fraction (60 μg/ml) plus L-NAME (30 μM) for 90 min. Perfusates were collected for 8 minutes at 20 min-intervals. Other legends are the same as in Fig. 4 and 12. **: $P < 0.01$. ns: Statistically not significant.

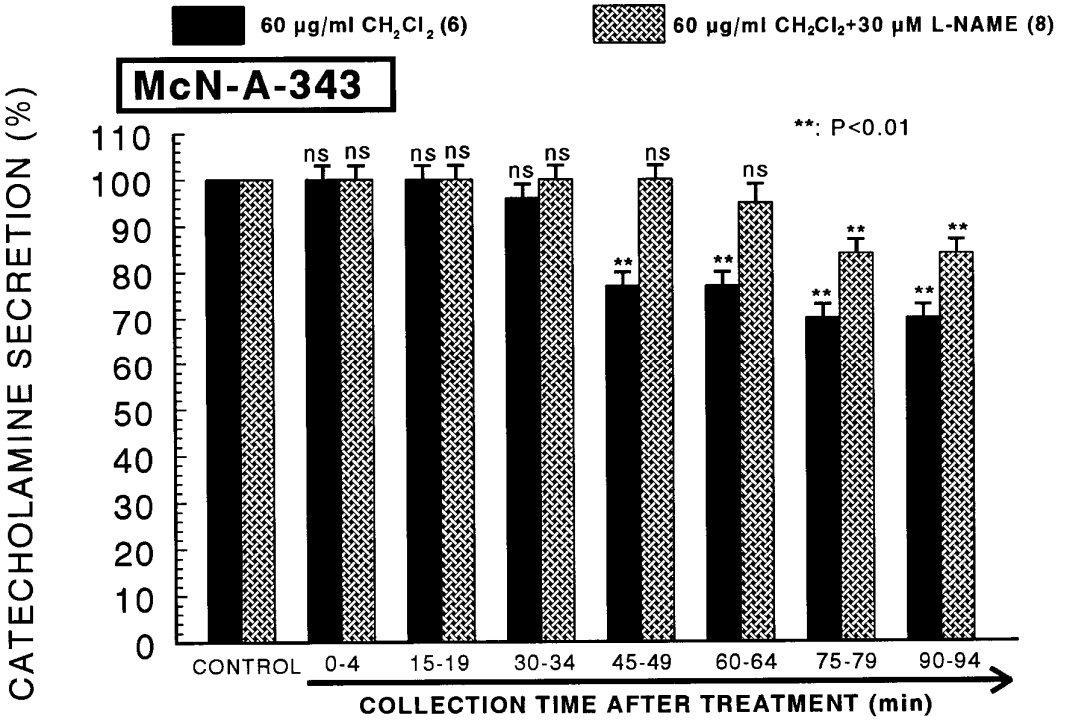

Fig. 15. Effect of CH_2Cl_2 fraction plus L-NAME on the McN-A-343-evoked CA secretory responses from the perfused rat adrenal glands. The CA secretion by perfusion of McN-A-343 (10^{-4} M) for 2 min was induced at 15 min intervals after preloading with CH_2Cl_2 fraction (60 μg/ml) plus L-NAME (30μM) for 90 min. Other legends are the same as in Fig. 4 and 12. **: $P < 0.01$. ns: Statistically not significant.

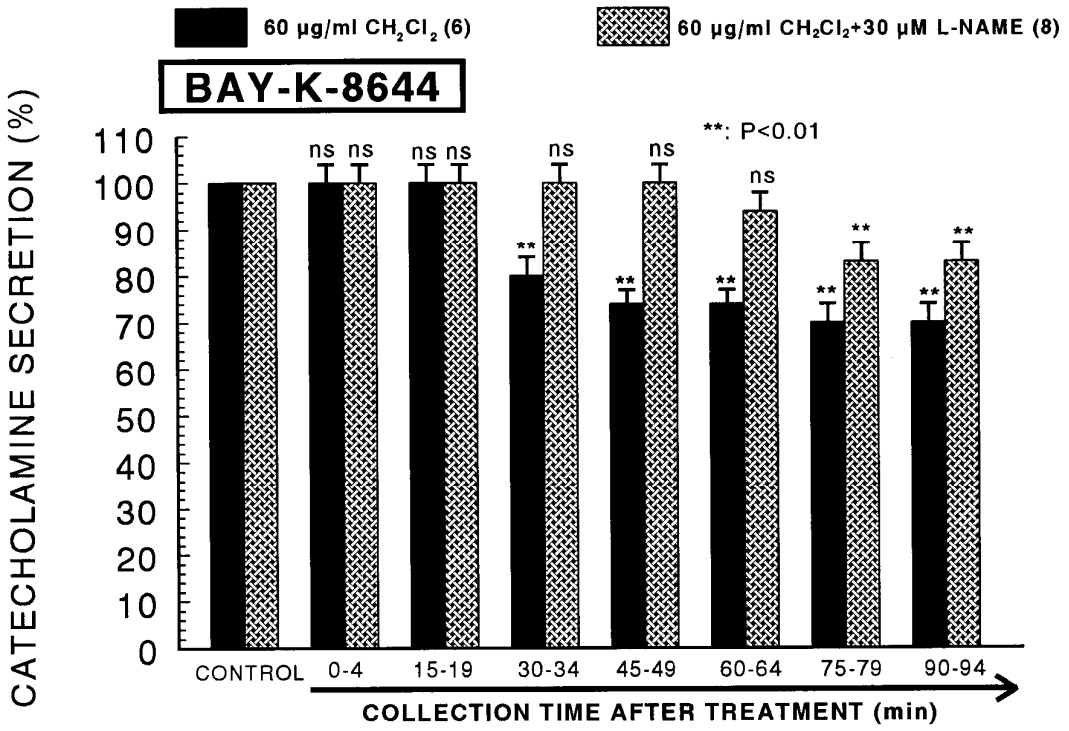

Fig. 16. Effects of CH_2Cl_2 fraction plus L-NAME on the Bay-K-8644-evoked CA secretory responses from the rat adrenal glands. Bay-K-8644 (10^{-5} M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with CH_2Cl_2 (60 μg/ml) plus L-NAME (30μM) for 90 min. Other legends are the same as in Fig. 4 and 12. **: $P < 0.01$. ns: Statistically not significant.

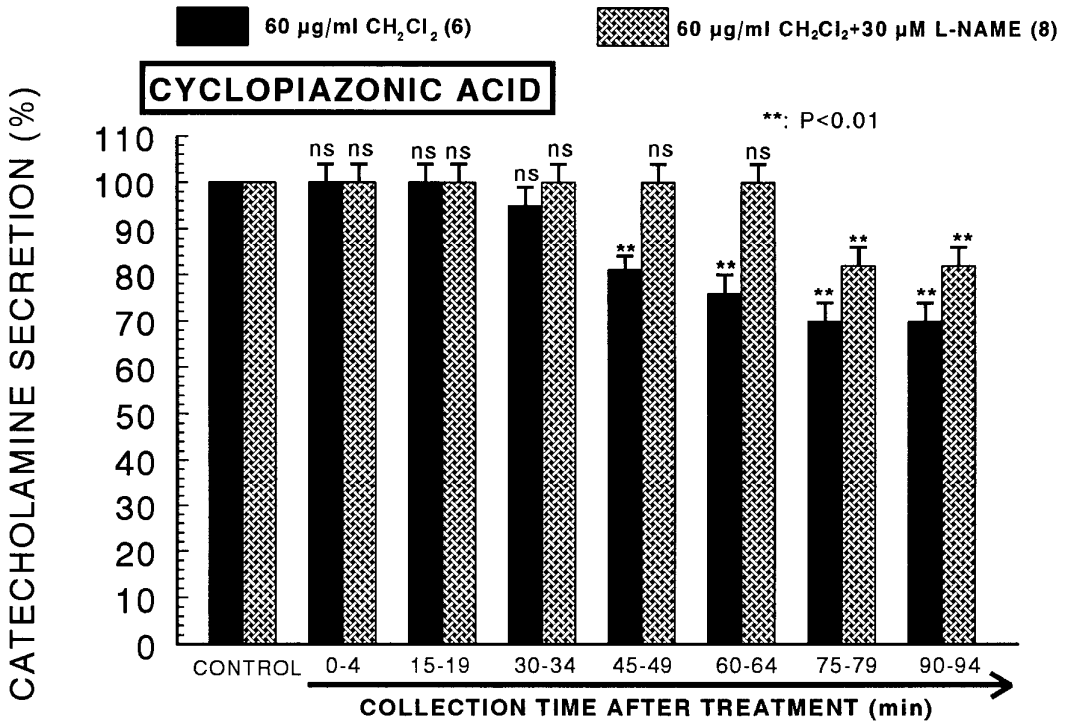

Fig. 17. Effects of CH_2Cl_2 fraction plus L-NAME on the cyclopiazonic acid-evoked CA secretory responses from the rat adrenal glands. Cyclopiazonic acid (10^{-5} M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with CH_2Cl_2 fraction (60 μg/ml) for 90 min. Other legends are the same as in Fig. 4 and 12. **: $P < 0.01$. ns: Statistically not significant.

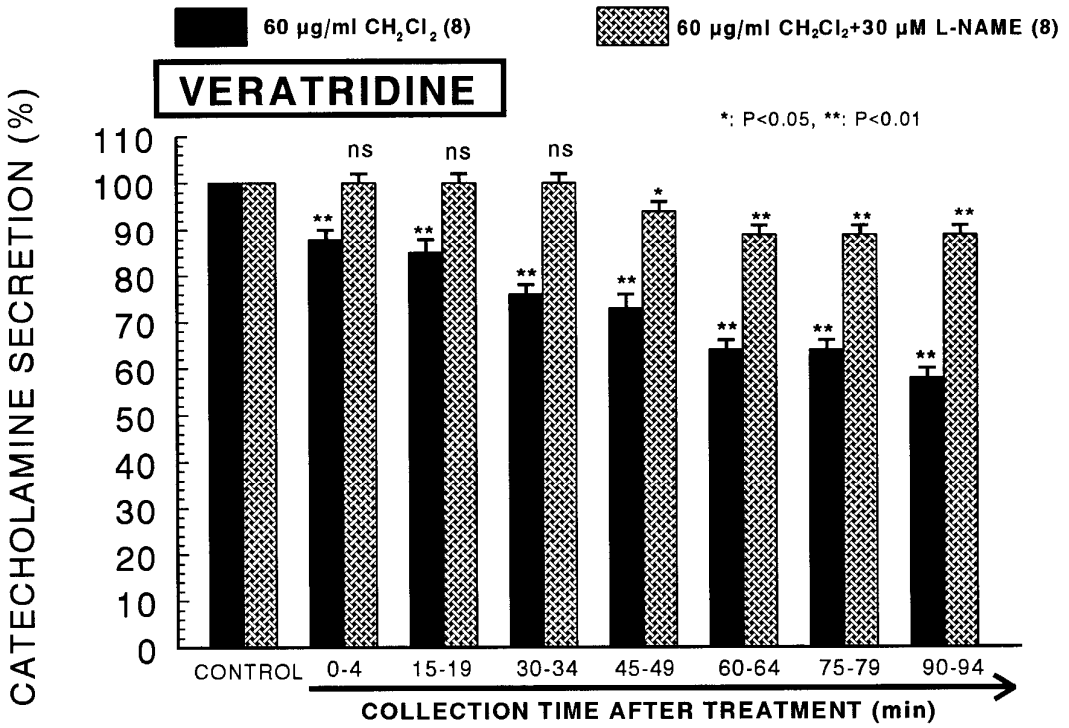

Fig. 18. Effects of CH_2Cl_2 fraction plus L-NAME on the veratridine-evoked CA secretory responses from the rat adrenal glands. Veratridine (10^{-4} M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with CH_2Cl_2 fraction (60 μg/ml) for 90 min. Other legends are the same as in Fig. 4 and 12. **: $P < 0.01$. ns: Statistically not significant.

Effect of CH_2Cl_2 fraction on the level of nitric oxide released from the perfused rat adrenal medulla

As shown in Fig. 12~18, the inhibitory effects of CH_2Cl_2 fraction on cholinergic stimulation- and direct membrane depolarization-evoked CA secretory responses were significantly reduced in the presence of L-NAME. Therefore, it was decided directly to determine the level of NO released from the perfused rat adrenal medulla after the treatment of CH_2Cl_2 fraction. Moreover, It has been shown that PCRC inhibits the CA secretion evoked by several seretagogues is exerted through the increased NO production due to the activation of nitric oxide synthase in the perfused adrenal medulla in normotensive rats and SHRs (Kee and Lim, 2007; Yu et al., 2009). It is also found that red wines and grapes exhibit endothelium-dependent relaxation of blood vessels via enhanced generation and/or increased biological activity of NO, leading to the elevation of cGMP levels (Fitzpatrick et al., 1993; Fitzpatrick et al., 1995; Fitzpatrick et al., 2000; Zenebe et al., 2003). In 10 adrenal glands, the basal amount of NO released from medulla prior to administration of CH_2Cl_2 fraction was 7.5±1.0 picomoles. However, during 8 min after loading with CH_2Cl_2 fraction it was greatly elevated to 169.7±8.3 picomoles, which was 2263% of the basal release, as shown in Fig. 19.

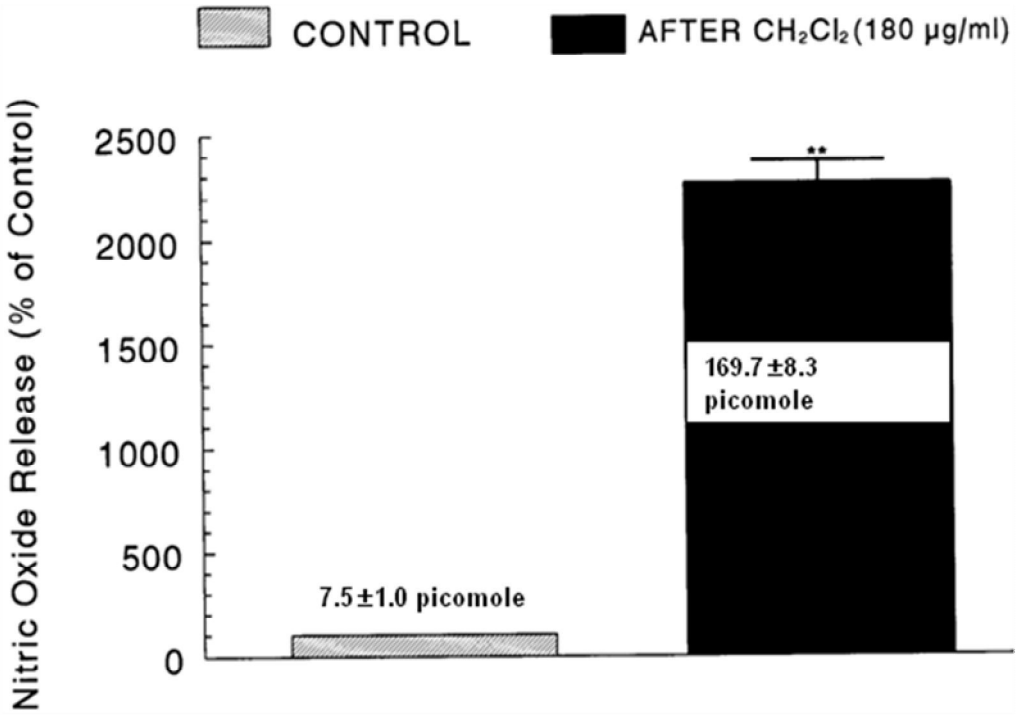

Fig. 19. Effect of CH_2Cl_2 fraction on the nitric oxide (NO) production in the perfused rat adrenal medulla. "CONTROL" and "AFTER" indicate NO release before and after administration of CH_2Cl_2 fraction in the perfused rat adrenal medulla, respectively.

