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Resveratrol Inhibits Nicotinic Stimulation-Evoked Catecholamine Release from the Adrenal Medulla

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Resveratrol has been known to possess various potent cardiovascular effects in animal, but there is little information on its functional effect on the secretion of catecholamines (CA) from the perfused model of the adrenal medulla. Therefore, the aim of the present study was to determine the effect of resveratrol on the CA secretion from the isolated perfused model of the normotensive rat adrenal gland, and to elucidate its mechanism of action. Resveratrol (10∼100 μM) during perfusion into an adrenal vein for 90 min 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) in both a time- and dose-dependent fashion. Also, in the presence of resveratrol (30 μM), the secretory responses of CA evoked by veratridine 8644 (an activator of voltage-dependent Na^+ channels, 100 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. In the simultaneous presence of resveratrol (30 μM) and L-NAME (an inhibitor of NO synthase, 30 μM), the CA secretory evoked by ACh, high K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid were recovered to a considerable extent of the corresponding control secretion compared with the inhibitory effect of resveratrol alone. Interestingly, the amount of nitric oxide (NO) released from the adrenal medulla was greatly increased in comparison to its basal release. Taken together, these experimental results demonstrate that resveratrol can inhibit the CA secretory responses evoked by stimulation of cholinergic nicotinic receptors, as well as by direct membrane-depolarization in the isolated perfused model of the rat adrenal gland. It seems that this inhibitory effect of resveratrol is exerted by inhibiting an influx of both ions through Na^+ and Ca^{2+} channels into the adrenomedullary cells as well as by blocking the release of Ca^{2+} from the cytoplasmic calcium store, which are mediated at least partly by the increased NO production due to the activation of NO synthase.

Key Words: Resveratrol, Catecholamine secretion, Adrenal medulla, Cholinergic receptors, Nitric oxide

INTRODUCTION

Resveratrol, 3,4',5-trihydroxystilbene, is a naturally occurring polyphenolic compound present in variety of plants, such as *Yucca schidigera* (Uenobe et al, 1997), a South Africa medicinal plant *Erythrophleum lasianthum* (Orisini et al, 1997), and grapes (Celotti et al, 1996; Sato et al, 1997). Interest in resveratrol has expanded in recent years, focusing on its potentially beneficial effects on the cardiovascular as well as neoplastic diseases. It has been shown that resveratrol mainly produced endothelium-dependent and nitric oxide-mediated vasodilation in human internal mammary arteries, and only partially in saphenous vein rings, and improved their endothelial reactivity (Rakici et al, 2005). Several 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 also found that red wine polyphenolic compounds (PCRW) promote the endothelium-dependent relaxation, activate NO synthase, inhibit platelet aggregation, and prevent oxidation of LDL-cholesterol (Fitzpatrick et al, 1993; Frankel et al, 1993; Demrow and Slane, 1995; Andriambeloson et al, 1997; Flesh et al, 1998; Leikert et al, 2002).

The polyphenolic compound resveratrol contained in red wine is thought to be a responsible factor for its beneficial cardiovascular effects. Resveratrol has similar effects to PCRW, such as promotion of vasodilation, activation of nitric oxide synthase, inhibition of platelet aggregation and

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ABBREVIATIONS: CA, catecholamines; NO, nitric oxide; DMPP, 1.1-dimethyl-4-phenyl piperazinium iodide, methyl-1,4-dihydro-2; ACh, acetylcholine; McN-A-343, 3-(m-chlloro-phenyl-carbamoyl-oxy-2-butynyl-trimethyl ammonium chloride; BAY-K8644, 6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-carboxylate.

leukocyte activation, prevention of oxidation of LDL-cholesterol and reduction of cholesterol synthesis (Frankel et al, 1993; Pace-Asciak et al, 1995; Chen and Pace-Asciak, 1996; Rotondo et al, 1998; Wallerath et al, 2002).

On the other hand, it has been observed that these natural polyphenols, like a number of antidepressant drugs (Slotkin et al, 1986; Gareri et al, 2000; To et al, 2005), inhibit the uptake of 5-hydroxytryptamine (5-HT) by human platelets. In recent studies (Yáñez et al, 2006), both *cis*-resveratrol and trans-resveratrol (5～200 μM) in a concentration-dependent manner inhibited the uptake of [^{3}H]NA and [3H]5-HT by synaptosomes from rat brains and the uptake of [^{3}H]5-HT by human platelets. Both *cis*-resveratrol and *trans*-resveratrol (5～200 μM) in a concentration-dependent manner also inhibited the enzymatic activity of commercial (human recombinant) MAO (monoamine oxidase) isoform (MAO-A and MAO-B) activity.

Resveratrol has various potent cardiovascular effects in animals but there is so far no evidence about its functional effect on the CA secretion from the perfused model of the adrenal gland. Therefore, the aim of the present study was to examine the ability of resveratrol on the CA secretion in the isolated perfused model of the rat adrenal gland, and to elucidate its mechanism of action.

