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Effects of resveratrol, a grape polyphenol, on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells

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ABSTRACT

We report the effects of resveratrol, a polyphenol found in the skins of red grapes, on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells. Resveratrol suppressed catecholamine secretion and $^{22}Na^{+}$ and $^{45}Ca^{2+}$ influx induced by acetylcholine, an agonist of nicotinic acetylcholine receptors, in a concentration-dependent manner (IC_{50} = 20.4, 11.0, and 62.8 μM, respectively). Resveratrol also inhibited catecholamine secretion induced by veratridine, an activator of voltage-dependent Na^{+} channels, and 56 mM K^{+}, an activator of voltage-dependent Ca^{2+} channels, at concentrations similar to those for $^{45}Ca^{2+}$ influx. Resveratrol directly inhibited the current evoked by acetylcholine in *Xenopus* oocytes expressing α3β4 neuronal nicotinic acetylcholine receptors (IC_{50} = 25.9 μM). Furthermore, resveratrol (IC_{50} = 5.32 μM) attenuated ^{14}C-catecholamine synthesis induced by acetylcholine. The present findings suggest that resveratrol inhibits acetylcholine-induced catecholamine secretion and synthesis through suppressing ion influx in cultured bovine adrenal medullary cells.

1. Introduction

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a natural phytoestrogen found in grapes, berries, and red wine [1,2]. Resveratrol has been reported to be implicated in the beneficial action of red wine, i.e., the lower incidence of coronary artery disease in certain populations such as the French and the Greek, despite a diet rich in saturated fat and high smoking. This effect has been dubbed the 'French Paradox', and has been attributed to the regular consumption of red wine in moderate amounts by these populations [3,4]. Indeed, resveratrol is thought to protect against atherosclerosis and coronary heart disease through various mechanisms, including vasorelaxation [5,6] and anti-platelet effects [7,8]. The precise mechanism of resveratrol for cardioprotective effects, however, remains to be determined.

Adrenal medullary cells are derived from the embryonic neural crest and share many properties with sympathetic postganglionic neurons. In cultured bovine adrenal medullary cells, there are at least three distinct types of ionic channels involved in catecholamine secretion [9]; these are (1) nicotinic acetylcholine receptor-ion channels, (2) voltage-dependent Na^{+} channels, and (3) voltage-dependent Ca^{2+} channels. In these cells, our previous studies have shown that either

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Abbreviations: KRP, Krebs–Ringer phosphate; Eagle's MEM, Eagle's minimum essential medium; PCA, perchloric acid; DMSO, dimethyl sulfoxide.

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carbachol-induced $^{22}Na^+$ influx via nicotinic acetylcholine receptor-ion channels or veratridine-induced $^{22}Na^+$ influx via voltage-dependent Na^+ channels increases $^{45}Ca^{2+}$ influx via voltage-dependent Ca^{2+} channels, a prerequisite for secretion [9] and synthesis [10] of catecholamines; in contrast, high K^+ directly gates voltage-dependent Ca^{2+} channels to increase $^{45}Ca^{2+}$ influx without increasing $^{22}Na^+$ influx. Since catecholamine secretion mediated by stimulation of these ion channels and the mechanism of stimulation of catecholamine synthesis in adrenal medullary cells are thought to be similar to those of norepinephrine in the sympathetic neurons, adrenal medullary cells have provided a good model for detailed analysis of the actions of cardiovascular drugs, such as α_2-adrenergic agonists [10], natriuretic peptides [11], carvedilol [12] and pimobendan [13].