B. Influence of CH_2Cl_2 fraction on contractile responses of the thoracic aortic strips of normotensive rats and SHRs

Effects of four fractions extracted from Rubus coreanum on phenylephrine-induced contractile responses in the thoracic aortic strips of normotensive rats

The resting (basal) tension from the isolated rat aortic strips with intact endothelium reaches a steady state after the perfusion with oxygenated Krebs-bicarbonate solution for 90 min before the experimental protocol is initiated. The resting tension was adjusted to 0.5 g. The effects of four fractions extracted from *Rubus coreanum* on phenylephrine (PE)-induced contractile responses in the rat aorta with intact endothelium were examined. In the present study, CH_2Cl_2 fraction itself did not produce any effect on the resting tension in the aortic strips with intact endothelium isolated from rats (data not shown). In previous study, it has been found that PCRC causes vascular relaxation in the isolated aortic strips of SHRs at least partly by the increased NO production through the activation of NO synthase of vascular endothelium, but not through the activation of cyclooxygenase (Lim, 2008). Therefore, it was attempted to examine effects of four fractions (ethylacetate [EtOAc], methylene chloride [CH_2Cl_2], n-butanol [BuOH], and water [H_2O]) isolated from *Rubus coreanum M.* on PE-induced contractile responses in the isolated rat aortic strips. As shown in Fig. 20, in the presence of CH_2Cl_2 (400 μg/mL), EtOAc (400 μg/mL), BuOH (400 μg/mL), and H_2O (400 μg/mL) 5 min before addition of phenylephrine, the contractile responses of phenylephrine (10^{-5} M) were significantly reduced to 50±1% ($P< 0.01$, n=6), 58±11% ($P< 0.01$, n=6), 90±18% ($P< 0.01$, n=8), and 78±2% ($P< 0.05$,

n=6) of the corresponding control response (1.6±0.1 g), respectively. Based on these results, for the PE-induced contractile response, the following rank order of inhibitory potency was obtained: CH_2Cl_2**>**EtOAc**>>**H_2O**>>**BuOH. Therefore, in all subsequent experiments, CH_2Cl_2 fraction (400 µg/ml) only was used.

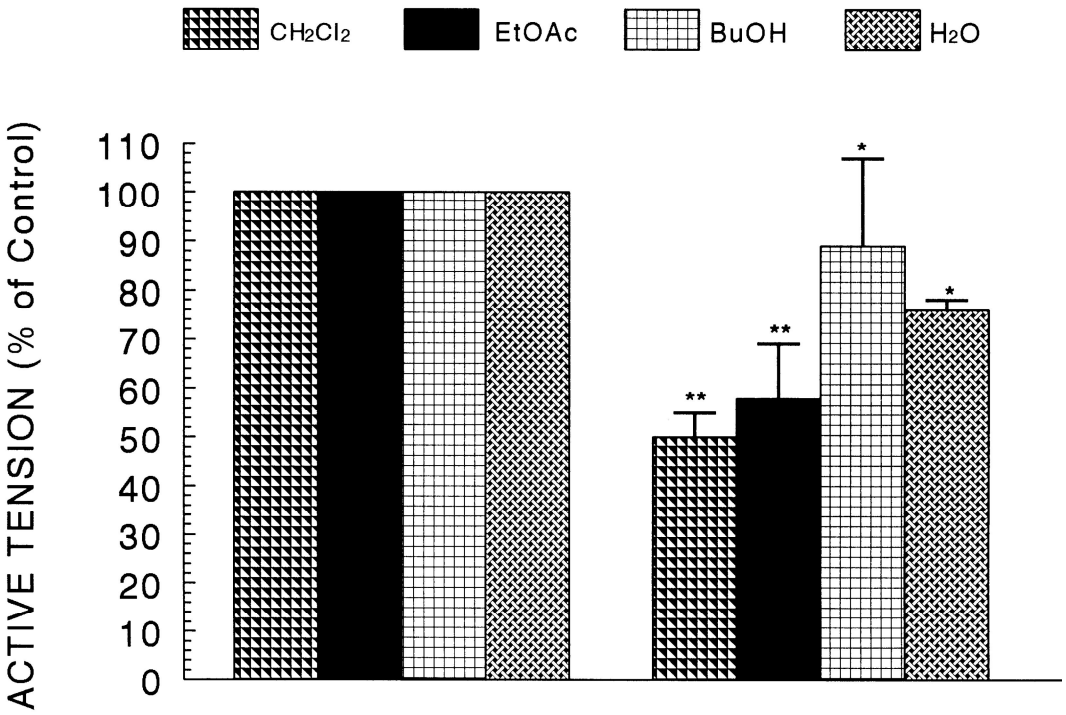

Fig. 20. Comparative effects of four fractions (water [H_2O], butanol [BuOH], ethylacetate [EtOAc], and methylene chloride [CH_2Cl_2]) extracted from *Rubus coreanum* on the inhibition of phenylephrine-induced contractile responses in the isolated thoracic aortic strips of rats. The contractile responses were induced by adding 10 μM PE at 120 min interval after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol. Each column denotes active tension induced evoked by 10 μM PE before and after adding fractions (400 μg/ml) of CH_2Cl_2, EtOAc, BuOH and H_2O, respectively. Vertical bars represent the standard error of the mean (S.E.M). Ordinate: the active tension (% of control, 1.6±0.1g [10 μM]). Abscissa: Concentrations of CH_2Cl_2 fraction. Statistical difference was obtained by comparing the control with the CH_2Cl_2 fraction-pretreated group. *: $P< 0.05$, **: $P< 0.01$.

Effects of CH_2Cl_2 fraction isolated from Rubus coreanum on contractile responses induced by phenylephrine and high K^+ in the thoracic aortic strips of normotensive rats and SHRs

Phenylephrine is a selective agonist of adrenergic α_1 receptors, which exhibits vasoconstriction. To establish the inhibitory effect of CH_2Cl_2 fraction on phenylephrine (10^{-5} M)-induced contractile responses, in the presence of CH_2Cl_2 fraction at 200, 400 and 800 μg/ml, 5 min before addition of phenylephrine, the contractile responses of phenylephrine (10^{-5} M) were dose-dependently reduced to 60±9% ($P< 0.01$, n=8), 50±5% ($P< 0.01$, n=10) and 36±5% ($P< 0.01$, n=9) of the corresponding control response (1.6±0.1 g), respectively (Fig. 21 and 22).

High K^+ exerts two distinct effects on cells: (1) depolarization of cell membrane, and (2) depolarization- induced influx of calcium via voltage-dependent calcium channels (Wada et al., 1985). When added through the bath, high potassium at 5.6 x 10^{-2} M, which is a membrane-depolarizing agent, caused an increase in aortic contraction (1.72±0.2 g). As shown in Fig. 22 and 23, high potassium (5.6 x 10^{-2} M)-induced contractile responses after pre-loading with 200, 400 and 800 μg/ml of CH_2Cl_2 fraction 5 min before high potassium were dose-dependently reduced to 55±9% ($P< 0.01$, n=12), 48±5% ($P< 0.01$, n=10) and 23±5% ($P< 0.01$, n=9) of the corresponding control response (1.72±0.2 g), respectively. In all subsequent experiments, a single dose of CH_2Cl_2 fraction (400 μg/ml) was used.

As shown in Fig. 20~23, CH_2Cl_2 fraction inhibited phenylephrine - and high K+-induced contractile responses of thoracic aortic strips isolated from normotensive rats in a dose-dependent fashion. Therefore, it is of interest to examine effect of CH_2Cl_2 fraction on phenylephrine- and high K^+-induced

contractile responses of thoracic aortic strips isolated from SHRs. In the presence of CH_2Cl_2 fraction (400 μg/ml), phenylephrine (10^{-5} M) and high K^+ (5.6×10^{-2} M)-induced contractile responses were significantly inhibited (Fig. 24 and 25)

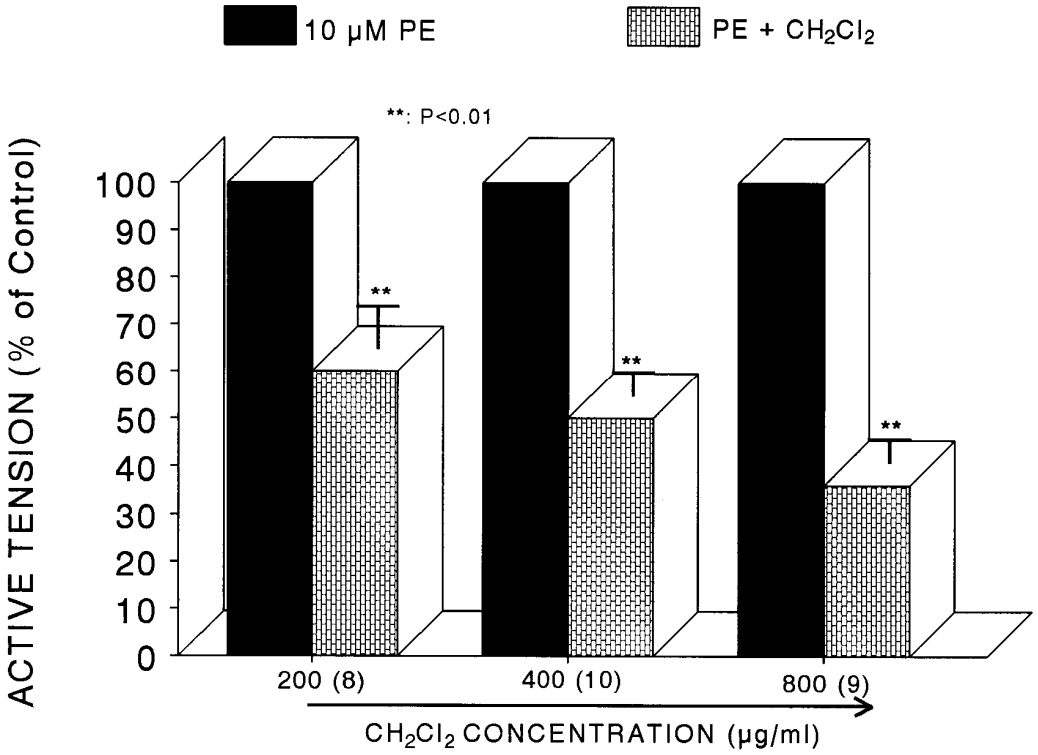

Fig. 21. Dose-dependent effects of CH_2Cl_2 fraction on phenylephrine (PE)-induced contractile responses in the isolated rat aortic strips. The contractile responses were induced by adding 10 μM PE at 120 min interval after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol. "Black column" and "Brick column" denote active tension induced evoked by 10 μM PE before and after adding 200, 400 and 800 μg/ml of CH_2Cl_2 fraction, respectively. Other legends and methods are the same as in Fig. 20. **: $P < 0.01$.

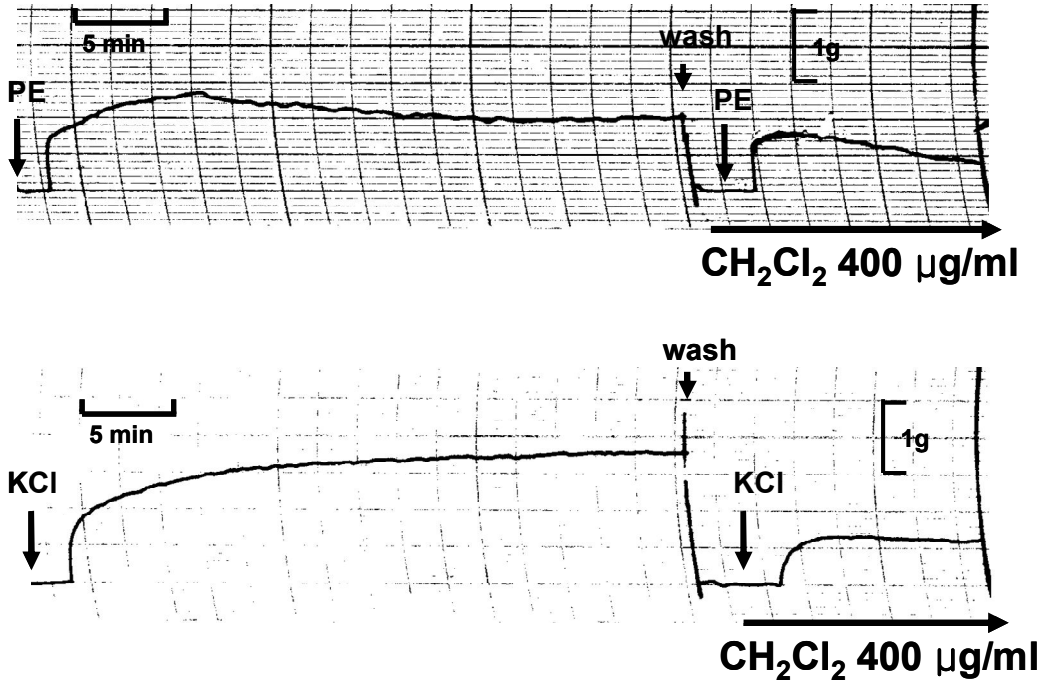

Fig. 22. The typical tracing showing the effect of CH_2Cl_2 fraction on phenylephrine (PE)- and high potassium (KCl)-induced contractile response in the isolated rat aortic strip. **[Upper panel]** Left: PE-induced contractile response (Control). Right: PE-induced contractile response in the presence of CH_2Cl_2 (400 μg/ml). **[Lower panel]** Left: KCl-induced contractile response (Control). Right: KCl-induced contractile response in the presence of CH_2Cl_2 (400 μg/ml). At arrow mark, the indicated dose of PE (10 μM) and KCl (56 mM) was added into the bath, respectively. The chart speed was 5 mm/min.

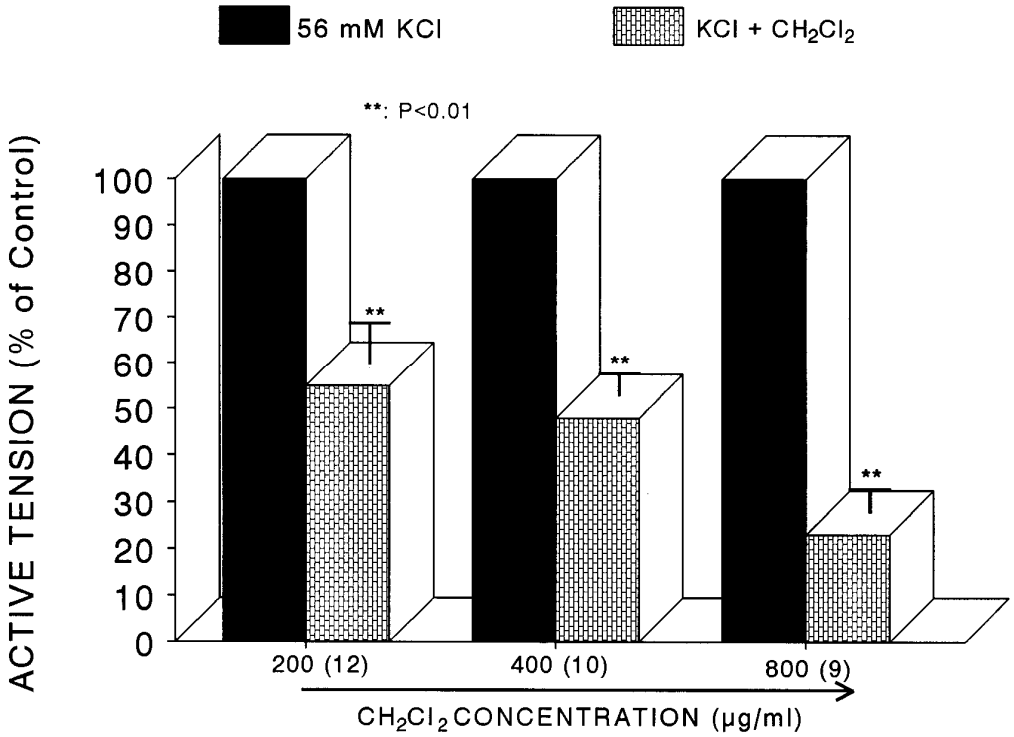

Fig. 23. Dose-dependent effects of CH_2Cl_2 fraction on high potassium (KCl)-induced contractile responses in the isolated rat aortic strips. The contractile responses were induced by adding 56 mM KCl at 120 min interval after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol. “Black column" and “Brick column" denote active tension induced evoked by 56 mM KCl before and after adding 200, 400 and 800 μg/ml of EtOAc fraction, respectively. Other legends and methods are the same as in Fig. 20. **: P< 0.01.