METHODS

Experimental procedure

Male Sprague-Dawley rats, weighing 180 to 300 grams, were anesthetized with thiopental sodium (40 mg/kg) intraperitoneally. The adrenal gland was isolated by the methods described previously (Wakade, 1981). The abdomen was opened by a midline incision, and the left adrenal gland and surrounding area were exposed by placing 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 gauze 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 were 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.

Perfusion of adrenal gland

The adrenal glands were perfused by means of ISCO pump (WIZ Co.) at a rate of 0.33 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 CA.

Drug administration

The perfusions of DMPP (10^{-4} M) and McN-A-343 (10^{-4} M) for 2 minutes and/or a single injection of ACh (5.32 x 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. 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 returned 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 resveratrol on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing quinine for 20 min, 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 resveratrol, 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

CA content of perfusate was measured directly using the fluorometric method of Anton and Sayre (1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using fluorospectrophotometer (Kontron Co., Milano, Italy).

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.

Measurement of NO release

NO release was measured using a NO-selective microelectrode (amiNO-700, innovative Instruments Inc) and an amplifier (inNO meter, Innovative Instruments). Platelet NO production was quantified as the integrated signal detected by the microelectrode after platelet activation, as previously described (Freedman et al, 2000). The electrode

was calibrated by producing standardized concentrations of NO in 0.5% (wt/vol) KI in 0.1 mol/L H_2SO_4 from $NaNO_2$ standards. NO release was quantified as the current detected at the electrode 30 min after the presence of Provinol at room temperature. NO release was calculated as picomole. NO production was also measured indirectly by measuring nitrite content in the supernatant.

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 the means and the standard errors of the mean (SEM). The statistical analysis of the experimental results was made using the computer program described by Tallarida and Murray (1987).

Drugs and their sources

The following drugs were used: resveratrol hydrochloride, 1.1-dimethyl-4 -phenyl piperazinium iodide (DMPP), acetylcholine chloride (ACh), 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 acid, veratridine hydrochloride (Sigma Chemical Co., USA), and (3-(m-chloro-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 used are expressed in terms of molar base.

RESULTS

Effect of resveratrol on CA secretion evoked by ACh, excess 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 23.1±2.2 ng/2 min (n=6). Since some papers have demonstrated that resveratrol relaxes isolated vascular arteries (Fitzpatrick et al, 1993; Jager and Nguyen-Duong, 1999; Naderali et al, 2000; Naderali et al, 2001), it was attempted initially to examine the effects of resveratrol itself on CA secretion from the perfused model of the rat adrenal glands. However, in the present study, resveratrol ($10^{-5}\sim10^{-4}$ M) 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 resveratrol on cholinergic receptor stimulation- as well as membrane depolarization-mediated CA secretion. Secretagogues were given at intervals of 15 min. Resveratrol was perfused for 90 min after completion of control responses to various secretagogues.

When ACh (5.32×10^{-2} M) in a volume of 0.05 ml was given into the adrenal vein, the amount of CA secreted was

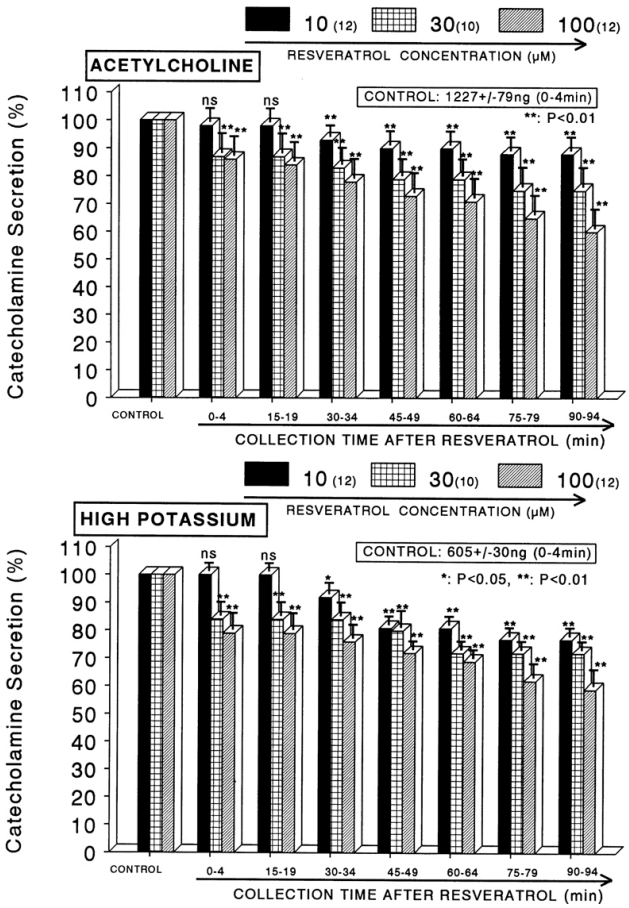

Fig. 1. Dose-dependent effect of resveratrol on the secretory responses of catecholamines (CA) evoked by acetylcholine (upper) and high potassium (lower) from the isolated perfused rat adrenal glands. The CA secretion by a single injection of ACh (5.32×10^{-3} M) and K^+ (5.6×10^{-2} M) in a volume of 0.05 ml was evoked at 15 min intervals during loading with 10, 30 and 100 μM of resveratrol for 90 min as indicated by the arrow marks, respectively. The numbers in parentheses indicate the number of rat adrenal glands. Vertical bars on the columns represent the standard error of the mean (SEM). 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 each concentration- treated group of resveratrol. ACh- and high K^+-induced perfusates were collected for 4 minutes, respectively. **p<0.01. ns: not statistically significant.