Several lines of evidence have shown that resveratrol has inhibitory effects on some ion channels such as L-type voltage-dependent Ca^{2+} channels in rat ventricular myocytes [14], human cardiac voltage-dependent Na^+ channels [15], and voltage-activated potassium currents in rat hippocampal neurons [16]. Evidence has accumulated that aberrant acute or chronic activation of the sympathoadrenal system is involved causally in the development of various cardiovascular diseases such as heart failures [17]. Furthermore, a recent study reported that upregulation of adrenal medullary G protein-coupled receptor kinase 2 mediates sympathetic hyperactivity in heart failure through an increase in catecholamine secretion in two different models of heart diseases [18]. These evidences suggest that catecholamines play an important role in the pathological events of cardiovascular diseases. There is, however, little evidence regarding the effects of resveratrol on ion channel-mediated secretion and synthesis of catecholamines. In the present study, we examined the direct effects of resveratrol on acetylcholine-induced synthesis and secretion of catecholamines in cultured bovine adrenal medullary cells and acetylcholine-induced currents in the *Xenopus* oocytes expressing cloned $\alpha3\beta4$ nicotinic acetylcholine receptors. We found that resveratrol inhibits acetylcholine-induced catecholamine synthesis and secretion via suppression of nicotinic acetylcholine receptor-ion channels.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: Eagle's minimum essential medium (MEM), Nissui Pharmaceutical (Tokyo, Japan); calf serum, Nacalai Tesque (Kyoto, Japan); collagenase, Nitta Zerachin (Osaka, Japan); resveratrol, acetylcholine, veratridine, histamine, nitrendipine, α-scorpion venom (α-ScTx) (*Leiurus quinquestriatus*), and β-scorpion venom (β-ScTx) (*Centruroides sculpturatus*), Sigma (St. Louis, MO); *Ptychodiscus brevis* toxin-3 (PbTx-3), Latoxan (Westburg, NY), ω-conotoxin-GVIA and ω-agatoxin-IVA, Alomone Labs (Jerusalem, Israel); [^{45}Ca]Cl_2 and L-[U-^{14}C]tyrosine, GE Healthcare UK Ltd. (Little Chalfont, Bucks., UK); [^{22}Na]Cl, PerkinElmer Life Sciences (Boston, MA). Resveratrol was dissolved in 100% ethanol, and then diluted in a reaction medium before use at a final ethanol concentration not exceeding 0.5%, unless otherwise specified.

2.2. Isolation and primary culture of bovine adrenal medullary cells

Bovine adrenal medullary cells were isolated by collagenase digestion of adrenal medullary slices, as described previously [19]. Cells were suspended in Eagle's MEM containing 10% calf serum, 3 μM cytosine arabinoside and several antibiotics, and maintained in monolayer culture at a density of 4×10^6 cells/dish (Falcon, 35 mm, Becton Dickison Labware, Franklin Lakes, NJ) in 5% CO_2/95% air. The cells were used for experiments between 2 and 5 days of culture.

2.3. Catecholamine secretion from cultured bovine adrenal medullary cells

Oxygenated Krebs–Ringer phosphate (KRP) buffer (153 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaCl_2$, 0.85 mM NaH_2PO_4, 2.15 mM Na_2HPO_4 and 10 mM glucose, adjusted to pH 7.4) was used throughout. After preincubation for 10 min, cells were incubated with or without resveratrol and various secretagogues at 37 °C for 10 min. After the reaction, the incubation medium was transferred immediately to a test tube containing perchloric acid (PCA) (final concentration, 0.4 M). Catecholamines (norepinephrine and epinephrine) secreted into the medium were adsorbed onto aluminum hydroxide and estimated by the ethylenediamine condensation method using a fluorescence spectrophotometer (F-4010; Hitachi, Tokyo, Japan) with excitation and emission wavelengths of 420 and 540 nm, respectively [19].

2.4. $^{22}Na^+$ and $^{45}Ca^{2+}$ influx by the cells

The influx of $^{22}Na^+$ and $^{45}Ca^{2+}$ was measured as reported previously [9,13]. After preincubation for 10 min, cells were incubated with 1.5 μCi of $^{22}NaCl$ or 1.5 μCi of $^{45}CaCl_2$ at 37 °C for 10 min with or without the various secretagogues and resveratrol in KRP buffer. Then, the cells were washed 4 times with ice-cold KRP buffer, solubilized in 10% Triton X-100, and counted for radioactivity of $^{22}Na^+$ and $^{45}Ca^{2+}$.