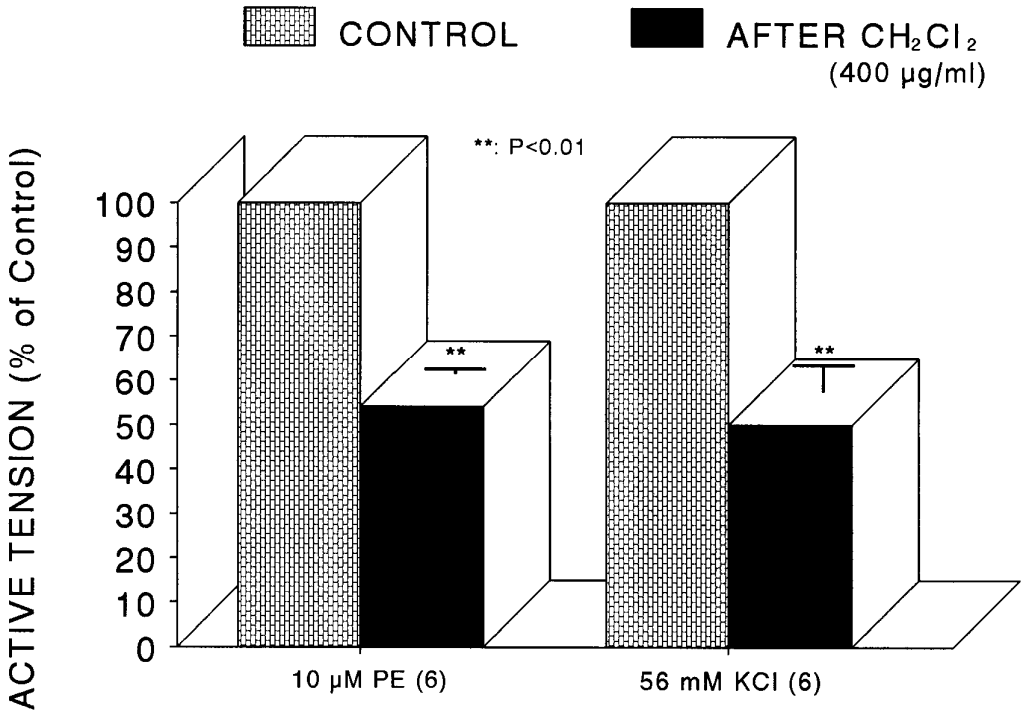

Fig. 24 Influence of CH_2Cl_2 fraction on contractile responses induced by phenylephrine (PE) and high potassium (KCl) in the aortic strips isolated from SHRs. The contractile responses were induced by adding 10 μM PE and 56 mM KCl at 120 min interval after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol, respectively. "CONTROL" and "AFTER" denote active tension induced evoked by PE anf KCl before and after adding 400 μg/ml of CH_2Cl_2 fraction. Statistical difference was obtained by comparing the control with the CH_2Cl_2 fraction -pretreated group. Other legends are the same as in Fig. 20. **: $P< 0.01$.

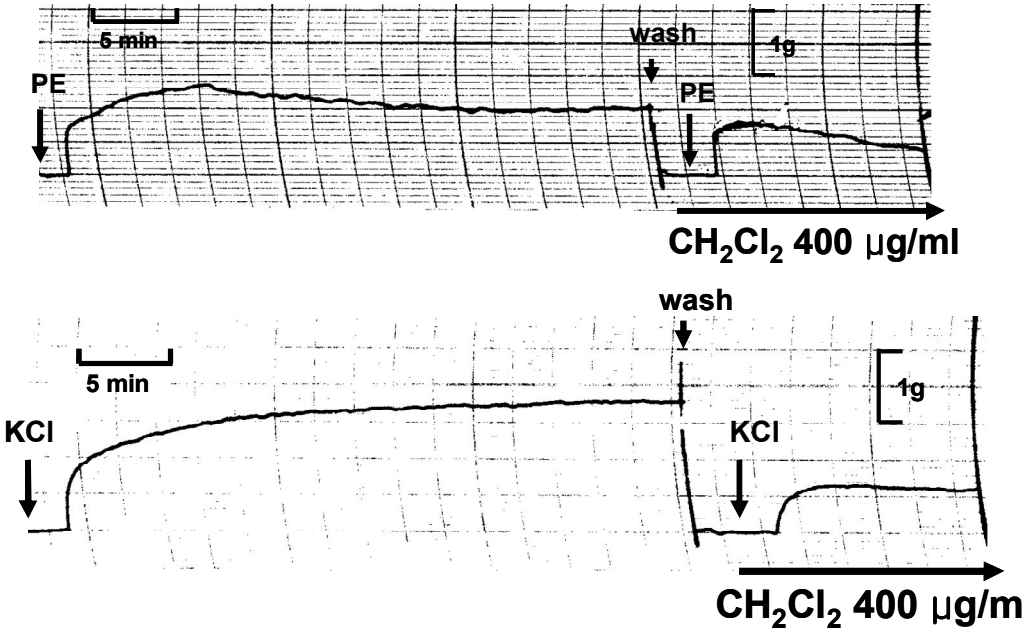

Fig. 25. The typical tracing showing the effect of CH_2Cl_2 fraction on phenylephrine (PE)- and high potassium (KCl)-induced contractile response in the aortic strip isolated from the SHR. **[Upper panel]** Left: PE-induced contractile response (Control). Right: PE-induced contractile response in the presence of CH_2Cl_2 fraction (400 μg/ml). **[Lower panel]** Left: KCl-induced contractile response (Control). Right: KCl-induced contractile response in the presence of CH_2Cl_2 fraction (400 μg/ml). At arrow mark, the indicated dose of PE (10 μM) and KCl (56 mM) was added into the bath, respectively. The chart speed was 5 mm/min.

Influence of CH_2Cl_2 fraction plus L-NAME on CH_2Cl_2 fraction -induced inhibition to the contractile responses evoked by phenylephrine (PE) and high potassium (KCl) in the thoracic aortic strips of normotensive rat

In previous study, it has been demonstrated that PCRC inhibits the CA secretion evoked by cholinergic stimulation and direct membrane-depolarization from the perfused adrenal medulla of SHRs, which was blocked in the presence of L-NAME, a NO synthase inhibitor (Yu et al., 2009). These results suggest that PCRC can inhibit the CA release at least partly through the activation of nNOS in the adrenal medulla of SHRs. Therefore, in the presence of L-NAME, it was likely interesting to compare the effects of CH_2Cl_2 fraction on the contractile responses induced by high potassium and phenylephrine.

In the simultaneous presence of CH_2Cl_2 fraction (400 μg/ml) and L-NAME (300 μM), the aortic contractile response evoked by phenylephrine (10^{-5} M) was 94±11% ($P< 0.05$, n=8) of the control in comparison with the inhibitory response of CH_2Cl_2 fraction-treatment alone (50±5%) from the resting tension level as shown in Fig. 26 and 27.

High potassium (5.6×10^{-2} M)-induced contractile response in the simultaneous presence of CH_2Cl_2 fraction (400 μg/ml) and L-NAME (300 μM) was recovered to 54±4% ($P< 0.01$, n=7) of the corresponding control compared with the inhibitory response of CH_2Cl_2 fraction-treatment alone (38±4%) from the resting tension level (Fig. 26 and 27).

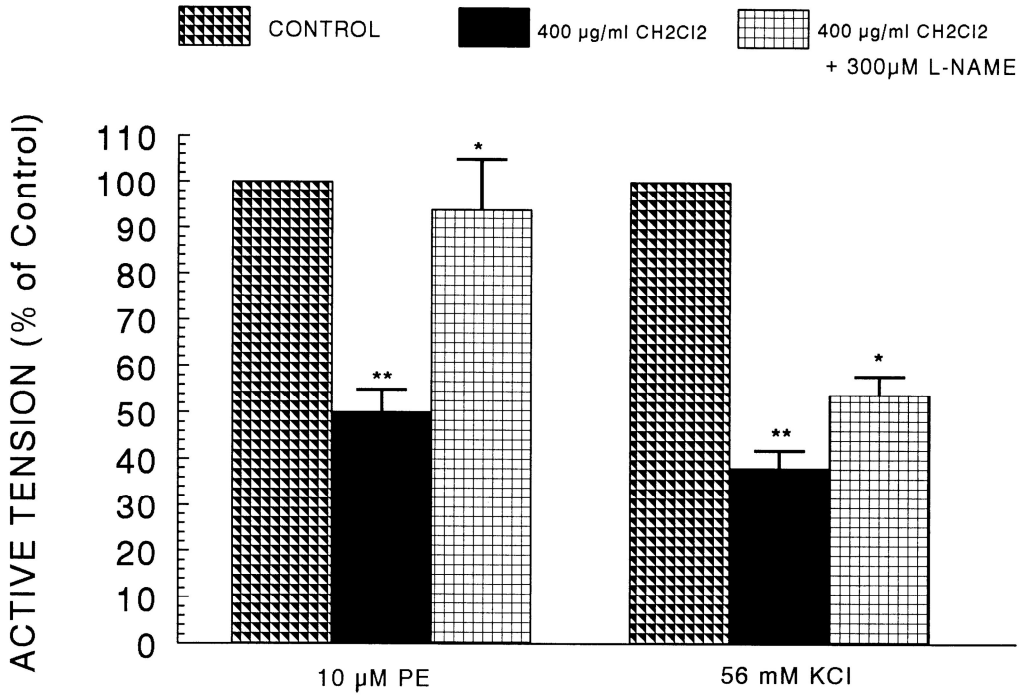

Fig. 26. . Influence of CH_2Cl_2 fraction plus L-NAME on the contractile responses evoked by phenylephrine (PE) and high potassium (KCl) in the isolated rat aortic strips. Statistical difference was obtained by comparing the control with the CH_2Cl_2 fraction-pretreated group or CH_2Cl_2 fraction (400 μg/ml) plus L-NAME (300 μM). Other legends are the same as in Fig. 20. *: $P < 0.05$, **: $P < 0.01$.

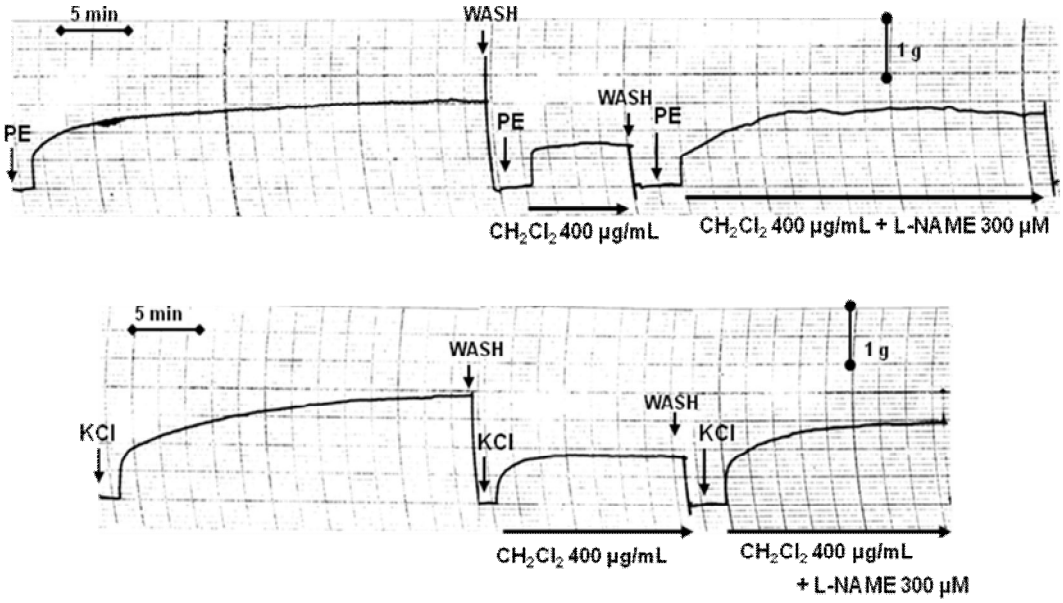

Fig. 27. The typical tracing showing the effect of CH_2Cl_2 fraction plus L-NAME on phenylephrine (PE, upper pannel)- and high potassium (lower pannel)-induced contractile response in the isolated rat aortic strips. **(Upper panel)**; Left: PE-induced contractile response (Control). Middle: PE-induced contractile response in the presence of CH_2Cl_2 fraction (400 µg/mL). Right: PE-induced contractile response in the presence of CH_2Cl_2 fraction (400 µg/mL) plus L-NAME (300 µM). **(Lower panel)**; Left: KCl-induced contractile response (Control). Middle: KCl-induced contractile response in the presence of CH_2Cl_2 fraction (400 µg/mL). Right: KCl-induced contractile response in the presence of CH_2Cl_2 fraction (400 µg/mL) plus L-NAME (300 µM). At arrow mark, the indicated doses of PE (10 µM) and KCl (56 mM) were added to the bath. The chart speed was 5 mm/min.

Influence of CH_2Cl_2 fraction plus CHAPS on contractile responses induced by phenylephrine (PE) and high potassium (KCl) in the thoracic aortic strips of normotensive rat

As shown in Fig. 26 an 27, CH_2Cl_2 fraction-induced vasorelaxation was markedly blocked in the presence of L-NAME, a NO synthase inhibitor. Therefore, it is likely interesting to examine the effects of CHAPS, a detergent which suppresses endothelial function (Moore et al., 1990), on CH_2Cl_2 fraction-induced inhibitory responses to the contractile active tension evoked by high potassium and phenylephrine.

In the presence of CH_2Cl_2 fraction (400 µg/ml) after pretreatment with 0.4% CHAPS, the aortic contractile response evoked by phenylephrine (10^{-5} M) exhibited 67±11% ($P< 0.01$, n=6) of the control in comparison with the corresponding control response (100%) from the resting tension level as shown in Fig. 28 and 29.

High potassium (5.6×10^{-2} M)-induced contractile response in the simultaneous presence of CH_2Cl_2 fraction (400 µg/ml) after pretreatment with CHAPS elicted 56±11% ($P< 0.01$, n=6) of the control in comparison with the corresponding control response (100%) from the resting tension level (Fig. 28 and 29).