1227±79 ng for 4 min. However, the pretreatment with resveratrol in the range of $10^{-5}\sim10^{-4}$ M for 90 min concentration- and time-dependently inhibited ACh-evoked CA secretion. As shown in Fig. 1 (Upper), in the presence of resveratrol, ACh-evoked CA releasing responses were inhibited maximally by 60% of the corresponding control release. Also, it has been found that a depolarizing agent, high K^+ stimulates CA secretion (605±30 ng for 0~4 min). However, in the presence of resveratrol (10^{-5} M~10^{-4} M), excess K^+ (5.6×10^{-2} M)-evoked CA secretion was significantly inhibited by 59% of the control release (Fig. 1-Lower). When perfused through the rat adrenal gland, DMPP (10^{-4} M), which is a selective neuronal nicotinic receptor agonist, evoked a sharp and rapid increase in CA secretion (1189±54 ng for 0~8 min). However, as shown

in Fig. 2 (Upper), DMPP-evoked CA secretion during treatment with resveratrol was greatly reduced to 63% of the control release. McN-A-343 (10^{-4} M), which is a selective muscarinic M1-agonist (Hammer and Giachetti, 1982), perfused into an adrenal gland for 4 min caused an increased CA secretion (4894±21 ng for 0～4 min). However, McN-A-343-evoked CA secretion in the presence of resveratrol was markedly depressed to 55% of the corresponding control secretion as depicted in Fig. 2 (Lower).

Effect of resveratrol on CA secretion evoked by veratridine, 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 resveratrol 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 resveratrol (3×10^{-5} M) was greatly blocked to 72% of the control except for the early 45 min as compared to the corresponding control release (461±21 ng for 0～4 min) from 10 glands as shown in Fig. 3 (Upper).

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 resveratrol on cyclopiazonic acid-evoked CA secretory response was observed as shown in Fig. 3 (Lower). In the presence of resveratrol (3×10^{-5} M) in 12 rat adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was significantly depressed by 71% of the control secretory response (448±19 ng for 0～4 min).

It has also 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 secretion of CA in cultured bovine

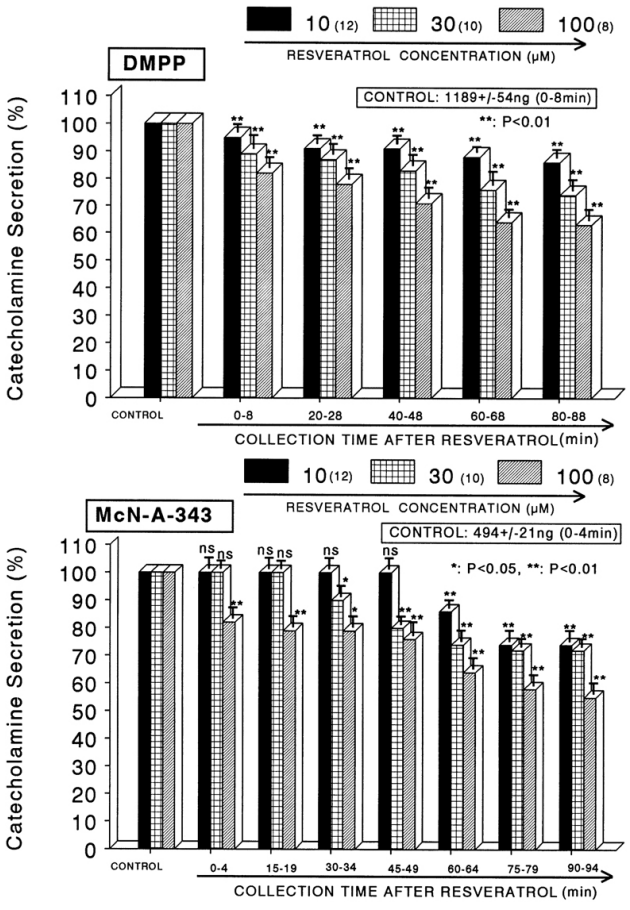

Fig. 2. Dose-dependent effect of resveratrol on the CA secretory responses evoked by DMPP (upper) and McN-A- 343 (lower) from the isolated perfused rat adrenal glands. The CA secretion by perfusion of DPPP (10^{-4} M) and McN-A-343 (10^{-4} M) for 2 min was induced at 20 and 15 min intervals during loading with 10, 30 and 100 μM of resveratrol for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONTROL) with each concentration- pretreated group of resveratrol. DMPP- and McN-A-343-induced perfusates were collected for 8 and 4 minutes, respectively. Other legends are the same as in Fig. 1. *p<0.05, **p<0.01. ns: not statistically significant.

