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Influence of Polyphenolic Compounds Isolated from *Rubus coreanum* MIQUEL on Catecholamine Release in the Isolated Adrenal Gland

조선대학교 대학원

의학과

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복분자에서 분리한 Polyphenolic Compounds 가 흰쥐 적출 부신에서 카테콜아민 유리작용에 미치는 영향

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이 논문을 의학박사 학위신청논문으로 제출함.

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복분자에서 분리한 Polyphenolic Compounds가 흰쥐 적출 부신에서 카테콜아민 유리작용에 미치는 영향

기 영 우

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본 연구의 목적은 복분자 *(Rubus coreanum* MIQUEL, 覆盆子)로 양조한 복분자주에서 분리한 폴리페놀 화합물(PCRK)이 정상혈압 흰쥐로부터 분리 적출한 부신의 관류모델에서 카테콜아민 (catecholamines, CA) 분비작용에 미치는 영향을 검색하여 그 작용기전을 규명하고, 나아가 적포도주에서 분리한 폴리페놀 화합물(PCRW)과 작용상의 차이유무를 비교 검색코자 본 연구를 시행하여 다음과 같은 결과를 얻었다. PCRK (20~180 μg/ml)을 부신정맥내로 90분간 관류시 비교적 용량 및 시간 의존적으로 ACh (5.32 mM), 고칼륨 (56 mM, 막탈분극제), DMPP (100 μM, 선택성 니코틴수용체 작용제), 및 McN-A-343 (100 μM, 선택성 무스카린수용체 작용제)에 의한 CA 분비반응을 억제하였다. 그러나, PCRK자체는 기초 CA 분비량에 영향을 미치지 않았다. 또한, PCRK (60 μg/ml) 존재 하에서, L형 칼슘통로 활성화제인 Bay-K-8644 및 세포질에서 Ca^{2+}-ATPase 억제제인 cyclopiazonic acid에 의한 CA 분비반응이 억제되었다. 흥미롭게도,

PCRW(60 μg/ml) 존재 하에서 ACh, 고칼륨, DMPP, McN-A-343, Bay-K-8644 및 cyclopiazonic acid에 의한 CA분비작용도 시간 의존적으로 억제되었다. 또한 PCRK (60 μg/ml)과 L-NAME (100 μM)을 90분간 동시 처치하였을 때 ACh, 고농도의 K^+, DMPP, 및 Bay-K-8644의 CA 분비효과가 PCRK 단독처치 시 나타나는 억제효과에 비교하여 상응하는 대조치의 수준까지 회복되었다.

이와 같은 연구결과를 종합하여 보면, 정상혈압 흰쥐의 적출 관류 부신수질에서 PCRK및 PCRW는 콜린성(니코틴 및 무스카린 수용체)흥분작용 및 막탈분극에 의한 CA 분비작용에 대하여 억제작용을 나타내었다. 이러한 PCRK의 억제작용은 흰쥐 적출 부신수질에서 NO Synthase의 활성화에 의한 NO 생성증가로 인하여 크롬친화세포내로 칼슘유입과 세포내 칼슘저장고로부터 칼슘유리를 억제하며, 이는 적어도 니코틴수용체 자체와의 상호작용에 기인되는 것으로 생각된다. 또한 PCRK 와 PCRW은 CA분비 억제작용에 대해여 유사한 효력을 나타내는 것으로 사료된다.

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 (覆盆子酒). *Rubus coreanum* has beeb found to possess several polyphenolic compounds, such as (-)-epicatechin, (+)-catechin, proanthocyanidin, etc. There are no reports about its cardiovascular effects so far, while there are too many reports about those of polyphenolic compound isolated from red wine

In *in vivo* studies, red wine polyphenolic compounds (PCRWs) were shown to reduce blood pressure in normotensive and hypertensive rats (Mizutani et al., 1999; Diebolt et al., 2001). 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). 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). PCRWs enhanced NO synthesis and cGMP accumulation only in the presence of functional endothelium. In denuded aortic rings, 103-fold higher concentration PCRWs was necessary to induce relaxation (Ndiaye et al., 2003; Corder et al., 2001). 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; Rakici et al., 2005).