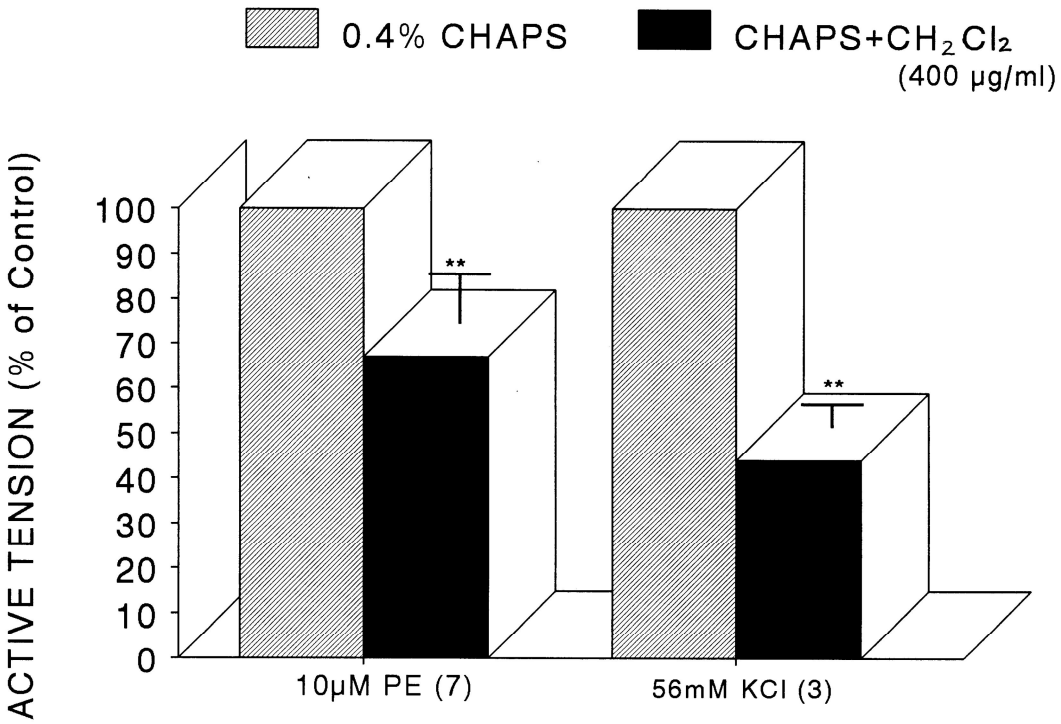

Fig. 28. Influence of CHAPS plus CH_2Cl_2 fraction on contractile responses induced by phenylephrine (PE) and high potassium (KCl) in the isolated rat aortic strips. The contractile responses were induced by adding 10 μM PE and 56 mM KCl at 120 min interval after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol, respectively. Statistical difference was obtained by comparing the control (% of control) with the CH_2Cl_2 fraction (400 μg/ml)-treated group after pretreatment of 0.4% CHAPS. Other legends are the same as in Fig. 20. **: $P< 0.01$.

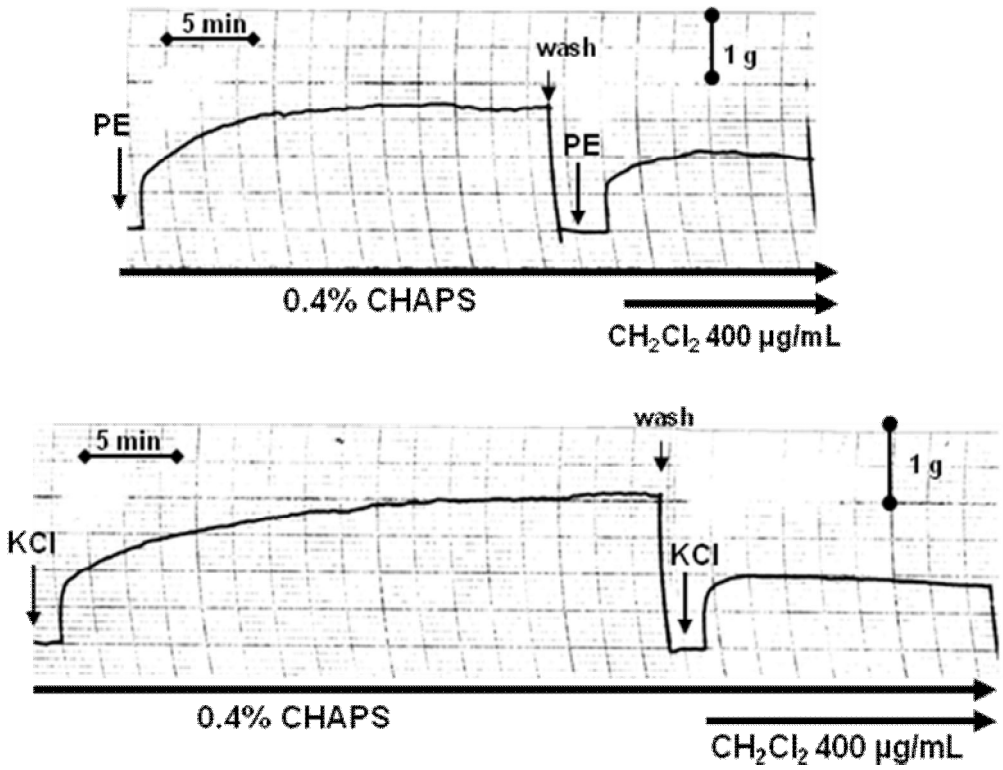

Fig. 29. The representative tracing of CHAPS plus CH_2Cl_2 fraction effect on contractile responses induced by phenylephrine and high potassium in the isolated rat aortic strips. At arrow marks, phenylephrine (10 μM) and high potassium (56 mM) were added into a CHAPS-pretreated aortic strips. Upper: Phenylephrine-induced contractile response after CH_2Cl_2 fraction (400 μg/ml)-treatment in a CHAPS-pretreated aortic strip. Lower: High potassium-induced contractile response after CH_2Cl_2 fraction (400 μg/ml)-treatment in a CHAPS-pretreated aortic strip. The chart speed was 5 mm/min.

C. Influence of CH_2Cl_2 fraction on arterial blood pressure of normotensive rats and SHRs

Effects of intravenous CH_2Cl_2 fraction on blood pressure in the anesthetized normotensive rats and SHRs

All of rats used in this study were allowed to be stabilized at least for 60 min before experimental protocols were initiated. When cardiovascular parameters were stabilized, CH_2Cl_2 fraction (0.3-3.0 mgkg) was given into a femoral vein of the normotensive rat anesthetized with thiopental sodium and urethane. CH_2Cl_2 fraction produced a dose-related and potent fall in arterial blood pressure. However, an equivalent volume of 0.9% saline given into a femoral vein did not produce any changes in blood pressure of the normotensive rats. As shown in Fig. 30 and 31, intravenous 0.3mg of CH_2Cl_2 fraction induced a fall in mean arterial pressure by 9.3±1.1 mmHg from the original baseline of 119.5±7.2 mmHg, but increasing doses of CH_2Cl_2 fraction to 1.0 and 3.0 mg/kg, i.v. showed the decreased mean arterial pressure of 15.0±1.8 and 25.5±2.7 mmHg, respectively from the pre-injection level of the baseline from 16 rats. All of the above experimental results were statistically significant from the corresponding pre-injection values ($p< 0.01$).

In SHRs, CH_2Cl_2 fraction at 1.0, 3.0 and 10.0 mg/kg, i.v. dose-dependently reduced the blood pressure by 16.8±0.4, 27.7±0.9 and 43.0±0.6 mmHg from the pre-injection level of the baseline from 8 rats, respectively, as shown in Fig. 32 and 33.

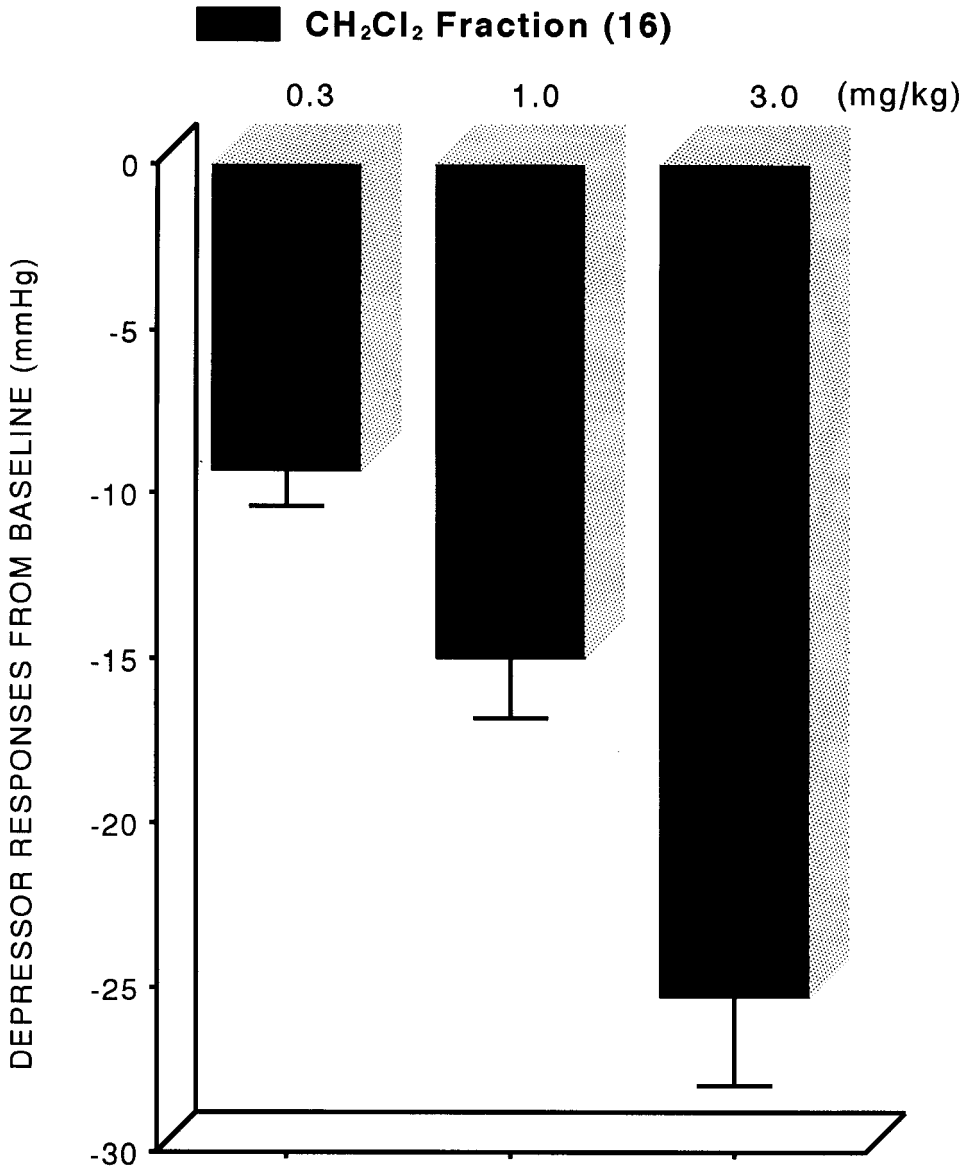

Fig. 30. Dose-dependent hypotensive effects of CH_2Cl_2 fraction in the anesthetized rats. CH_2Cl_2 fraction (0.3, 1.0 and 3.0 mg/kg, respectively) was administered into a femoral vein. Arterial blood pressure from pre-injection level was expressed in mmHg.

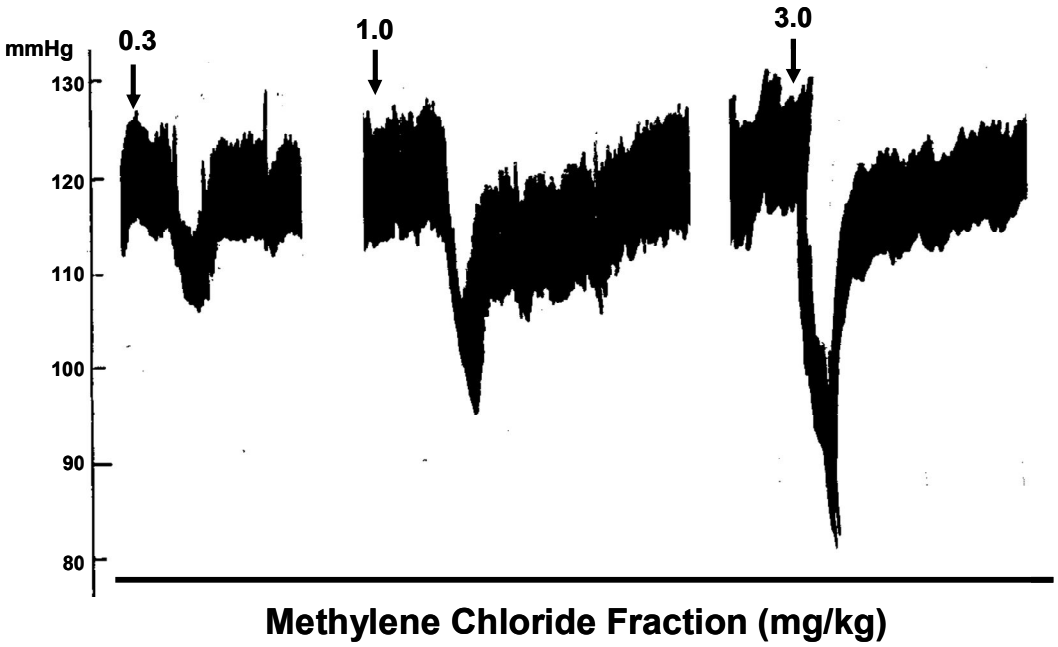

Fig. 31. The typical tracings of CH_2Cl_2 fraction-induced hypotensive action in an anesthetized rat. CH_2Cl_2 fraction at the indicated doses (0.3, 1.0 and 3.0 mg/kg) was injected intravenously at the arrow marks.