2.5. ^{14}C-Catecholamine synthesis from [^{14}C]tyrosine in the cells

After preincubation for 10 min, cells were incubated with 20 μM L-[U-^{14}C] tyrosine (1 μCi) in KRP buffer in the presence or absence of various concentrations of resveratrol and 0.3 mM acetylcholine at 37 °C for 20 min. After removing the incubation medium by aspiration, cells were harvested in 0.4 M PCA and centrifuged at 1600 × *g* for 10 min. ^{14}C-Labelled catechol compounds were separated further by ion exchange chromatography on Duolite C-25 columns (H^+-type, 0.4 × 7.0 cm) and counted for radioactivity [20]. ^{14}C-Catecholamine synthesis was expressed as the sum of the ^{14}C-catecholamines (epinephrine, norepinephrine and dopamine), because the ratio of ^{14}C-epinephrine plus ^{14}C-norepinephrine/^{14}C-dopamine was not changed significantly by resveratrol and/or acetylcholine.

2.6. Expression of acetylcholine receptors in Xenopus oocytes and electrophysiological recordings

Isolation and microinjection of *Xenopus* oocytes were performed as described previously [21]. The cDNAs encoding the α3 and β4 subunits of rat neuronal nicotinic acetylcholine receptor, subcloned into pcDNAI/Neo (Invitrogen, Carlsbad, CA) vector, were kindly provided from Dr. James W. Patrick (Division of Neuroscience, Baylor College of Medicine, TX). Oocytes were injected with cDNAs (1.5 ng/30 nl) and electrophysiological recordings were performed 2–3 days after injection. Each oocyte was perfused (2 ml min^{-1}) with Ba^{2+}-Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$ and 10 mM HEPES, pH 7.4) containing 1 μM atropine sulfate, to minimize the effects of secondarily activated Ca^{2+}-dependent Cl^- currents, then impaled with two glass electrodes (1–5 MΩ) filled with 3 M KCl and clamped at −70 mV using the OC-725C Oocyte Clamp Amplifier (Harvard Apparatus, Inc., Holliston, MA). Acetylcholine was applied for 30 s to obtain the maximum (peak) current used as a measure of drug response. We tested the capacity of resveratrol to modify the effect of a concentration of acetylcholine that produced 50% of the maximal effect (EC_{50}) of acetylcholine. This EC_{50} was determined individually for each oocyte, using 1 mM acetylcholine to produce a maximal current. Resveratrol was first dissolved in dimethyl sulfoxide (DMSO), then diluted in Ba^{2+}-Ringer's solution before use at a final DMSO concentration not exceeding 0.05%, which had no effect on acetylcholine-evoked responses (data not shown). Resveratrol was pre-applied for 2 min before coapplication of acetylcholine to allow for complete equilibration of the oocytes with resveratrol. In all cases, a 10- to 15-min washout period was allowed following application of the resveratrol and acetylcholine solutions.