Recently, it has been shown that green tea extract inhibits the secretory responses of catecholamines (CA) evoked by cholinergic (nicotinic and muscarinic) stimulation and direct membrane-depolarization in the perfused adrenal medulla isolated from the rat (Lim et al., 2003) and the rabbit (Lim, 2005). However, epigallocatechin-3-gallate, one of potent catechins isolated from green tea, did not affect the secretion of catecholamines (CA) release evoked by the above serectagogues. As aforementioned, there are so far many reports about the effects of red wine on cardiovascular system. Despite of these studies, there are no reports on in vitro functional effects of polyphenolic compounds (PCRK) from wine, which is brewed from *Rubus coreanum* MIQUEL (覆盆子酒), on the cardiovascular system. Therefore, the aim of the present study was to investigate the ability of PCRK on secretion of catecholamines (CA) in the perfused model of the rat adrenal gland, to establish its mechanism of action, and additionally to compare its effect with that of PCRW.

Ⅱ. MATERIALS AND METHODS

Experimental procedure

Mature male Sprague-dowley rats, weighing 200 to 350 grams, were used in the experiment. The animals were housed individually in separate cages, and food (Cheil Animal Chow) and tap water were allowed *ad libitum* for at least a week to adapt to experimental circumstances. On the day of experiment, a rat was anesthetized with thiopental sodium (50 mg/kg) intraperitoneally, and tied in supine position on fixing panel.

Isolation of adrenal glands: The adrenal gland was isolated by the modification of previous method (Wakade, 1981). The abdomen was opened by a midline incision, and the left adrenal gland and surrounding area were exposed by the placement of three-hook retractors. The stomach, intestine and portion of the liver were not removed, but pushed over to the right side and covered by saline-soaked gauge pads and urine in bladder was removed in order to obtain enough working space for tying blood vessels and cannulations. A cannula, used for perfusion of the adrenal gland, was inserted into the distal end of the renal vein after all branches of adrenal vein (if any), vena cava and aorta were ligated. Heparin (400 IU/ml) was injected into vena cava to prevent blood coagulation before ligating vessels and cannulations. A small slit was made into the adrenal cortex just opposite entrance of adrenal vein. Perfusion of the gland was started, making sure that no leakage was present, and the perfusion fluid escaped only from the slit made in adrenal cortex. Then the adrenal gland, along with ligated blood vessels and the cannula, was carefully removed from the animal and

placed on a platform of a leucite chamber. The chamber was continuously circulated with water heated at 37 ±1 ^{o}C (Fig. 1).

Perfusion of adrenal gland

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

Drug administration

The perfusions of DMPP (10^{-4} M) for 2 minutes and/or a single injection of ACh (5.32×10^{-3} M) and KCl (5.6×10^{-2} M) in a volume of 0.05 ml were made into perfusion stream via a three-way stopcock, respectively. McN-A-343 (10^{-4} M), Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were also perfused for 4 min, respectively.

In the preliminary experiments, it was found that upon administration of the above drugs, secretory responses to ACh, KCl, McN-A-343, Bay-K-8644 and cyclopiazonic acid returned to preinjection level in about 4 min, but the responses to DMPP in 8 min.

Collection of perfusate

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

To study the effect of PCRK or PCRW on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing PCRK or PCRW 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 PCRK or PCRW, and the perfusates were collected for the same period as that for the background sample. The adrenal gland's perfusate was collected in chilled tubes.

Measurement of catecholamines

The content of perfusate was measured directly by the fluorometric method of Anton and Sayre (Anton & Sayre, 1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using 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 content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

Isolation of polyphenolic compounds

Polyphenolic compounds were prepared as described by Caderni et al (2000), using adsorption chromatography from a 1-year old wine brewed from Rubus coreanum Miquel (覆盆子酒) at the Research Institute of Bokbunja, Gochang County, Cheollabukdo Province, Korea or a 2-year-old red a cabernet sauvignon red wine made from Cabernet Sauvignon grapes by standard red wine making procedures at the Arzens Cooperative winery (Arzens, Aude, France), as follows (Fig. 2): alcohol was eliminated by distillation of 40 l batches and the remaining solution was deposited on a Diaion HP-20 column (Mitsubish Chemical Industries, Japan). After rinsing with water to remove sugars and organic acids, the phenolic pool of chemicals present in wine was eluted with 100% ethanol in water, concentrated by vacuum, evaporation and atomized, lyophilized by (Coldvac -80, Hanil R & D, Korea). About 2.9 g PCRK was obtained from 1 L Bokbunja wine, and 2.1 g PCRW from 1 L red grape wine. The working solution of this PCRK or PCRW was prepared by dissolving in 0.9% NaCl solution on the day of each experiment and filtered before administration.