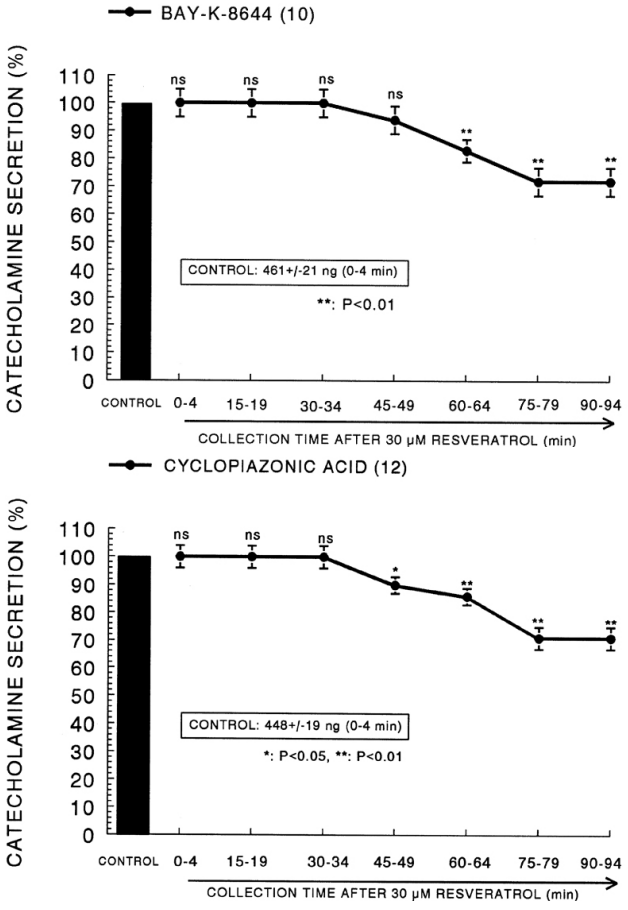

Fig. 3. The time-dependent effects of resveratrol on the CA release evoked by Bay-K-8644 (upper) and cyclopiazonic acid (lower) from the rat adrenal glands. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals during loading with resveratrol (30 μM) for 90 min. Other legends are the same as in Fig. 1. *p<0.05, **p<0.01. ns: not statistically significant.

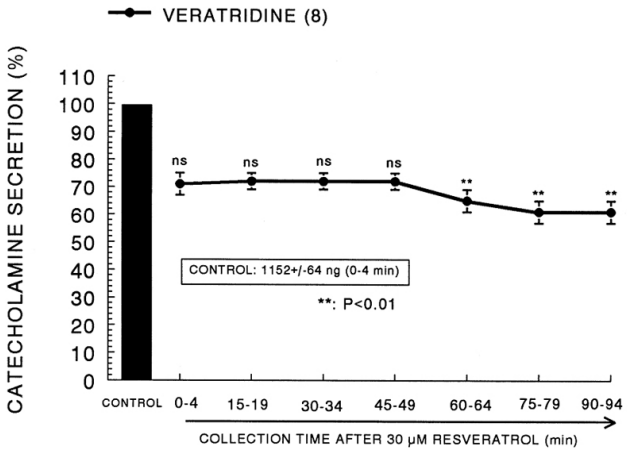

Fig. 4. Time-course effects of resveratrol on the CA release evoked by veratridine from the rat adrenal glands. Veratridine (10^{-4} M) was perfused into an adrenal vein for 4 min at 15 min intervals during loading with resveratrol (30 μM) for 90 min. Other legends are the same as in Fig. 1. **p < 0.01.

adrenal medullary cells (Wada et al, 1985). To characterize the pharmacological action of PCRW on voltage-dependent Na^+ channels, the effect of PCRW on the CA secretion induced by veratridine was examined here. As shown in Fig. 4, veratridine greatly produced CA secretion (1,152±64 ng for 0-4 min). However, in the presence of resvertarol (30 μM), veratridine (100 μM)-evoked CA secretion was greatly inhibited to 61% of the corresponding control release in a time-dependent manner.

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

In the present work, resveratrol caused inhibition in the CA secretion by cholinergic receptor stimulation from the perfused rat adrenal gland. Therefore, in order to establish the relationship between NO and resveratrol-induced inhibitory action on the CA release, we tried to examine the effect of L-NAME on resveratrol-induced inhibitory responses of CA secretion evoked by ACh, high K^+, DMPP and McN-A-343.

In the simultaneous presence of resveratrol (30 μM) and L-NAME (30 μM) for 90 min, ACh-evoked CA release had recovered by 87~100% of the corresponding control release compared to the inhibitory effect of resveratrol-only treatment as illustrated in Fig. 5 (Upper). High K^+ (56 mM)-evoked CA release in the simultaneous presence of resveratrol (30 μM) and L-NAME (30 μM) for 90 min had also recovered by 79~100% of the corresponding control release during all periods in comparison to the data of the treatment with resveratrol alone (Fig. 5-Lower).