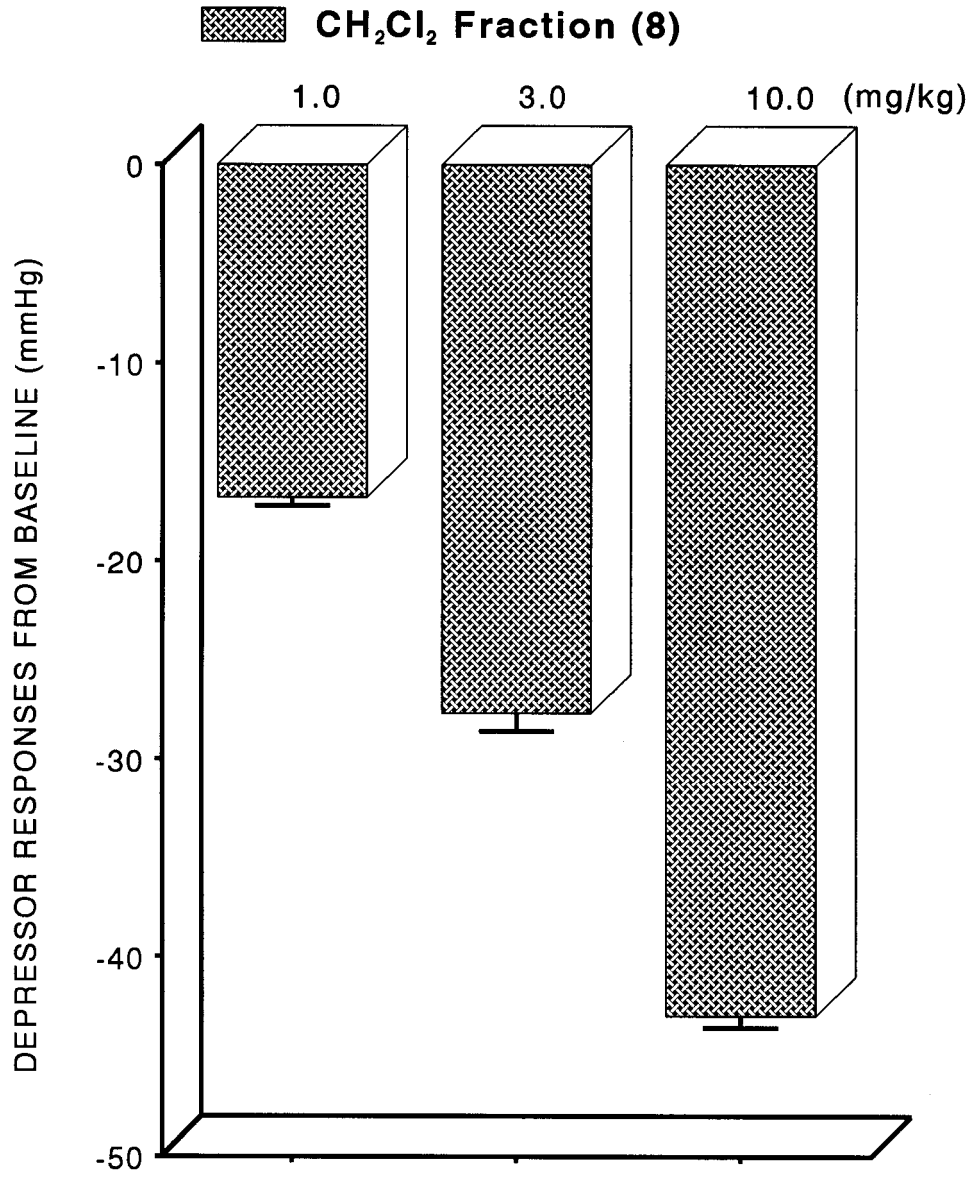

Fig. 32. Dose-dependent hypotensive effects of CH_2Cl_2 fraction in the anesthetized SHRs. CH_2Cl_2 fraction (1.0, 3.0 and 10.0 mg/kg, respectively) was administered into a femoral vein. Arterial blood pressure from pre-injection level was expressed in mmHg.

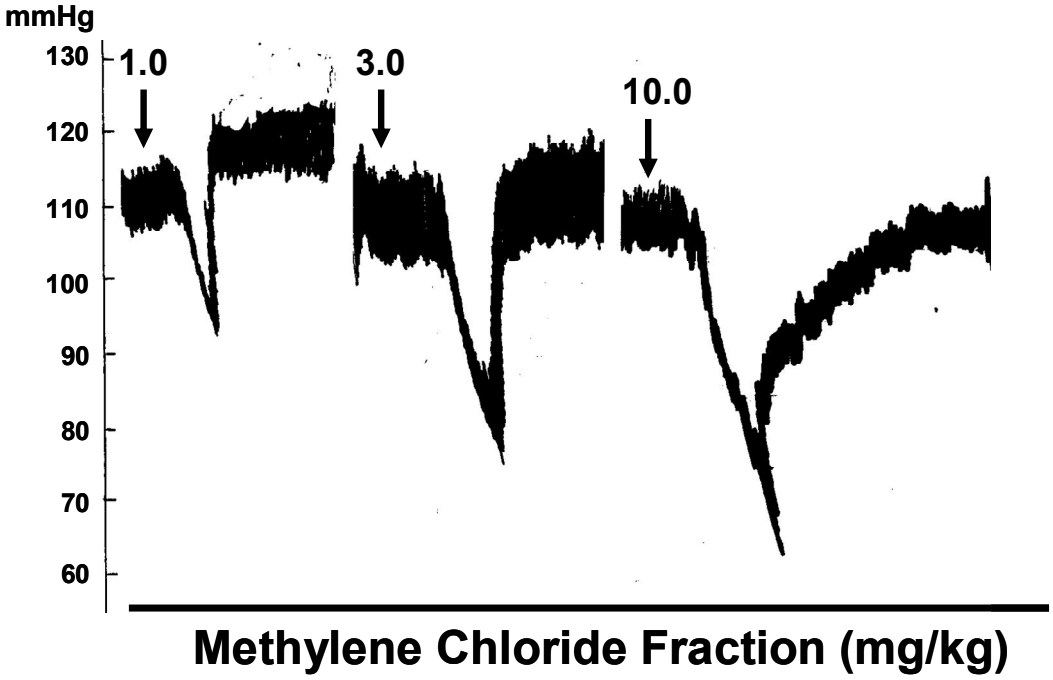

Fig. 33. The typical tracings of CH_2Cl_2 fraction-induced hypotensive action in an anesthetized SHR. CH_2Cl_2 fraction at the indicated doses (0.3, 1.0 and 3.0 mg/kg) was injected intravenously at the arrow marks.

Influence of phentolamine, chlorisondamine, L-NAME and sodium nitroprusside on CH_2Cl_2 fraction-induced depressor action

In 8 rats, in order to examine the relationship between adrenergic α-receptors and CH_2Cl_2 fraction-induced depressor action, phentolamine (1.0 mg/kg) was given intravenously after obtaining the control responses of intravenous CH_2Cl_2 fraction. In the presence of phentolamine effect, depressor response induced by intravenous CH_2Cl_2 fraction (1.0 mg/kg) were greatly depressed to 5.8±1.7 mmHg ($P< 0.01$) from the pre-injection level of the baseline as compared with the control depressor response (19.1±2.0 mmHg) as shown in Fig. 34 and 35. Chlorisandamine (1.0 mg/kg), an autonomic ganglionic blocking agent was given slowly into a femoral vein. Following the administration of chlorisondamine, the baseline of blood pressure was reduced from 119.5±7.2 mmHg to 70.5±4.6 mmHg. In 10 rats, intravenous CH_2Cl_2 fraction (1.0 mg/kg)-induced depressor response after chlorisondamine-treatment was markedly inhibited by 0.4±0.02 mmHg ($P<0.01$) as compared with the control depressor response (19.0±3.3 mmHg), as shown in Fig. 34 and 35. Intravenous infusion of L-NAME (3 mg/kg/30min), an inhibitor of NO synthase, into a femoral vein resulted in a significant decrease in the blood pressure by 4.7±0.8 mmHg ($P<0.01$, $n=20$) as compared with the control depressor response (15.4±1.5 mmHg), as shown in Fig. 34 and 36. In 8 rats, in order to examine the relationship between NO and CH_2Cl_2 fraction-induced depressor action, sodium nitroprusside (30μg/kg/30min) was infused intravenously after obtaining the control responses of intravenous CH_2Cl_2 fraction. In the presence of sodium nitroprusside effect, depressor response induced by intravenous CH_2Cl_2 fraction (1.0 mg/kg) were greatly

depressed to 9.0±3.5 mmHg (P< 0.01) as compared with the control depressor response (22.3±4.3 mmHg) as shown in Fig. 34 and 36.

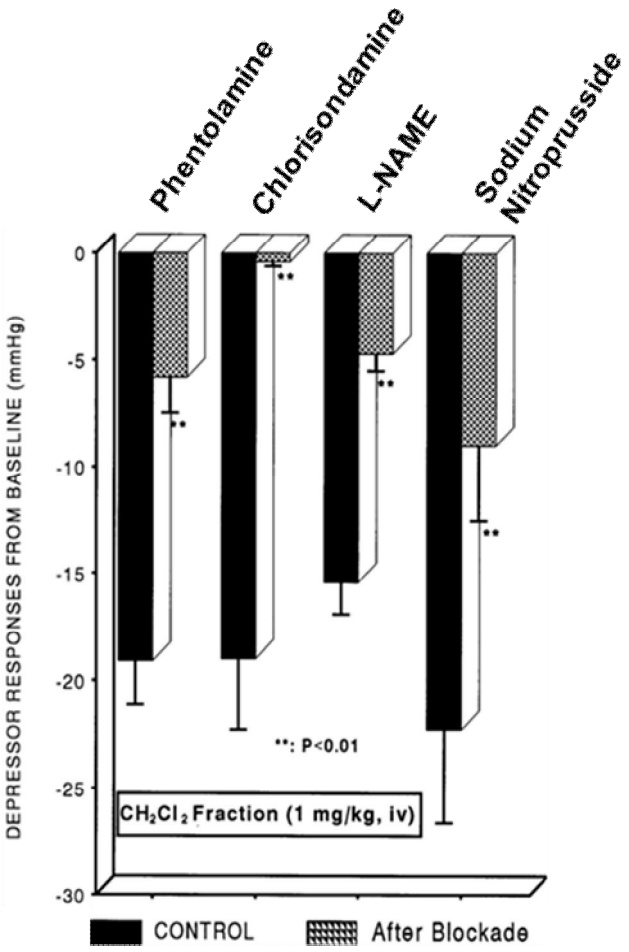

Fig. 34. Effects of phentolamine, chlorisondamine, L-NAME and nitroprusside on CH_2Cl_2 fraction–induced hypotensive action in the anesthetized rats. Phentolamine (1 mg/kg), chlorisondamine (1 mg/kg), L-NAME (3 mg/kg/30min) and sodium nitroprusside (30 μg/kg/30min) were given intravenously, respectively, after obtaining CH_2Cl_2 fraction-induced hypotensive action. Statistical difference was analyzed by comparing control response with that after treatment with each blockade. **: P< 0.01.

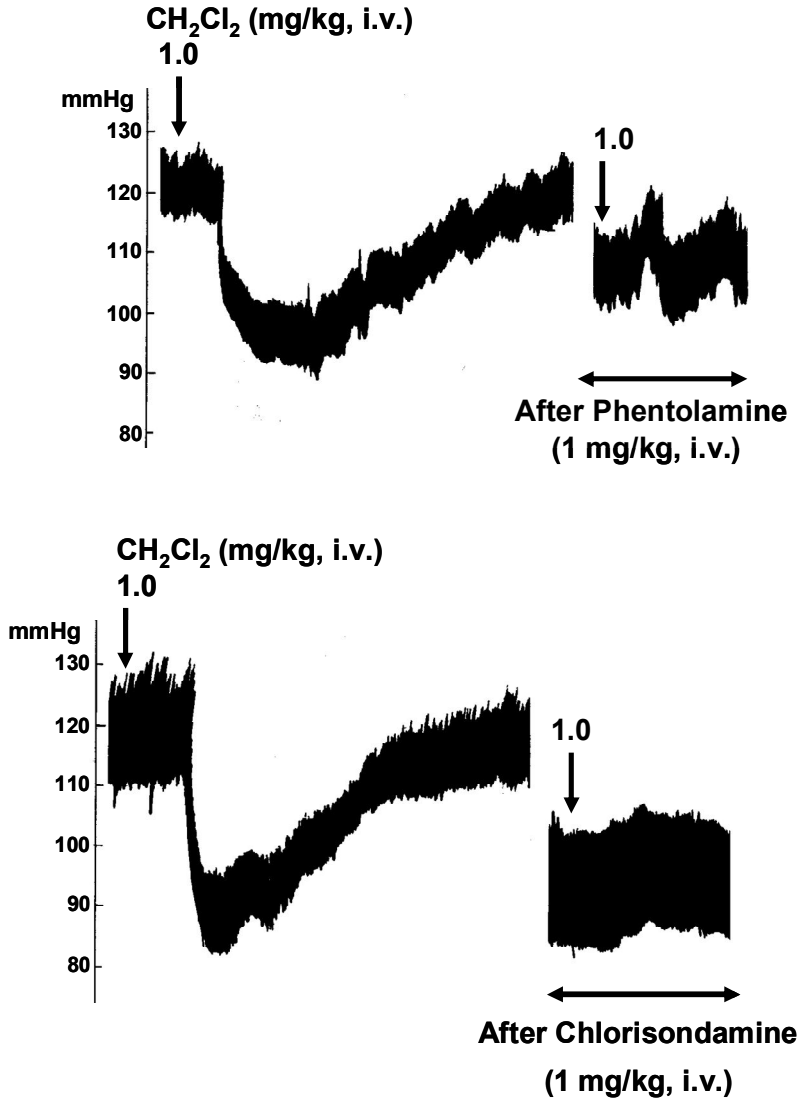

Fig. 35. A typical tracing showing the effects of phentolamine (left) and chlorisondamine (right) on hypotensive action of CH_2Cl_2 fraction in the anesthetized rat Phentolamine (1 mg/kg) and chlorisondamine (1 mg/kg) were given intravenously after obtaining control hypotensive responses of CH_2Cl_2 fraction, respectively.

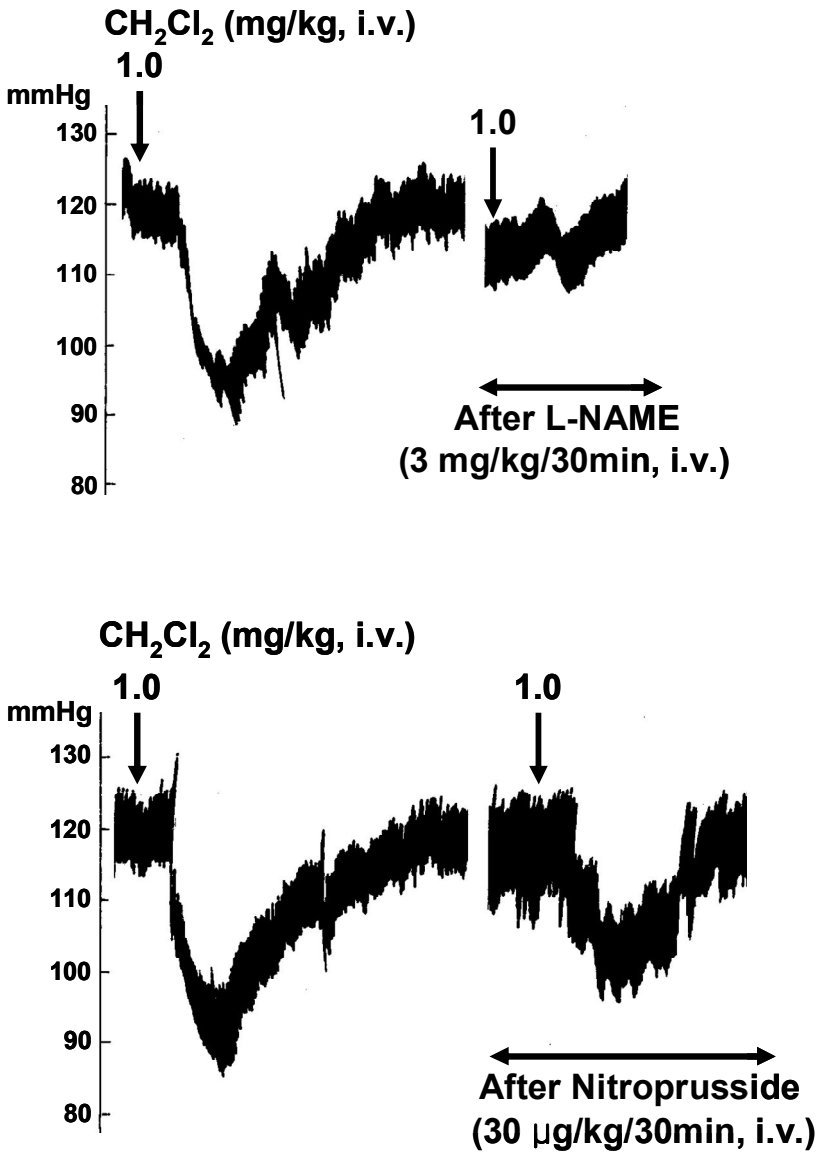

Fig. 36. A typical tracing showing the effects of L-NAME (left) and nitroprusside (right) on hypotensive action of CH_2Cl_2 fraction in the anesthetized rat. L-NAME (3 mg/kg/30min) and nitroprusside (30 μg/kg/30min) were given intravenously after obtaining control hypotensive responses of CH_2Cl_2 fraction, respectively.