Statistical analysis

The statistical difference between the control and pretreated groups was determined by the Student's *t* and ANOVA tests. A P-value of less than 0.05 was considered to represent statistically significant changes unless specifically noted

in the text. Values given in the text refer to means and the standard errors of the mean (S.E.M.). The statistical analysis of the experimental results was made by computer program described by Tallarida and Murray (1987).

Drugs and their sources

The following drugs were used: PCRK and PCRW (gifted from professor Young-Hong Baik, Department of Pharmacology, College of Medicine, Chonnam National University, Gwangju, Korea), 1.1-dimethyl-4 -phenyl piperazinium iodide (DMPP), acetylcholine chloride, norepinephrine bitartrate, potassium chloride (KCl), N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), methyl-1,4 -dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-carboxylate (BAY-K-8644), cyclopiazonic acd, (Sigma Chemical Co., U.S.A.), 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, which was dissolved in 99.5 % ethanol and diluted appropriately with Krebs-bicarbonate solution (final concentration of alcohol was less than 0.1 %). Concentrations of all drugs except PCRK and PCRW used are expressed in terms of molar base.

III. RESULTS

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

After the perfusion with oxygenated Krebs-bicarbonate solution for 1 hr, basal CA release from the isolated perfused rat adrenal glands amounted to 20±2 ng for 2 min (n=12). Since in *in vivo* study, PCRWs were shown to reduce blood pressure in normotensive and hypertensive rats (Mizutani et al., 1999; Diebolt et al., 2001), it was attempted initially to examine the effects of PCRK itself on CA secretion from the perfused model of the rat adrenal glands. However, in the present study, PCRK (20 ~ 180 μg/ml) itself did not produce any effect on basal CA output from perfused rat adrenal glands (data not shown). Therefore, it was decided to investigate the effects of PCRK on cholinergic receptor stimulation- as well as membrane depolarization-mediated CA secretion. Secretagogues were given at 15 to 20 min-intervals. PCRK 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 1371±120 ng for 4 min. However, the pretreatment with PCRK in the range of 20 ~ 180 μg/ml for 90 min concentration- and time-dependently inhibited ACh-stimulated CA secretion. As shown in Fig. 3 and table 1~3, in the presence of PCRK, CA releasing responses were inhibited by 71% of the corresponding control release (100%). Also, it has been found that depolarizing agent like KCl stimulates markedly CA secretion (746±43 ng for 0-4 min). High K^+ (5.6×10^{-2} M)-stimulated CA secretion after the

pretreatment with 20 μg/ml PCRK was not affected for the first 60 min as compared with its corresponding control secretion (100%) (Fig. 4 and Table 1~3). However, following the pretreatment with higher concentrations of PCRK (60 ~ 180 μg/ml), high K^+ ($5.6x10^{-2}$ M)-stimulated CA secretion was significantly inhibited to 74% of the control after 75 min period, although it was not initially affected by PCRK. DMPP (10^{-4} M), which is a selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion (1182±53 ng for 0-8 min). However, as shown in Fig. 5, DMPP-stimulated CA secretion after pretreatment with PCRK was greatly reduced to 84% of the control release (Table 1~3). 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 (578±26 ng for 0-4 min). However, McN-A-343-stimulated CA secretion in the presence of PCRK was markedly depressed to 71% of the corresponding control secretion (100%) as depicted in Fig. 6 and table 1~3.

Effect of PCRK on CA secretion evoked by Bay-K-8644 and cyclopiazonic acid 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 PCRK 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 PCRK was greatly blocked to 69% of the control except for the early 30 min as compared to the corresponding

control release (491±27 ng for 0-4 min) from 12 rat adrenal glands as shown in Fig. 7 and table 4.

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 PCRK on cyclopiazonic acid-evoked CA secretory response was observed as shown in Fig. 8 and table 4. However, in the presence of PCRK in 12 rat adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was also inhibited to 70% of the control response (427±18 ng for 0-4 min).