As shown in Fig. 6 (Upper), the simultaneous perfusion of resveratrol and L-NAME for 90 min got over the DMPP-evoked CA release to 88~100% of the control response compared to the corresponding control response in comparison to the inhibitory effect of the resveratrol-only treatment. Moreover, in the presence of resveratrol (30 μM) and L-NAME (30 μM), the CA secretory response evoked

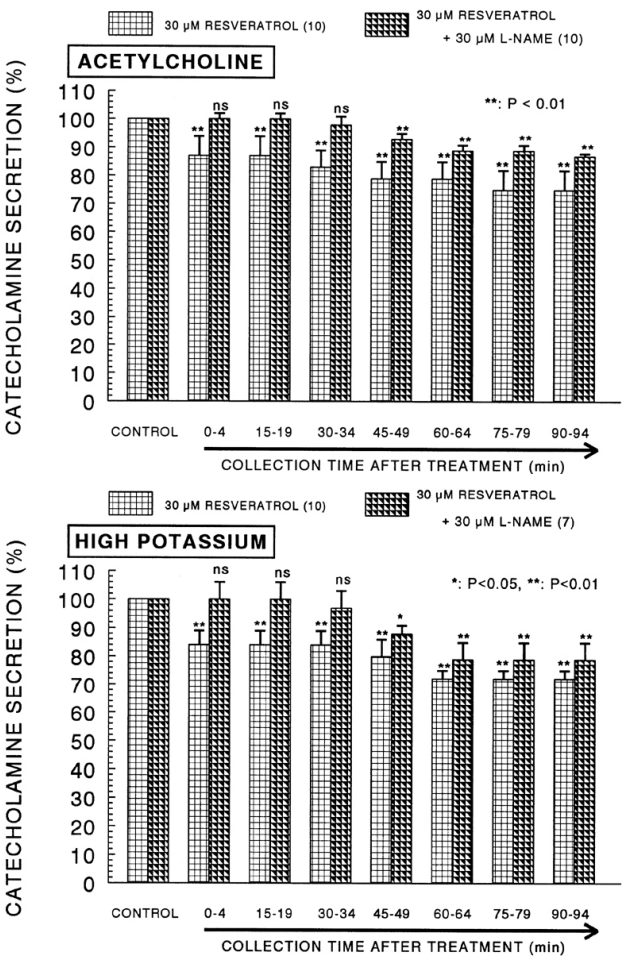

Fig. 5. Effect of resveratrol plus L-NAME on the CA secretory responses evoked by acetylcholine (upper) and high potassium (lower) from the perfused rat adrenal glands. The CA secretion by a single injection of ACh (5.32×10^{-3} M) and K^+ (5.6×10^{-2} M) in a volume of 0.05 ml was evoked at 15 min intervals during simultaneous loading with resveratrol (30 μM) plus L-NAME (30 μM) for 90 min. Statistical difference was obtained by comparing the corresponding control (CONTROL) with resveratrol-treated group or group treated with resveratrol+L-NAME. Other legends are the same as in Fig. 1. *p < 0.05, **p < 0.01. ns: not statistically significant.

by McN-A-343 (10^{-4} M for 4 min) had recovered to 76~100% of the corresponding control release compared to results of the resveratrol-only treatment, as shown in Fig. 6 (Lower).

As shown in Fig. 7 (Upper), the simultaneous perfusion of resveratrol (30 μM) and L-NAME (30 μM) for 90 min had restored the CA release evoked by Bay-K-644 (10 μM, an activator of voltage-dependent L-type calcium channel) to 75~100% of the corresponding control response compared to the inhibitory effect of the resveratrol-only treatment. After the simultaneous perfusion with resveratrol and L-NAME, cyclopiazonic acid (10 μM, an inhibitor of Ca^{2+}-ATPase of endoplasmic reticulum)-evoked CA release had also recovered by 77~100% of the control release in comparison to the results following the treatment with resveratrol alone (Fig. 7-Lower).