Influence of intravenous CH_2Cl_2 fraction on norepinephrine (NE)-evoked pressor responses in the anesthetized rats

As shown in Fig. 21~36, CH_2Cl_2 fraction greatly inhibited phenylephrine-induced contractile response of the aortic strip of normotensive rats and SHRs, and also CH_2Cl_2 fraction-induced depressor responses were significantly reduced by phentolamine and chlorisondamine, it suggests that CH_2Cl_2 fraction might cause hypotension through the blockade of peripheral adrenergic α-receptors. It is also of interest to examine the effect of CH_2Cl_2 fraction on norepinephrine-evoked pressor responses. When cardiovascular parameters were stabilized for 30 min before the experimental protocols were initiated, the administration of physiological saline solution in a volume of 0.2 ml into a femoral vein did not cause any changes in arterial blood pressure. Then, it was tried to test the effect of CH_2Cl_2 fraction on norepenephrine-induced hypertensive responses in the anesthetized rats.

In 12 rats, as shown in Fig. 37 and 38, norepinephrine at doses of 0,3, 1.0 and 3.0 μg/kg, i.v. caused dose-dependent pressor responses of 9.6±1.4 mmHg, 20.0±2.3 mmHg and 35.7±3.2 mmHg from the original baseline (119.5±4.6 mmHg), respectively. After infusion of CH_2Cl_2 fraction with a rate of 1.0, 3.0 and 10.0 mg/kg/30min, hypertensive responses of norepinephrine at doses of 0,3, 1.0 and 3.0 μg/kg were inhibited maximally to 48±6% ($P< 0.01$), 57±3% ($P< 0.01$) and 60±5% ($P< 0.01$) of control responses at the above same doses, respectively.

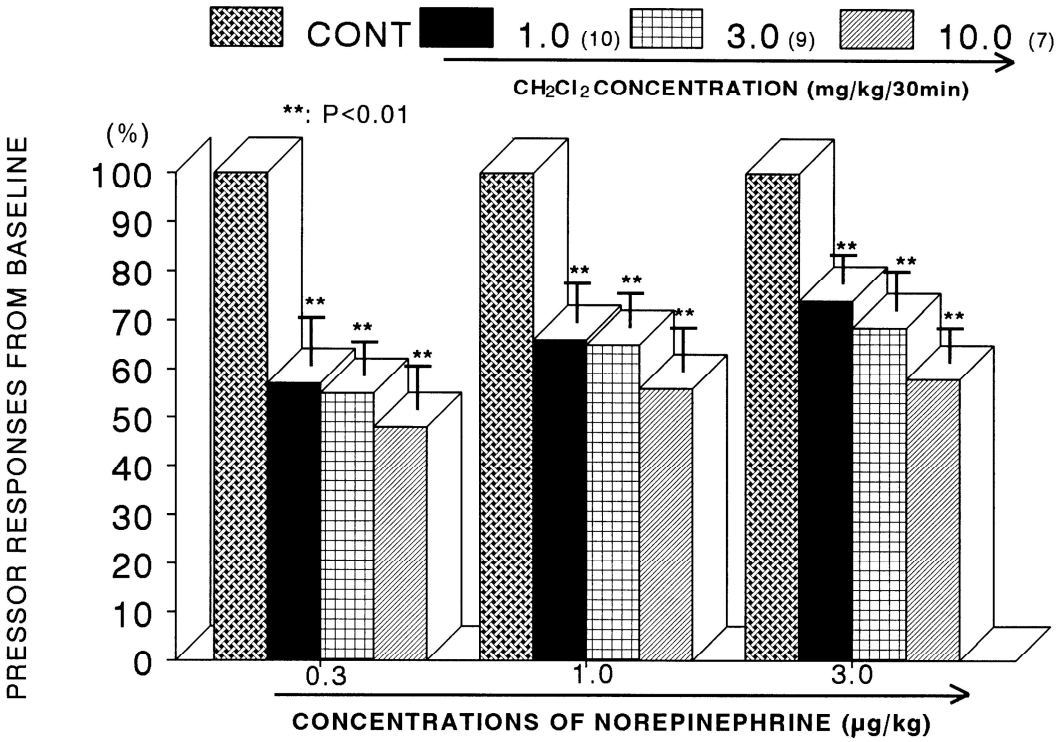

Fig. 37. Influence of intravenous CH_2Cl_2 fraction on norepinephrine (NE)-evoked pressor responses in anesthetized rats. CH_2Cl_2 fraction (1.0, 3.0 and 10.0 mg/kg/30 min, respectively) was given intravenously after obtaining the corresponding control responses of intravenous norepinephrine (0.3, 1.0 and 3.0 μg/kg, respectively). **: $P < 0.01$.

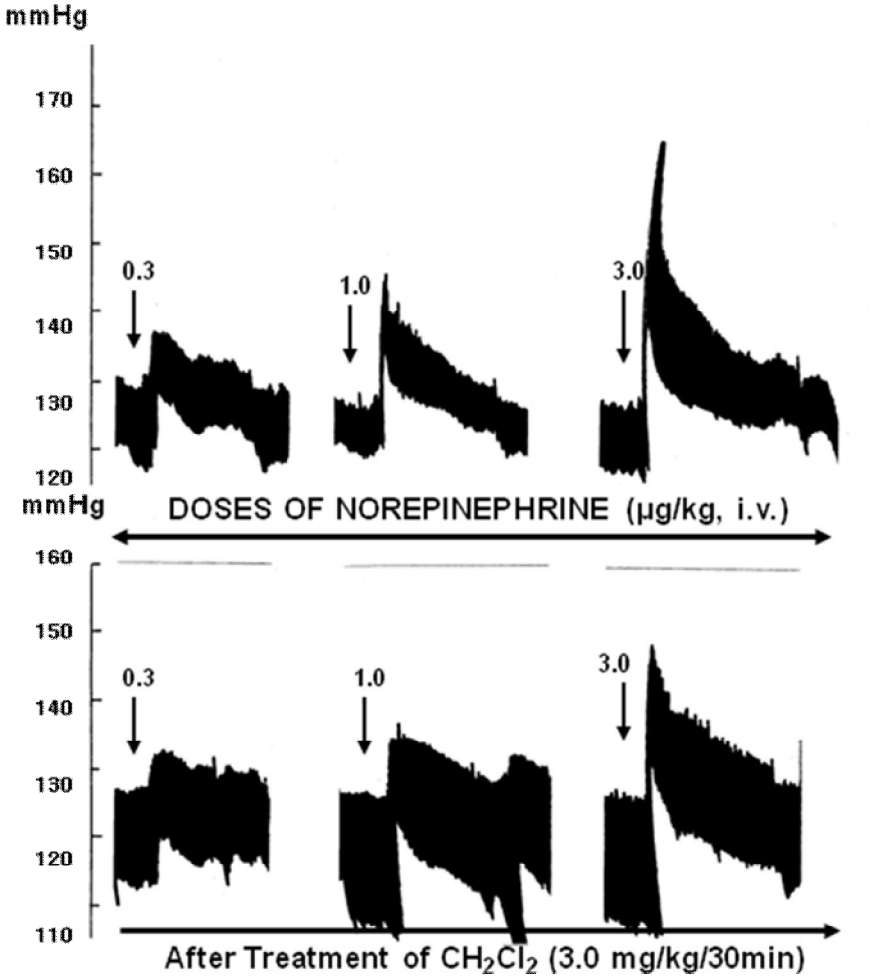

Fig. 38. The representative tracing of effect of CH_2Cl_2 fraction on intravenous norepinephrine (NE)-induced pressor responses in the anesthetized rat. At arrow marks, the indicated doses (0.3, 1.0 and 3.0 μg/kg) of NE were administered into a femoral vein. Upper: NE-induced hypertensive responses in a non-treated rat. Lower: NE-induced hypertensive responses in a CH_2Cl_2 fraction-pretreated rat. CH_2Cl_2 fraction was infused into a femoral vein with a rate of 3 mg/kg/30 min. Arterial blood pressure from pre-injection level was expressed in mmHg. The chart speed was 10 mm/min.

IV. DISCUSSION

Influence of CH_2Cl_2 fraction on adrenal CA secretion

The present experimental results have demonstrated that CH_2Cl_2 fraction extracted from *Rubus coreanum* inhibits the CA secretory responses evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused rat adrenal glands. It seems that this inhibitory effect of CH_2Cl_2 fraction is exerted by inhibiting both the Ca^{2+} and Na^+ influx into the rat adrenal medullary chromaffin cells as well as the release of Ca^{2+} from the cytoplasmic calcium store partly through the activation of NO production.

In the present study, in the simultaneous presence of CH_2Cl_2 fraction and L-NAME (NO synthase inhibitor), the CA secretory responses evoked by ACh, DMPP, high K^+ and Bay-K-8644 were considerably recovered to the extent of the corresponding control secretion compared to those of CH_2Cl_2 fraction treatment alone. This result is well consistent with the report that polyphenolic compounds isolated from red wine produced the endothelium-NO-dependent relaxation through an extracellular Ca^{2+}-dependent mechanism (Andriambeloson et al., 1999). Amongst the different classes of polyphenolic compounds present in PCRW, anthocyanins and oligomeric condensed tannins had the same pharmacological profile as PCRW (Andriambeloson et al., 1998). Of different anthocyanins identified in wine, only delphinidin caused endothelium-dependent relaxation, although it was slightly less potent than PCRW (Andriambeloson et al., 1998). Moreover, in this study, following treatment of CH_2Cl_2 fraction into

perfused rat adrenal medulla, NO production was greatly elevated as shown in Fig. 19. Taking into account these findings, in the present study, it is likely that CH_2Cl_2 fraction inhibits the CA secretory response evoked by various secretagogues through increasing NO production in adrenal chromaffin cells since CH_2Cl_2 fraction -induced inhibitory responses of CA secretion were significantly reduced in the presence of L-NAME, an inhibitor of NO synthase, and CH_2Cl_2 fraction practically enhanced NO release from adrenal medulla of SHRs.

It has also been shown that (-) epicatechin, one of polyphenolic components of green tea, 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). It was 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).

Some epidemiological studies indicate an association between moderate

consumption of red wine and reduced risk of coronary heart disease (Renaud and de Lorgeril, 1992; German and Walzem, 2000). It has been shown that PCRW promote the endothelium-dependent relaxation, activate NO synthase, inhibit platelet aggregation, and prevent oxidation of LDL-cholesterol (Fitzpatrick, et al, 1993; Andriambeloson, et al., 1997; Flesh, et al., 1998; Leikert, et al., 2002; Demrow and Slane, 1995; Frankel, wt al., 1993). The polyphenolic compound resveratrol presented in red wine is thought to be responsible factor for its beneficial cardiovascular effects. Since resveratrol has similar effects to RWPC such as promotion of vasodilation, activation of nitric oxide synthase, inhibition of platelet aggregation and leukocyte activation, prevention of oxidation of LDL-cholesterol and reduction of cholesterol synthesis (Chen and Pace-Asciak, 1996; Wallerath, et al., 2002; Pace-Asciak, et al., 1995; Rotondo, et al., 1998; Frankel, et al., 1993).

Furthermore, these effects of resveratrol and PCRW are agreement with the present result that CH_2Cl_2 fraction can inhibit the CA secretory responses evoked by cholinergic stimulation and membrane depolarization at least partly by activation of nitric oxide synthase in the isolated perfused adrenal medulla of SHRs, since this inhibitory effect of CH_2Cl_2 fraction on the CA secretory responses was significantly attenuated in the presence of L-NAME, an inhibitor of nitric oxide synthase.

In support of this idea, generally, NO is produced enzymatically from the terminal guanidino nitrogen of L-arginine by the action of NO synthase (NOS) (Palmer, et al., 1988; Sakuma, et al., 1988). There are at least three isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The adrenal medulla possesses characteristic postganglionic sympathetic neurons,

and the presence of nNOS has been demonstrated (Marley, et al., 1995; Oset-Gasque, et al., 1994; Palacios, et al., 1989; Schwarz, et al., 1998). In vitro studies using NOS inhibitors and NO donors were performed to examine the role of NO in modulating CA secretion from the adrenal medulla but the results remain controversial. In the present work, in presence of L-NAME, the inhibitory responses of CH_2Cl_2 fraction on the CA secretion were recovered to the considerable extent of the control secretion compared with the inhibitory effects of CH_2Cl_2 fraction -treatment alone. This result demonstrates that CH_2Cl_2 fraction can inhibit the CA release at least partly through the activation of nNOS in the rat adrenal medulla. In the support of this finding, it has been reported that the NOS inhibitor, L-NAME enhances K^+-stimulated CA secretion in cultured bovine chromaffin cells (Torres, et al., 1994) and that sodium nitroprusside (SNP) inhibits ACh-induced CA secretion in bovine chromaffin cells (Rodriguez-Pascual, et al., 1996). These studies suggest that NO may play an inhibitory role in the control of the CA secretion. Moreover, the presence of endothelial cells has been reported to inhibit 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 contrast, it has been reported that L-NAME inhibits acetylcholine (ACh)-induced CA secretion in bovine chromaffin cells (Uchiyama, et al., 1994) and that the NO donor sodium nitroprusside (SNP) enhances nicotine-induced CA secretion in cultured bovine chromaffin cells (O'Sullivan and Burgoyne, 1990). These findings suggest that NO may facilitate 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;

Breslow, et al., 1993). Based on these reports, the present studies suggest that CH_2Cl_2 fraction possesses the ability partly to activate nNOS in the rat adrenomedullary chromaffin cells, in addition to the direct inhibitory effects on the CA secretion.

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 activates 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, and then activates nicotinic the CA secretion. Based on this fact, the present findings that CH_2Cl_2 fraction inhibited the CA secretory responses evoked by nicotinic receptor stimulation as well as by membrane depolarization in the rat adrenal medulla seem to be able to support the fact that, in *in vivo* studies, PCRW lowers blood pressure in normotensive and hypertensive rats (Mizutani et al., 1999; Diebolt et al., 2001). It has been reported that red wines and grapes exhibit endothelium-dependent relaxation of blood vessels via enhanced generation and/or increased biological activity of NO, leading to the elevation of cGMP levels (Fitzpatrick et al., 1993; Fitzpatrick et al., 1995; Fitzpatrick et al., 2000; Zenebe et al., 2003).