Effect of PCRW on CA secretion evoked by ACh, excess K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

As shown in Fig. 3 ~ 8, PCRK significantly inhibited the CA secretory responses evoked by cholinergic stimulation and membrane depolarization form the perfused rat adrenal glands. Therefore, in order to investigate the effect of dopaminergic D_1 receptor antagonist on CA release, it was likely of interest to examine effect of PCRW, polyphenolic compounds isolated from red wine, on CA secretion evoked by cholinergic stimulation and membrane depolarization from the isolated perfused rat adrenal glands. In order to test the effect of PCRW on cholinergic receptor-stimulated CA secretion as well as membrane depolarization-mediated secretion, PCRW (60 μg/ml) was loaded into the adrenal medulla for 90 min. PCRW itself also did not give any effects on basal CA output from perfused rat adrenal glands (data not shown). Therefore, in the subsequent

experiments, the effects of PCRW on the CA seretory responses evoked by ACh, high K^+, DMPP and McN-A-343 were examined. As illustrated in Fig. 9 and table 5, ACh (5.32×10^{-3} M)-evoked CA release prior to the perfusion with PCRW was 1328±68 ng (0-4 min). In the presence of PCRW (60 μg/ml) for 60 min, it was significantly inhibited by 81% of the control release (100%). High potassium (56 mM KCl), a direct membrane-depolarizing agent, stimulates CA secretion (691±21 ng, 0-4 min). In the present work, high K^+ (5.6×10^{-2} M)-evoked CA release in the presence of PCRW (60 μg/ml) for 90 min was also reduced by 81% of the corresponding control secretion (100%) after 30 min period, as shown in Fig. 10 and table 5. DMPP (10^{-4} M), a selective nicotinic receptor agonist in autonomic sympathetic ganglia, when perfused through the rat adrenal gland, evoked a sharp increase in CA secretion. As shown in Fig. 11 and table 5, DMPP (10^{-4} M)-stimulated CA secretion following the loading with PCRW (6×10^{-5} M) was inhibited by 89% compared to the corresponding control secretion (1109±29 ng, 0-8 min). As illustrated in Fig. 12, McN-A-343 (10^{-4} M), which is a selective muscarinic M_1-receptor agonist (Hammer and Giachetti, 1982), perfused into an adrenal vein for 4 min caused an increased CA secretion to 183 ± 5 ng (0-4 min). However, in the presence of PCRW (60 μg/ml), McN-A-343-evoked CA secretion was significantly reduced by 77% of the corresponding control release (Table 5).

Bay-K-8644 (10^{-5} M)-stimulated CA secretion in the presence of PCRW was greatly inhibited to 73% of the corresponding control release (480±20 ng for 0-4 min) from 8 rat adrenal glands, as shown in Fig. 13 and table 6.

As depicted in Fig. 14 and table 6, in the presence of PCRW from 8 rat adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was reduced to 71% of the control response (448±24 ng for 0-4 min).

Effect of PCRK plus L-NAME on CA release evoked by ACh, high K^+, DMPP, and BAY-K-8644 from the perfused rat adrenal glands

It has also been found that, in this study, PCRK inhibits the CA secretory response evoked by cholinergic stimulation in the perfused rat adrenal gland. Therefore, to study the relationship between NO and PCRK-induced inhibitory effects on the CA release from the rat adrenal glands, the effect of L-NAME on PCRK-induced inhibitory responses of CA secretion evoked by cholinergic receptor-stimulation as well as membrane depolarization was examined. In the present study, ACh (5.32 mM)-evoked CA release before perfusion with PCRK plus L-NAME was 1050±50 ng (0-4 min) from 10 rat adrenal glands. In the simultaneous presence of PCRK (60 μg/ml) and L-NAME (30 μM) for 90 min, it was initially not affected at 0-34 min, but later rather inhibited by 85% of the corresponding control release at the period of 90-94 min as illustrated in Fig. 15 and table 7. High K^+ (56 mM)-evoked CA release in the presence of PCRK (60 μg/ml) and L-NAME (30 μM) for 90 min was also not changed for 0-64 min, but later rather inhibited to 82% of the corresponding control release only at the last period of 90-94 min period in comparison to the control secretion (717±51 ng, 0-4 min) from 10 glands (Fig. 16 and Table 7). As shown in Fig. 17 and table 7, DMPP-evoked CA release prior to the perfusion with PCRK and L-NAME was 1126±57 ng (0-8 min). The simultaneous perfusion of PCRK and L-NAME for 90 min no longer inhibited DMPP-evoked CA release for the period of 0-48 min from 10 experiments while later rather depressed to 91% of the control release at the period of 80-84 min. As shown in Fig. 18 and table 7, the simultaneous perfusion

of PCRK (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 from 10 experiments, but later rather depressed to 79% of the control release at the last period of 60-64 min in comparison to their corresponding control responses (448±25 ng, 0-4 min).