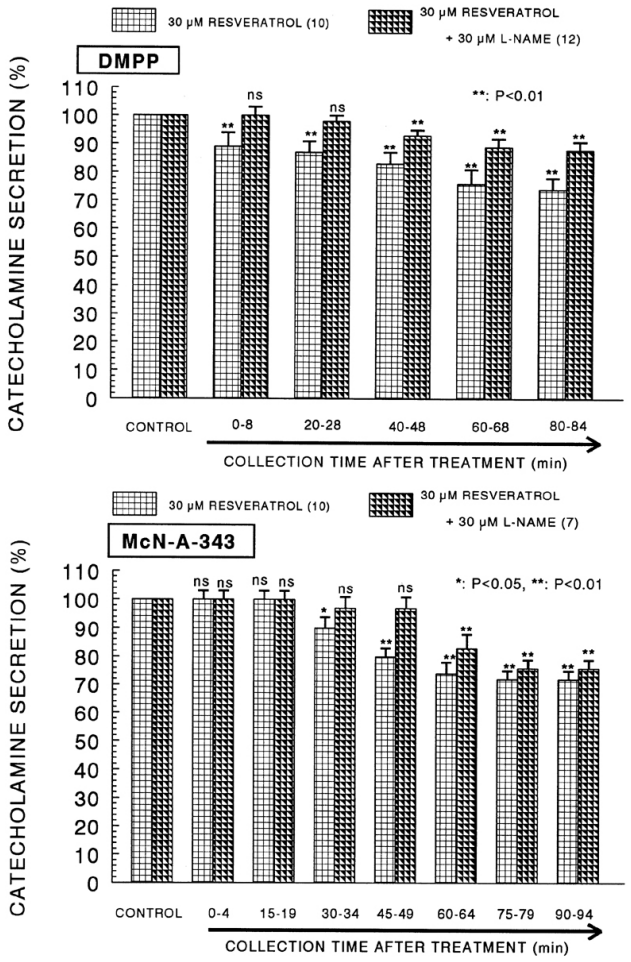

Fig. 6. Effect of resveratrol plus L-NAME on the CA secretory responses evoked by DMPP (upper) and McN-A-343 (lower) from the perfused rat adrenal glands. The CA secretion by perfusion of DPPP (10^{-4} M) and McN-A-343 (10^{-4} M) for 2 min was induced at 20 and 15 min intervals after preloading with resveratrol (30 μM) plus L-NAME (30 μM) for 90 min, respectively. Other legends are the same as in Fig. 1 and 5. **p<0.01. ns: not statistically significant.

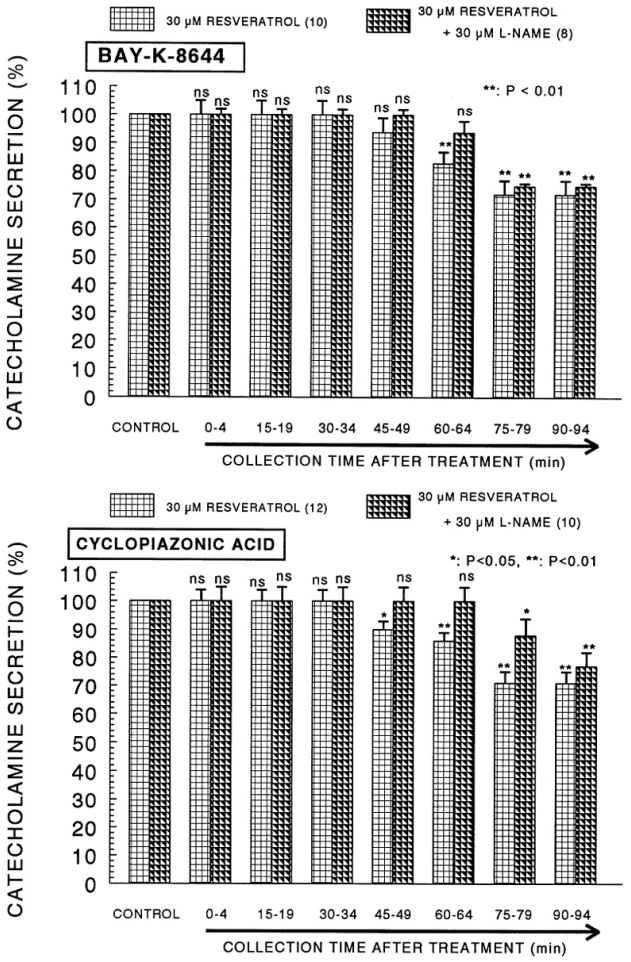

Fig. 7. Effects of resveratrol plus L-NAME on the CA secretory responses evoked by Bay-K-8644 (upper) and cyclopiazonic acid (lower) from the rat adrenal glands. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals during simultaneous loading with resveratrol (30 μM) for 90 min. Other legends are the same as in Fig. 1 and 5. *p<0.05, **p<0.01. ns: not statistically significant.

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

As shown in Fig. 5~7, it has been shown that, in the simultaneous presence of resveratrol and L-NAME (an inhibitor of NO synthase), the CA secretory responses evoked by ACh, high K^+, DMPP, McN-A-343, BAY-K-8644 and cyclopiazonioc acid were greatly recovered to the extent of the corresponding control release compared to those effects of the resveratrol-only treatment. Therefore, we attempted to determine directly the amount of nitric oxide released from adrenal medulla following the perfusion of resveratrol-containing Krebs-bicarbonate solution. As shown in Fig. 8, the basal level of NO before the loading of resveratrol was 27.4±3 picomole. However, 30 min after the presence of resveratrol (100 μM), it was greatly enhanced to 241% of the control release. Consequently, it was confirmed that resveratrol practically increase the release of NO from the rat adrenal medulla.