These experimental results indicate that CH_2Cl_2 fraction-induced inhibitory activity of the 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 the CA, and induces dopamine β-hydroxylase by calcium-dependent secretory process (Dixon et al, 1975; Viveros et al, 1968). In terms of this fact, the present results suggest that CH_2Cl_2 fraction 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 occurring 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 and Hwang, 1991). In support of this idea, it has been found that green tea extract inhibits the CA secretory responses evoked by cholinergic stimulation and membrane depolarization in the adrenal medulla isolated from the rat (Lim et al., 2003) and the rabbit (Lim, 2005). In this study, CH_2Cl_2 fraction inhibited the secretory responses of CAs evoked by ACh, DMPP, McN-A-343 and high K^+. It suggests that CH_2Cl_2 fraction can produce the similar effect in adrenal medulla of the normotensive rats with that of green tea extract 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., 1995) 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 systemic blood pressure (Stensvold et al., 1992). In terms of these findings, the results of the present study seem likely that CH_2Cl_2 fraction 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., 2002) 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 present study, CH_2Cl_2 fraction also time-dependently depressed the 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 CH_2Cl_2 fraction 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 CA secretion (Fisher et al., 1981; Yanagihara et al, 1979). It has also been known that the activation of nicotinic receptors stimulates the 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 K^+-induced CA secretory response was depressed by pretreatment with CH_2Cl_2 fraction indicates that this inhibitory effect of CH_2Cl_2 fraction is exerted through the direct inhibition of calcium influx into the adrenal chromaffin cells. Furthermore, slight elevation in the extracellular potassium concentration increases both the frequency of spontaneous action potentials and the CA secretion (Kidokoro & Ritchie, 1980), suggesting that the influx of calcium that occurs during action potentials is directly linked to the rate of secretion. These findings that CH_2Cl_2 fraction inhibited the CA secretion evoked by Bay-K-8644 as well as by high K^+ suggest that CH_2Cl_2 fraction inhibits directly the voltage-dependent Ca^{2+} channels. 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 voltage-dependent Ca^{2+} channels (Burgoyne, 1984; Oka et al., 1979). Therefore, it seems that these inhibitory effects of CH_2Cl_2 fraction on the CA secretion evoked by DMPP and veratridine may be mediated by inhibiting Ca^{2+} influx through voltage-dependent Ca^{2+} channels due to activation of nicotinic receptor-associated ionic channels, responsible for carbachol-induced Na^+ influx, as well as of voltage-dependent Na^+ channels, responsible for veratridine-induced Na^+ influx, respectively.

The present study has also shown that CH_2Cl_2 fraction inhibits the CA secretion evoked by cyclopiazonic acid. Cyclopiazonic acid is known to be a

highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Goeger & 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). Therefore, it is felt that the inhibitory effect of CH_2Cl_2 fraction on the CA secretion evoked by cholinergic stimulation as well as by membrane-depolarization may be associated with the mobilization of intracellular Ca^{2+} from the cytoplasmic calcium store. This indicates that the CH_2Cl_2 fraction has an inhibitory effect on the release of Ca^{2+} from the intracellular pools induced by stimulation of muscarinic ACh receptors, which is weakly responsible for the CA secretion. It has been shown that Ca^{2+}-uptake into intracellular storage sites susceptible to caffeine (Ilno, 1989) is almost completely abolished by treatment with cyclopiazonic acid during the proceeding of Ca^{2+} load (Suzuki et al., 1992). 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 cylopiazonic acid (Uyama et al., 1992). Suzuki and his coworkers (1992) have shown 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. Moreover, in bovine adrenal chromaffin cells, stimulation of muscarinic ACh receptors is also proposed to cause activation of phosphoinositide 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; Challis et al., 1991). The present results suggest that CH_2Cl_2 fraction-induced depression of the CA secretion evoked by McN-A-343

and cyclopiazonic acid may be due to the inhibition of Ca^{2+} release from the intracellular pools induced by stimulation of muscarinic ACh receptors. However, in the present study, it is uncertain whether the inhibitory effect of CH_2Cl_2 fraction on Ca^{2+} movement from intracellular pools is due to its direct effect on the PI response or the indirect effects.

In conclusion, as shown in Fig. 39, the results of the present study have suggest that CH_2Cl_2 fraction inhibits the CA secretion by stimulation of cholinergic nicotinic receptors as well as by membrane depolarization in the isolated perfused rat adrenal glands. It seems that this inhibitory effect of CH_2Cl_2 fraction is exerted by blocking influx of sodium and calcium into the rat adrenal medullary chromaffin cells as well as the release of Ca^{2+} from the cytoplasmic calcium store at least partly via the increased NO production due to the activation of nitric oxide synthase. Based on these experimental results, the ingestion of CH_2Cl_2 fraction may be helpful to prevent or alleviate the cardiovascular diseases, through inhibition of CA secretion from adrenomedullary chromaffin cells and consequent reduction of the CA level in the circulation.

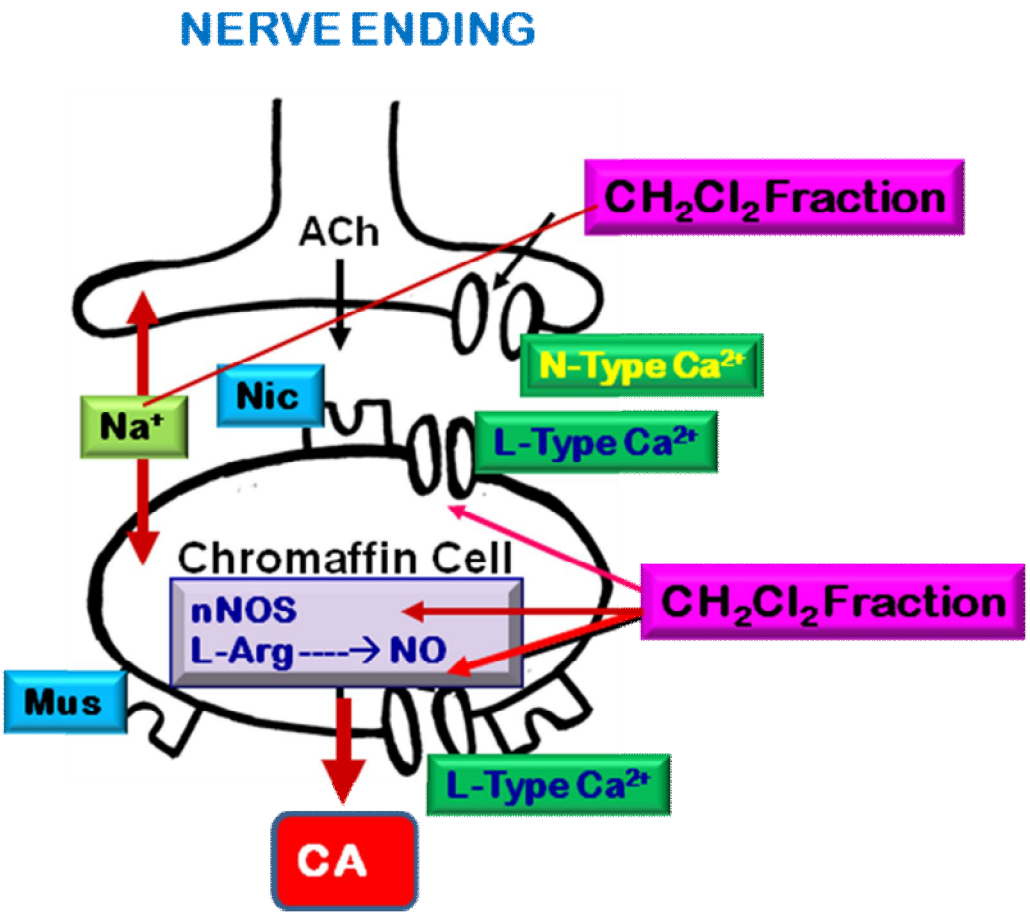

Fig. 39. Probable site of action of CH_2Cl_2 fraction at cholinergic nerve-chromaffin cell in the rat adrenal medulla.

Influence of CH_2Cl_2 fraction on thoracic aortic contractility and blood pressure

The present experimental results demonstrate that CH_2Cl_2 fraction causes vasorelaxation in the isolated aortic strips of SHRs as well as normotensive rats at least partly by the increased NO production through the activation of NO synthase of vascular endothelium, but not through the activation of cyclooxygenase. In support of this idea, recently, it has been demonstrated that CH_2Cl_2 fraction inhibits the CA secretory responses evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused adrenal gland of the normotensive rats (Kee and Lim, 2007) and spontaneously hypertensive rats (Lim and Hong, 2007). It seems that this inhibitory effect of CH_2Cl_2 fraction is exerted by inhibiting both the Ca^{2+} influx into the rat adrenal medullary chromaffin cells and the uptake of Ca^{2+} into the cytoplasmic calcium store partly through the increased NO production due to the activation of nitric oxide synthase (Kee and Lim, 2007; Lim and Hong, 2007*).* In the present study, CH_2Cl_2 fraction elicited a concentration-dependent inhibition in phenylehrine-induced contractile responses of rat aortic rings with functional endothelium. This effect was greatly abolished in the absence of functional endothelium by treatment with CHAPS, which is a detergent for removal of endothelium, indicating that the vasodilator effect of CH_2Cl_2 fraction is dependent on endothelium-derived relaxing factors. To evaluate the participation of NO in the vasorelaxant activity of CH_2Cl_2 fraction, aortic rings were treated with L-NAME, a classical NO synthase inhibitor. In the present experimental condition, the CH_2Cl_2 fraction-induced vasodilatation was

markedly blocked, as similarly observed in endothelium-denuded aortic rings by CHAPS, suggesting that NO is the main endothelium-derived relaxing factor involved in CH_2Cl_2 fraction activity. The present results are fully in accordance with previous those findings obtained from red wines and grapes. Previously, it has been reported that red wines and grapes exhibit endothelium-dependent relaxation of blood vessels via enhanced generation and/or increased biological activity of NO, leading to the elevation of cGMP levels (Fitzpatrick et al., 1993; Fitzpatrick et al., 1995; Fitzpatrick et al., 2000; Zenebe et al., 2003). *In vivo* the polyphenol compounds of red wine (PCRW) were shown to reduce blood pressure in normotensive and hypertensive rats (Mizutani et al., 1999; Diebolt et al., 2001; Bernátová et al., 2002). The administration of purple grape juice improved the endothelium dependent, flow-mediated vasodilation in coronary artery disease patients with impaired endothelial function (Stein et al., 1999). The amplitude of vasorelaxation changed depending on the variability of wine constituents according to grape varieties, area of cultivation, and vinification methods. Consequently, the vasodilatatory effect does not apply to all wines and the degree of vasorelaxation is correlated to the content and type of phenols. Endothelium-dependent relaxation was greatest for red wines produced "en barrique", a procedure leading to high concentration of phenolic compounds (Flesch et al., 1998). A correlation between the phenolic content with vasodilatatory effect was later confirmed by Burns and his colleagues (2000). While the antioxidant activity was associated with different classes of phenols (gallic acid, resveratrol and catechins), vasodilatation activity was correlated only with the total content of anthocyanosides (ACs) (Burns et al., 2000). Investigations devoted to characterize PCRW responsible for the

endothelium-dependent relaxation activity (Fitzpatrick et al., 2000; Andriambeloson et al., 1997; Freslon et al., 1997; Andriambeloson et al., 1998; Fitzpatrick et al., 2002) agree that monomeric catechins and simple phenols (benzoic acid, gallic acid and hydroxycinnamic acids) are devoid of effect. On the contrary, AC enriched fractions and oligomeric proanthocyanidines (PAs: dimers, trimers and tetramers) were the active compounds. Threshold for relaxation by PAs oligomers was between 0.5 and 4 µg/ml (Fitzpatrick et al., 2000; Fitzpatrick et al., 2002). Much higher concentration (>0.1 mg/l) were required for ACs (Andriambeloson et al., 1998). The endothelium-dependent relaxation activity was lost when higher molecular weight polymers were assayed (Andriambeloson et al., 1998). PCRW enhanced NO synthesis and cGMP accumulation only in the presence of functional endothelium. In denuded aortic rings, PCRW concentration 103-fold higher was necessary to induce relaxation (Ndiaye et al., 2003; Corder et al., 2001). Besides NO, red wine affected the formation of other mediators of vascular tone, such as endothelium-derived hyperpolarizing factor (Ndiaye et al., 2003) and prostacyclin (Derek et al., 1997). The mechanisms underlining NO-dependent vasorelaxation caused by PCRW were investigated (Zenebe et al., 2003; Andriambeloson et al., 1999; Martin et al., 2002). In addition to the increased NO synthase activity, PCRW may prolong the half-life and increase the bioavailability of NO, by reducing its degradation mediated by reactive oxygen species (de aetano and Cerletti, 2001). It has also been that Provinol elicited endothelium-dependent relaxation of rat femoral artery by the Ca^{2+}-induced increase of NO synthase activity and by protecting NO from degradation (Zenebe et al., 2003). Recently, Yu and his colleagues (2008) have found that PCRW inhibits the CA secretory responses evoked by stimulation of

cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused adrenal gland of the normotensive rats. It seems that this inhibitory effect of PCRW is mediated by blocking the influx of both ions through Na^+ and Ca^{2+} channels into the rat adrenomedullary chromaffin cells as well as by inhibiting the release of Ca^{2+} from the cytoplasmic calcium store, which are due at least partly to the increased NO production through the activation of nitric oxide synthase.

Consumption of wine polyphenol-, quercetin- or catechin-enriched diets increased aortic NO production in rats (Benito et al., 2002). Oral administration of an alcohol-free hydroalcoholic grape skin extract (from vinifera grape, *Vitis labrusca*) significantly reduced systolic, mean and diastolic arterial pressure in two distinct models of hypertensive Wistar rats (Soares De Moura et al., 2002). Intragastric administration of resveratrol (3 mg/kg/day), red wine (4 ml/kg/day) or even dealcoholized red wine (4 ml/kg/day) for 12 weeks to hypercholesterolemic rabbits improved the endothelial function, reduced plasma endothelin-1 levels and induced a significant elevation in NO levels (Zou et al., 2003). Moreover, in human studies, in healthy volunteers, the coronary flow-velocity reserve was increased 30 min after drinking red wine (1 g/kg ethanol), but not after drinking the same quantity of alcohol in white wine or vodka (Shimada et al., 1999). The endothelium-dependent vasodilation was also improved after acute intake of 500 ml of red wine or red wine without alcohol in men, as determined by ultrasonography of the brachial artery (Hashimoto et al., 2001). Endothelium-derived NO plays an important role in the control of vascular homeostasis. NO modulates the vascular tone, the growth of vascular smooth muscle cells, and decreases platelet adhesion and aggregation. It also decreases

the adherence of other blood components (Moncada et al., 1991; Scott-Burden and Vanhoutte, 1994). A decrease in NO production or bioavailability is closely associated with endothelial dysfunction or injury, which is an important factor in pathologies such as atherosclerosis, restenosis and hypertension (Landmesser and Drexler, 2007). PCRW and a grape skin extract also reduced blood pressure in males in several models of experimental hypertension (Bernatova et al., 2002; Pechanova et al., 2004; Sarr et al., 2006; Soares de Moura et al., 2002; Jiménez et al., 2007), which was related to a combination of vasodilator and antioxidant actions. Pechanova and his colleagues (2004) also provided evidence that Provinols partially prevents L-NAME-induced hypertension, cardiovascular remodeling and vascular dysfunction via the increase of NO-synthase activity and prevention of oxidative stress. In the present study, intravenous CH_2Cl_2 fraction-induced hypotensive response was significantly inhibited by pretreatment with L-NAME or sodium nitroprusside. In light of these results, it seems that CH_2Cl_2 fraction may produce hypotensive action at least through the increased NO production by eNOS activation. Thus, in view of the beneficial effects of plant polyphenols, the present results of CH_2Cl_2 fraction should shed light on the fact that the unique components of CH_2Cl_2 fraction may contribte to the treatment or prevention of hypertension through their complex influence on the NO balance in the cardiovascular system.