IV. DISCUSSION

The present experimental results have suggested that PCRK inhibits the CA secretory responses from the isolated perfused adrenal gland of the normotensive rats evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization. It seems that this inhibitory effect of PCRK is exerted by inhibiting both the calcium influx into the adrenal medullary chromaffin cells of the normotensive rats and the uptake of Ca^{2+} into the cytoplasmic calcium store partly through the activation of NO production, which are at least partly relevant to the direct interaction with the nicotinic receptor itself.

In general, the adrenal medulla has been employed as a model system to study numerous cellular functions involving not only noradrenergic nerve cells but also neurons. During neurogenic stimulation of the adrenal medulla, ACh is released from splanchnic nerve endings and activated cholinergic receptors on the chromaffin cell membrane (Viveros, 1975). This activation initiates a series of events known as stimulus-secretion coupling, culminating in the exocytotic release of CA and other components of the secretory vesicles into the extracellular space. Usually, two mechanisms are involved in the secretion of adrenal medullary hormones. Upon excitation of splanchnic nerves, ACh is released from the nerve terminals, and then is activates nicotinic secretion of CA. Based on this fact, the present findings that PCRK 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 PCRK-induced inhibitory activity of CA secretory response evoked by stimulation of nicotinic receptors might contribute at least partly to its hypotensive mechanism. ACh, the physiological presynaptic transmitter at the adrenal medulla, which is released by depolarizing splanchnic nerve terminals and then activates nicotinic receptors, releases CA, and 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 PCRK may inhibit CA secretion evoked by nicotinic stimulation from the splanchnic nerve ending through the blockade of nicotinic receptors. The release of epinephrine from the adrenal medulla in response to splanchnic nerve stimulation or nicotinic agonist is mediated by activation of nicotinic receptors located on the chromaffin cells. The exocytotic CA release from the chromaffin cells appears to be essentially similar to that occuring in noradrenergic axons (Douglas, 1968; Sorimachi & Yoshida, 1979). ACh-evoked CA secretion has shown to be caused through stimulation of both nicotinic and muscarinic receptors in guinea-pig adrenal gland (Nakazato et al, 1988) as well as in the perfused rat adrenal glands (Lim & Hwang, 1991).

In support of this idea, recently, it has been found that 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, PCRK inhibited the secretory responses of CAs evoked by ACh, DMPP, McN-A-343 and high K^+. It suggests that PCRK 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 PCRK can cause the depressor effect by the inhibition of CA secretion from the adrenal medulla. The present findings appeared to contribute at least partly to the facts that extracts of tea (Fitzpatrick et al., 1992) and flavonoids found in tea (Fitzpatrick et al., 1993) produced vasodilator effects, but not to the fact that tea ingestion in the normotensive men caused larger acute increases in blood pressure than caffeine alone (Hodgson et al., 1999).

In the present study, both PCRK and PCRW 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 PCRK may inhibit Ca^{2+} influx to the rat adrenomedullary cells. In support of this idea, in cultured bovine adrenal medullary cells, nicotinic (but not muscarinic) receptors mediate the