DISCUSSION

The present results demonstrate that resveratrol 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 gland. It seems that this inhibitory effect of resveratrol is mediated at least by inhibiting influx of both ions through Ca^{2+} and Na^+ channels into the rat adrenomedullary cells as well as by blocking the Ca^{2+} release from the cytoplasmic calcium store, which are due to the increased NO production through the activation of neuronal NO synthase.

In support of this idea, it has been reported that PCRW from two different sources, with a similar pharmacological profile in different blood vessels, are able to produce

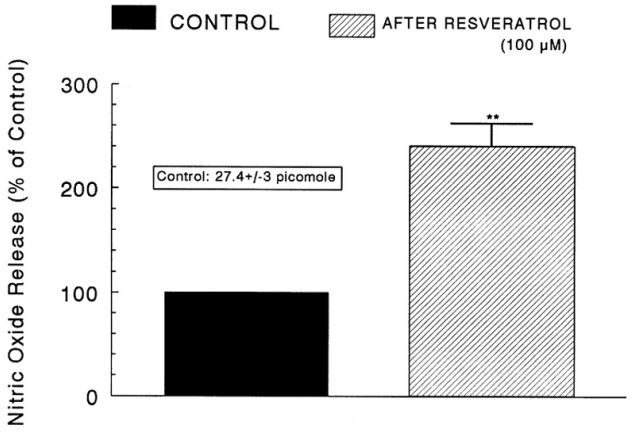

Fig. 8. Comparison of nitric oxide (NO) production before (CONTROL) and after administration of resveratrol in the isolated perfused rat adrenal medulla. Perfusate sample was taken for 8 min after loading the perfusion of resveratrol (100 μM) at a rate of 0.31 ml/min. Ordinate: the amounts of NO released from the adrenal medulla (% of control). Abscissa: treatment (before and after resveratrol). Statistical difference was made by comparing the control with resveratrol-treated group. **$p < 0.01$.

endothelium-dependent relaxation of the rat thoracic aorta (Andriambeloson et al, l997; l999). This effect was mediated by an increase in NO content due to enhancement of NO synthesis rather than protection against its breakdown by oxygen radicals associated with the antioxidant properties of PCRW (Andriambeloson et al, I997). Furthermore, studies in bovine aortic endothelial cells (BAEC) have shown that an increase in NO-synthase activity is associated with an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and the activation of tyrosine kinases (Martin et al, 2002). It has been shown that oral treatment with Provinols™ (polyphenol-containing product) accelerated the regression of blood pressure and prevented the development of cardiovascular remodeling, myocardial fibrosis and aortic stiffness in NO-deficient hypertensive rats. Furthermore, it has been found that oral administration of Provinols™ produced a decrease of systolic blood pressure in normotensive rats, which was accompanied with an enhanced endothelium-dependent relaxation and induction of gene expression within the arterial wall (Diebolt et al, 200l). This effect probably involved an NO pathway inasmuch N^{ω}-nitro-L-arginine-methyl-ester (L-NAME) treatment plus PCRW abolished the decrease in blood pressure and the improvement of endothelial function (Bernátová et al, 2002).

In the present study, in the simultaneous presence of resveratrol and L-NAME (NOS inhibitor), the CA secretory responses evoked by ACh, DMPP, high K^+, McN-A-343, Bay-K-8644 and cyclopiazonic acid were considerably recovered to the corresponding control level compared to the inhibitory effect of resveratrol treatment alone. This result is well consistent with report that PCRW 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).

Taking into account these findings, in this study, it is likely that resveratrol inhibits the CA secretory response evoked by various secretagogues through the increased NO production in adrenal chromaffin cells, but in the simultaneous presence of resveratrol and L-NAME, their secretory responses had recovered to the control level in comparison to the inhibition of resveratrol-only treatment. Furthermore, some epidemiological studies indicate that there is 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 also been shown that PCRW promotes the endothelium-dependent relaxation, activates NO synthase, inhibits platelet aggregation, and prevents 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 et al, 1993). The polyphenolic compound resveratrol present in red wine is thought to be the factor responsible for wine's beneficial cardiovascular effects. Since resveratrol has similar effects as PCRW 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). These effects are in agreement with the present result that resveratrol inhibits the CA secretory responses evoked by cholinergic stimulation and membrane depolarization at least by the increased NO production through activation of nitric oxide synthase in the isolated perfused rat adrenal medulla, since this inhibitory effect of resveratrol on the CA secretory responses was significantly attenuated in the presence of L-NAME, an inhibitor of nitric oxide synthase.

Generally, the adrenal medulla possesses characteristic postganglionic sympathetic neurons, and the presence of neuronal NOS (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 the simultaneous presence of resveratrol and L-NAME, the CA secretory responses evoked by cholinergic nicotinic and muscarinic stimulation and direct membrane-depolarization had recovered to the levels of the control secretion as compared with the inhibitory effects of resveratrol alone. This result demonstrates that resveratrol can inhibit the CA release at least partly by the inceased NO production 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 shown that L-NAME

inhibits ACh-induced CA secretion in bovine chromaffin cells (Uchiyama et al, 1994), and that the NO donor 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 resveratrol 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. The present findings that resveratrol inhibited the CA secretory responses evoked by both nicotinic receptor stimulation as well as by membrane depolarization from the rat adrenal medulla also seem 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). Therefore, these experimental results indicate that resveratrol-induced inhibitory activity of CA secretory response evoked by stimulation of nicotinic receptors may contribute at least partly to its hypotensive mechanism.