Generally, it is well known that potassium chloride (KCl) opens voltage-dependent calcium channels by depolarizing the cell membrane of vascular smooth muscle, resulting in increased influx of extracellualr Ca^{2+} (Bolton, 1979; Schwartz & Taira, 1983; Dube et al., 1985; 1988). Kim and his colleagues

(1989) have shown that the contractile responses of vascular smooth muscle induced by $CaCl_2$ and KCl may result most likely from the increased influx of extracellular Ca^{2+} through the voltage-dependent calcium channels (VDCCs). VDCCs are activated by depolarization of the plasma membrane when the extracellular K^+ concentration is increased. In the present work, incubation with CH_2Cl_2 fraction inhibited KCl concentration-dependent contractile response in rat aortic strips. This result is consistent with the effect of 17-β estradiol on a large elastic aorta as in previous report (Li et al., 2002; 2006) and is also supported by another study (Nevala et al., 1998). These findings suggest that CH_2Cl_2 fraction may have Ca^{2+} antagonistic properties and can inhibit extracellular Ca^{2+} influx through VDCCs, which are similar to those of 17-β estradiol or resveratrol. Generally, the mechanism of potassium-induced vasoconstriction has been shown to be through the calcium-influx by the opening of the voltage-dependent calcium channels (Spedding and Paoletti, 1992; Ryman et al., 1989). Voltage-dependent calcium channel blockers such as nifedipine or verapamil have been reported to attenuate potassium-induced vasoconstriction (Cortijo et al., 1986; Triggle et al., 1989). The contractile activity of vascular smooth muscle cells is mainly regulated by control over the cytoplasmic calcium concentration and both intracellular and extracellular calcium pools (Triggle et al., 1989; Johns et al., 1987). Based on these findings, the present results that CH_2Cl_2 fraction inhibited high K^+-evoked contractile responses, and that the inhibitory effect of CH_2Cl_2 fraction on high K^+-evoked contractile responses was enhanced, although their data are not shown here, indicate that CH_2Cl_2 fraction may block the VDCCs in aortic smooth muscle cells.

In the present study, the findings that CH_2Cl_2 fraction-induced hypotension is suppressed by the pretreatment with an autonomic ganglionic blocker (chlorisondamine), and adrenergic α-blocker (phentolamine) suggest strongly that the CH_2Cl_2 fraction-induced hypotension may be mediated through the inhibition of sympathetic tone. The action site of CH_2Cl_2 fraction seems to be the sympathetic ganglia or more higher level because its hypotensive response is inhibited by prior treatment of chlorisondamine. Furthermore, in terms of the fact that intravenous CH_2Cl_2 fraction-evoked hypotension is significantly attenuated by adrenergic α-receptor blockade (phentolamine), and that CH_2Cl_2 fraction inhibits greatly the pressor responses of norepinephrine, it is considered that CH_2Cl_2 fraction causes the hypotensive action via the blockade of adrenergic α_1-receptors. Among drugs which interfere with peripheral sympathetic function, adrenergic α-receptor blocking agents alone cause reversal of the epinephrine pressor response (Constantine et al., 1973). When epinephrine is administered to untreated animals, its α-agonist properties predominate, resulting in a rise in mean arterial pressure. However, in the presence of adrenergic α-receptor blockade, the peripheral β_2-agonist properties of epinephrine predominate and a fall in arterial pressure or reversal of the pressor response is observed. In contrast, the pressor responses to norepinephrine are impaired by adrenergic α-receptor blockade, but are not reversed (Freis et al., 1975) as this agent processes little β_2-agonist activity (Ablad et al., 1975). These previous facts support that CH_2Cl_2 fraction-induced depressor action may be due to the blockade of adrenergic α-receptors in the periphery. In the present work, CH_2Cl_2 fraction also inhibited the norepinephrine-induced pressor responses as well as phenylephrine-evoked contractile responses in aortic strips isolated from SHRs and normotensive rats. These results suggest that CH_2Cl_2 fraction may elicit the antagonistic activity of adrenergic α_1-receptors.

Based on all these results, many studies strongly support the view that polyphenol-rich diet, such as *Rubus coreanum* and red wine, could improve endothelial function and that the mechanisms of this beneficial effect found in above discussed *in vitro* studies (especially increased NO) might be involved *in vivo*, both in patients and in animals.

In conclusion, as shown in Fig. 40, the present study provides conclusive data showing for the first time that CH_2Cl_2 fraction elicits the endothelium- and NO-dependent vasorelaxation, which are due to unique polyphenolic constituents of CH_2Cl_2 fraction that may augment eNOS activity and thus facilitates endothelial NO output, and suggesting that CH_2Cl_2 fraction might be helpful in treating or alleviating cardiovascular diseases, such as hypertension and angina pectoris. The identification of the responsible constituents should help in the design of strategies to prevent or to improve cardiovascular diseases.

In addition, in terms of these data, it is expected that intake of sports beverage containing active components extracted from *Rubus coreanum* is helpful to physiologically stabilize cardiovascular system of athletes as well as beneficial to enhance athlete performance.

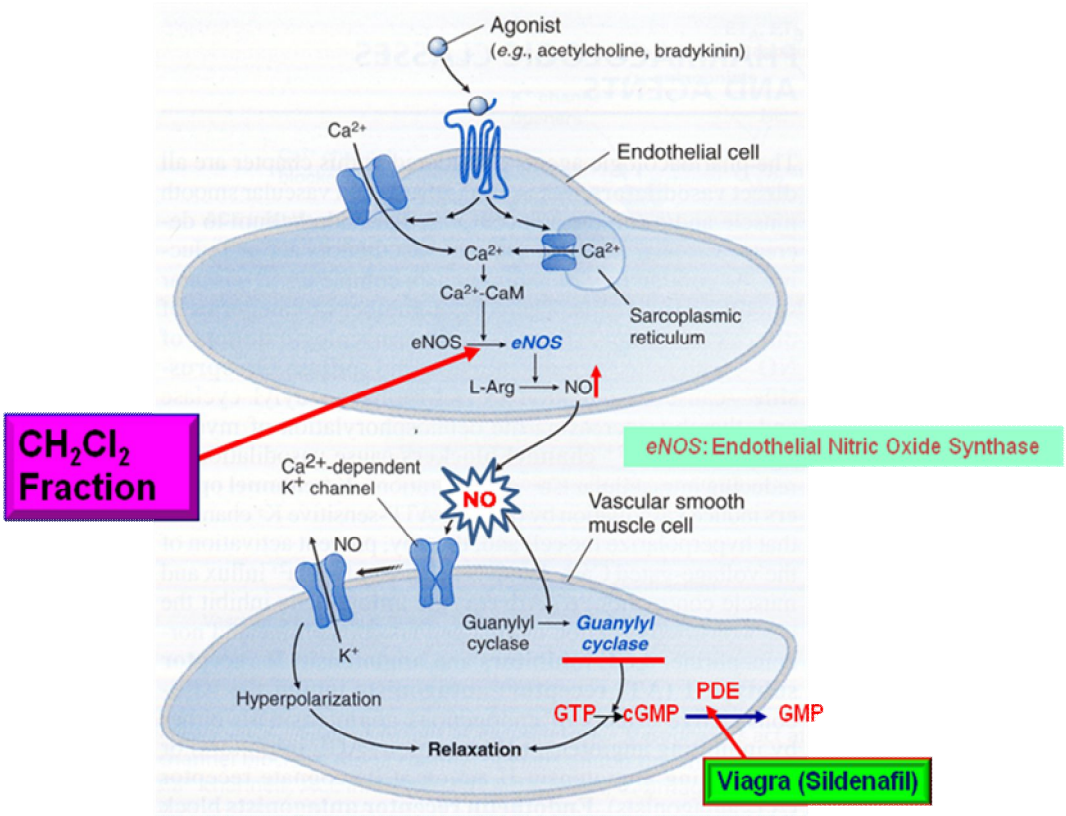

Fig. 40. Schematic diagram of probable action site of CH_2Cl_2 fraction in the isolated rat thoracic aorta.

V. SUMMARY

Effects of CH_2Cl_2 fraction on adrenal CA secretion:

The present study was attempted to investigate whether polyphenolic compounds isolated from wine, which is brewed from *Rubus coreanum* MIQUEL (覆盆子酒), may affect the release of catecholamines (CA) from the isolated perfused rat adrenal medulla, and to establish its mechanism of action.

CH_2Cl_2 fraction (20~180 μg/mL) perfused into an adrenal vein for 90 min relatively dose- and time-dependently inhibited the CA secretory responses evoked by ACh (5.32 mM), high K^+ (a direct membrane-depolarizer, 56 mM), DMPP (a selective neuronal nicotinic N_n receptor agonist, 100 μM) and McN-A-343 (a selective muscarinic M_1 receptor agonist, 100 μM). CH_2Cl_2 fraction itself did not affect basal CA secretion (data not shown). Also, in the presence of CH_2Cl_2 fraction (60 μg/mL), the secretory responses of CA evoked by veratridine (a selective Na^+ channel activator (10 μM), Bay-K-8644 (a L-type dihydropyridine Ca^{2+} channel activator, 10 μM), and cyclopiazonic acid (a cytoplasmic Ca^{2+}-ATPase inhibitor, 10 μM) were significantly reduced, respectively. Interestingly, in the simultaneous presence of CH_2Cl_2 fraction (60 μg/mL) and L-NAME (an inhibitor of NO synthase, 30 μM), the inhibitory responses of CH_2Cl_2 fraction on the CA secretion evoked by ACh, high K^+, DMPP, and Bay-K-8644 were considerably recovered to the extent of the corresponding control secretion compared with the inhibitory effect of CH_2Cl_2 fraction-treatment alone. Practically, the level of NO released from adrenal medulla after the treatment of CH_2Cl_2 fraction (60 μg/mL) was greatly elevated compared to the corresponding basal

released level.

Taken together, these results obtained from the present study demonstrate that CH_2Cl_2 fraction inhibits the CA secretory responses evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused rat adrenal medulla. It seems that this inhibitory effect of CH_2Cl_2 fraction is mediated by inhibiting both the influx of calcium and sodium through their ion channels into the rat adrenal medullary chromaffin as well as the release of Ca^{2+} from the cytoplasmic calcium store at least partly through the increased NO production due to the activation of nitric oxide synthase. Based on these effects, it is also thought that CH_2Cl_2 fraction may be beneficial to prevent or treat the cardiovascular diseases, suggesting that CH_2Cl_2 fraction also contains active components as an antihypertensive agent.

Effects of CH_2Cl_2 fraction on Blood pressure and contractile responses of the thoracic aortic strips:

Recently, Kee and Lim (2007) have demonstrated that PCRC, isolated from *Rubus coreanum* MIQUEL, inhibits the secretory responses of catecholamines (CA) evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused adrenal gland of the normotensive rats. It seems that this inhibitory effect of CH_2Cl_2 fraction is exerted by inhibiting both the calcium influx into the rat adrenal medullary chromaffin cells and the uptake of Ca^{2+} into the cytoplasmic calcium store partly through the increased NO production due to the activation of

endothelial nitric oxide synthase (eNOS), which are at least relevant to the direct interaction with the nicotinic receptor itself. The purpose of the present study was to investigate whether CH_2Cl_2 fraction may affect the contractility of the aortic strips isolated from normotensive arts and spontaneously hypertensive rats (SHRs), and to clarify its mechanism of action. CH_2Cl_2 fraction (200~800 μg/mL) concentration-dependently blocked phenylephrine (10 μM)-induced contractile responses of the isolated aortic strips of SHRs. CH_2Cl_2 fraction (400 μg/mL), added in to bath medium, also depressed the contractile active tension evoked by both phenylephrine (3 and 10 μM) and high potassium (25 and 56 mM). In the simultaneous presence of CH_2Cl_2 fraction (400 μg/mL) and L-NAME (a selective inhibitor of NO synthase, 300 μM), the contractile responses evoked by phenylephrine and high K^+ were recovered to considerable level of the corresponding control release compared with those effects of CH_2Cl_2 fraction-treatment alone. In the endothelium-denuded aortic strips by CHAPS-treatment, the contractile responses induced by phenylephrine or high potassium were considerably recovered in comparison to that of CH_2Cl_2 fraction-treatment alone. Intravenous CH_2Cl_2 fraction (1mg/kg)-induced hypotension was greatly inhibited by pretreatment with chlorisondamine (an autonomic ganglionic blocker, img/kg, i.v.), phentolamine (an adrenergic α-receptor blocker, 1mg/kg, i.v.), L-NAME (a selective inhibitor of NO synthase, 3mg/kg/30min), and sodium nitroprusside (a nitrosovasodilator, 30 μg/kg/30min), respectively. Interestingly, CH_2Cl_2 fraction (1.0, 3.0 and 10.0 mg/kg/ 30 min, i.v., respectively) dose-dependently suppressed intravenous norepinephrine-induced vasopressor responses in anesthetized SHRs as well as normotensive rats. Collectively, the present study provide these results demonstrate for the first time

that CH_2Cl_2 fraction causes vascular relaxation in the isolated aortic strips with intact endothelium of SHRs at least partly by the increased NO production through the activation of NO synthase of vascular endothelium. Based on these results, it seems that active components of CH_2Cl_2 fraction might be helpful to prevent or alleviate cardiovascular diseases, including hypertension and angina pectoris.

In addition, in terms of these data, it is expected that intake of sports beverage containing active components extracted from *Rubus coreanum* is helpful to physiologically stabilize cardiovascular system of athletes as well as beneficial to enhance athlete performance.

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Choi, Mee-Sung

감사의 글

위대한 일을 이루기 위해서는, 행동뿐 만 아니라 꿈을 갖고 실행에 옮겨야 하며, 계획만 세우기보다는 믿음을 갖고 실천해야 한다고 생각합니다. 나 자신에 대한 믿음을 실천에 옮긴 일에 대해 몇 마디 단어로 다 표현할 수 없을 것이라 생각됩니다.

이 논문은 저를 위해 많은 어려움을 겪은 제 가족들과 동료들의 꿈을 담았을 뿐만 아니라 이전의 전공분야와 상이한 분야인, 운동생리학에 대한 연구를 한 차원 높일 수 있는 계기가 되었습니다.

제가 포기하지 않고 계속 연구할 수 있도록 끊임없이 도와주시고 새로운 학문의 세계로 이끌어주신 지도교수 서영환 교수님, 많은 조언과 격려를 해주신 정명수 교수님, 본 논문이 완성되기까지 바쁜 중에도 처음부터 끝까지 돌보아 주신 임동윤교수님, 논문심사 마지막까지 섬세하게 조언해주신 안용덕 교수님, 이성노 교수님께도 진심으로 감사드립니다.

그리고 학위논문 준비로 가정에 소홀했음에도 많은 용기를 준 남편 김정빈박사와 아들 남우, 딸 명지에게 진심으로 감사하며, 여기까지 올 수 있도록 음과 양으로 도와준 여러 지인들과 연구실 동료들께 감사의 마음을 전합니다.

이와 같은 연구과정을 무사히 마칠 수 있도록 기회를 주시고 물심양면으로 성원해주신 동신대학교 김필식 총장님께 충심으로 감사드립니다.
박사과정 동안 내내 할 수 있다는 신념을 불어 넣어주신 김홍식 교수님을 비롯한 동신대학교 생활체육학과 교수님들께 감사의 뜻을 전하고 싶습니다.

처음으로 접해보는 실험을 당황하지 않고 실수하지 않도록 실험과정과 자료정리를 친절하게 도와준 조선대학교 의과대학 심혈관계 약리학 교실 연구원인 신혜경 선생님, 논문을 작성하는 동안 전공분야에 대해 기본적인 지식을 터득할 수 있도록 도와주신 백영홍 교수님, 김경근 교수님, 위승두 교수님 그리고 멀리서나마 끝까지 잘 해내도록 기원해주고 귀중한 조언을 아끼지 않은 미국 Cornell대학의 Susan P. Ashdown 교수께도 감사의 마음을 전합니다.

성공적인 삶을 성취하기 위해 모든 일에 항상 최선을 다하고자 합니다.
새롭게 뭔가를 시도해보지도 않고서 결코 자기 인생을 되돌아보기 어렵듯이,
새롭게 출발할 수 있는 자가 새로운 끝맺음도 할 수 있는 용기가 있다고 생각합니다.
저를 기억하시는 모든 분들에게 고개 숙여 진심으로 감사드립니다.

최미성 드림