Ca^{2+}-dependent secretion of CA (Fisher et al., 1981; Yanagihara et al, 1979). It has been also known that the activation of nicotinic receptors stimulates CA secretion by increasing Ca^{2+} entry through receptor-linked and/or voltage-dependent Ca^{2+} channels in both perfused rat adrenal glands (Wakade & Wakade, 1983; Lim & Hwang, 1991) and isolated bovine adrenal chromaffin cells (Kilpatrick et al, 1981; 1982; Knight & Kesteven, 1983). Wada and his coworkers (1985b) have found that the adrenomedullary chromaffin cells have (i) nicotinic receptor-associated ionic channels, responsible for carbachol-induced Na^+ influx, (ii) voltage-dependent Na^+ channels, responsible for veratridine-induced Na^+ influx and (iii) voltage-dependent Ca^{2+} channels, suggesting that the influx of Na^+ caused either by carbachol or by veratridine leads to activate voltage-dependent Ca^{2+} channels by altering membrane potentials, whereas high K^+ directly activates voltage-dependent Ca^{2+} channels without increasing Na^+ influx. In the present study, the finding that high K^+-induced CA secretory response was depressed by pretreatment with PCRK indicates that this inhibitory effect of PCRK is exerted through the direct inhibition of calcium influx into the rat adrenal chromaffin cells. Furthermore, slight elevation in the extracellular potassium concentration increases both the frequency of spontaneous action potentials and the secretion of CA (Kidokoro & Ritchie, 1980), suggesting that the influx of calcium that occurs during action potentials is directly linked to the rate of secretion. These findings that PCRK inhibited CA secretion evoked by Bay-K-8644 as well as by high K^+ suggest that PCRK 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 PCRK inhibits the DMPP-evoked CA secretion by inhibiting Ca^{2+} influx through voltage-dependent Ca^{2+} channels activated by nicotinic ACh receptors with DMPP.

The present study has also shown that PCRK 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 (Geoger & Riley, 1989; Siedler 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 PCRK on 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 PCRK 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 secretion of CA. 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 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 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 PCRK-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 PCRK on Ca^{2+} movement from intracellular pools is due to its direct effect on the PI response or the indirect effects.

However, in the present study, in the simultaneous presence of PCRK and L-NAME (NOS 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 PCRK treatment alone. This result is well consistent with 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).

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 PCRK 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 rat adrenal medulla, since this inhibitory effect of PCRK 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. 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 PCRK as well as PCRW on the CA secretion were recovered to the considerable extent of the control secretion compared with the inhibitory effects of PCRK alone. This result demonstrates that PCRK 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 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 PCRK possesses the ability partly to activate nNOS in the rat adrenal medullary chromaffin cells, in addition to the direct inhibitory effects on the CA secretion.

In conclusion, the results of the present study have suggest that both PCRK and PCRW inhibit the CA secretion by stimulation of cholinergic nicotinic receptors as well as by membrane depolarization in the isolated perfused adrenal glands of the normotensive rats. It seems that this inhibitory effect of PCRK is exerted by blocking both the calcium influx into the rat adrenal medullary chromaffin cells and the uptake of Ca^{2+} into the cytoplasmic calcium store at least partly via the increased NO production due to the activation of nitric oxide synthase, which are relevant to the direct interaction with the nicotinic receptor itself. These experimental results may contribute partly to the hypotensive effect of PCRK components, through inhibition of CA secretion from adrenomedullary

chromaffin cells and consequent reduction of the CA level in the circulation. It seems likely that there is no difference in mode of the CA-releasing action between PCRK and PCRW, based on the concentrations examined in the present work.

V. SUMMARY

The aim of the present study was to investigate whether polyphenolic compounds isolated from liquors, which is brewed from *Rubus koreanum* MIQUEL (覆盆子酒), may affect release catecholamine from the isolated perfused rat adrenal medulla, and to compare their effects with those effects of polyphenolic compounds isolated from red wine (PCRW).

Polyphenolic compounds (PCRK, 20~180 μg/mL) perfused into an adrenal vein for 90 min 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). PCRK itself did not affect basal CA secretion (data not shown). Also, in the presence of PCRK (60 μg/mL), the secretory responses of CA evoked by 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, PCRW (60 μg/mL) caused the inhibitory effects similar with PCRK (60 μg/mL) on the CA secretory responses evoked by ACh, high K^+, DMPP, McN-A-343, Bay-K-8644, and cyclopiazonic acid. In the simulataneous presence of PCRK (60 μg/mL) and L-NAME (an inhibitor of NO synthase, 30 μM), the inhibitory responses of PCRK 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 PCRK alone.

Taken together, these results obtained from the present study demonstrate that PCRK inhibits the CA secretory responses from the isolated perfused adrenal gland of the normotensive rats evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization. It seems that this inhibitory effect of PCRK is exerted by inhibiting both the calcium influx into the adrenal medullary chromaffin cells of the normotensive rats 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, which are at least relevant to the direct interaction with the nicotinic receptor itself. It is also thought that PCRK possesses the equi-potent inhibitory effect with PCRW in the CA secretion.

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