In the present study, resveratrol also depressed the CA secretory response evoked by Bay-K-8644, which is known to activate L-type voltage-dependent Ca^{2+} channels, in a time-dependent manner (Garcia et al, 1984; Schramm et al, 1983). This result indicates that resveratrol may inhibit Ca^{2+} influx into the rat adrenomedullary cells. In support of this idea, in cultured bovine adrenal medullary cells, nicotinic (but not muscarinic) receptors mediate the Ca^{2+}-dependent secretion of CA (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 and Wakade, 1983; Lim and Hwang, 1991) and isolated bovine adrenal chromaffin cells (Kilpatrick et al, 1981; 1982; Knight and Kesteven, 1983). Wada and his coworkers (1985) 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 resveratrol indicates that this inhibitory effect of resveratrol is exerted by inhibiting Ca^{2+} influx into the adrenomedullary cells through the blockade of voltage-dependent Ca^{2+} channels. Furthermore, slight elevation in the extracellular potassium concentration increases both the frequency of spontaneous action potentials and the secretion of CA (Kidokoro and Ritchie, 1980), suggesting that the influx of Ca^{2+} that occurs during action potentials is directly linked to the rate of secretion. These findings that resveratrol inhibited the CA secretion evoked by Bay-K-8644 as well as by high K^+ suggest that resveratrol directly inhibits 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, the finding that resveratrol inhibited the DMPP-evoked CA secretion seems to be associated with inhibition of Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

The present study has also shown that resveratrol also 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 and Riley, 1989; Seidler et al, 1989) and a valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic currents regulated by intracellular Ca^{2+} (Suzuki et al, 1992). Therefore, in the present work, it can be speculated that the inhibitory effect of resveratrol on CA secretion evoked by McN-A-343 may be associated with the mobilization of intracellular Ca^{2+} from the cytoplasmic calcium store. This indicates that the resveratrol 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 of Ca^{2+} load (Suzuki et al, 1992). This is consistent with the findings obtained in exposed 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 resveratrol-induced inhibition of the CA secretion evoked by McN-A-343 and cyclopiazonic acid may be due to the inhibition of Ca^{2+} release evoked by stimulation of muscarinic ACh receptors from the intracellular pools. Martin and his colleagues (2002) showed that PCRW stimulated a Ca^{2+}-dependent release of nitric oxide (NO) from bovine aortic endothelial cells accounting for the relaxation of endothelium-denuded rat aortic rings. PCRW, Provinols™ and delphinidin also increased cytosolic free calcium ($[Ca^{2+}]_i$), by releasing Ca^{2+} from intracellular stores and by increasing Ca^{2+} entry. However, in the present study, it is uncertain whether the inhibitory effect of resveratrol on Ca^{2+} movement from intracellular pools is due to its direct effect on the PI response or the indirect effects.

On the other hand, the present findings disagree with those obtained by Yanez and his coworkers (2006), who reported that both cis-resveratrol and trans-resveratrol (5～200 μM) concentration-dependently inhibited the uptake of [^{3}H]NA and [^{3}H]5-HT by synaptosomes from rat brain and the uptake of [^{3}H]5-HT by human platelets. Both *cis-*

resveratrol and trans-resveratrol (5～200 μM) also inhibited the enzymatic activity of commercial (human recombinant) MAO isoform (MAO-A and MAO-B) activity in a concentration dependent manner.

It has also been observed that these natural polyphenols, like a number of antidepressant drugs (Slotkin et al, 1986; Gareri et al, 2000; To et al, 2005), inhibit the uptake of 5-HT by human platelets. It is also well known that other classes of drugs used to treat major depressive disorders are inhibitors of monoamine oxidase (MAO) (Gareri et al, 2000; To et al, 2005). To date, however, the effects of resveratrol isomers on MAO isoform (MAO-A and MAO-B) activity have not been studied. Only Zhou and his coworkers (2001), in a preliminary structure-activity relationship study of a number of stilbenoids, have previously shown that trans-resveratrol exhibits a selective inhibitory effect on MAO-A activity but not on MAO-B.

In conclusion, the results of the present study have demonstrated that resveratrol 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 resveratrol is exerted by blocking influx of both ions through Ca^{2+} and Na^{+} channels into the adrenomedullary cells as well as by blocking the release of Ca^{2+} from the cytoplasmic calcium store, which are at least partly due to the increased NO production through the activation of nitric oxide synthase. These experimental results may contribute partly to the hypotensive effect of resveratrol, through inhibition of the CA secretion from adrenomedullary chromaffin cells and consequent reduction of the CA level in the circulation.

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