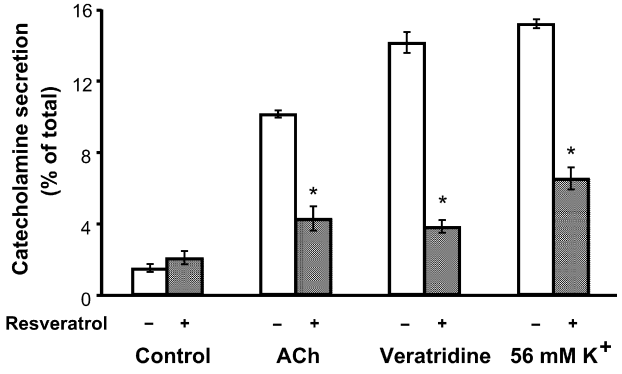

Fig. 1 – Effects of resveratrol on catecholamine secretion induced by acetylcholine, veratridine and 56 mM K^+. After preincubation with or without resveratrol (100 μM) for 10 min, cells were stimulated with or without acetylcholine (300 μM), veratridine (100 μM) and 56 mM K^+ in the presence or absence of resveratrol (100 μM) for 10 min at 37 °C. Catecholamines secreted into the medium were measured, and expressed as a percentage of the total catecholamines (3.17 ± 0.63 μg/10^6 cells) in the cells. Data are means ± S.E.M. from three to five separate experiments carried out in triplicate. *P < 0.001, compared with each secretagogue alone.

2.7. Statistics

All experiments were performed in duplicate or triplicate and each experiment was repeated at least three times. All values are given as means ± S.E.M. The significance of differences between means was evaluated using Student's *t*-test or two-way ANOVA. When a significant *F* value was found by ANOVA, Dunnett's or Scheffé's test for multiple comparisons was used to identify differences among the groups. Values were considered statistically different when *P* was less than 0.05. Statistical analyses were performed using StatView for Macintosh version 5.0J software (Abacus Concept, Berkely, CA). Curve fitting for concentration/response curves was performed using GraphPad Prism software (San Diego, CA).

3. Results

3.1. Effects of resveratrol on various secretagogue-induced catecholamine secretion in cultured bovine adrenal medullary cells

In bovine adrenal medullary cells, stimulation of nicotinic acetylcholine receptor-ion channels with acetylcholine, a physiological secretagogue, caused catecholamine secretion corresponding to 10.7 ± 0.6% of the total catecholamines in the cells (Fig. 1). Veratridine (0.1 mM), an activator of voltage-dependent Na^+ channels, or 56 mM K^+, an activator of voltage-dependent Ca^{2+} channels, caused catecholamine secretion corresponding to 14.0 ± 0.5% and 15.0 ± 0.5% of the total catecholamines, respectively. We examined the effect of resveratrol (100 μM) on catecholamine secretion induced by acetylcholine, veratridine and 56 mM K^+. Pretreatment of cells with resveratrol at 100 μM for 10 min significantly inhibited catecholamine secretion induced by acetylcholine, veratridine and 56 mM K^+ to 42.4, 27.3 and 43.1% of each secratogogue alone, respectively (Fig. 1). Resveratrol (100 μM) did not significantly affect basal secretion of catecholamines (control = 1.54 ± 0.23%; resveratrol = 2.11 ± 0.39% of the total catecholamines). We further examined the time course of resveratrol effect on catecholamine secretion induced by acetylcholine. Pretreatment of cells with resveratrol (100 μM) for 0, 5, 10 and 20 min decreased the secretion of catecholamines induced by acetylcholine to 75, 52, 41 and 37% of acetylcholine alone, respectively (unpublished observation).

3.2. Concentration-inhibition curves for the effects of resveratrol on various secretagogue-induced catecholamine secretion and $^{22}Na^+$ and $^{45}Ca^{2+}$ influx in cultured bovine adrenal medullary cells

Pretreatment of cells with resveratrol at 0.1, 1, 10, and 100 μM for 10 min significantly reduced acetylcholine-induced secretion of catecholamines to 82.4, 71.5, 64.9, and 42.4% of acetylcholine alone, respectively (IC_{50} = 20.4 μM) (Fig. 2A). Resveratrol suppressed $^{22}Na^+$ influx (IC_{50} = 11.0 μM) and $^{45}Ca^{2+}$ influx (IC_{50} = 62.8 μM) induced by acetylcholine in a concentration-dependent manner (Fig. 2B and C). Resveratrol also inhibited catecholamine secretion (IC_{50} = 5.83 μM), $^{22}Na^+$ influx (IC_{50} = 88.7 μM), and $^{45}Ca^{2+}$ influx (IC_{50} = 9.10 μM)

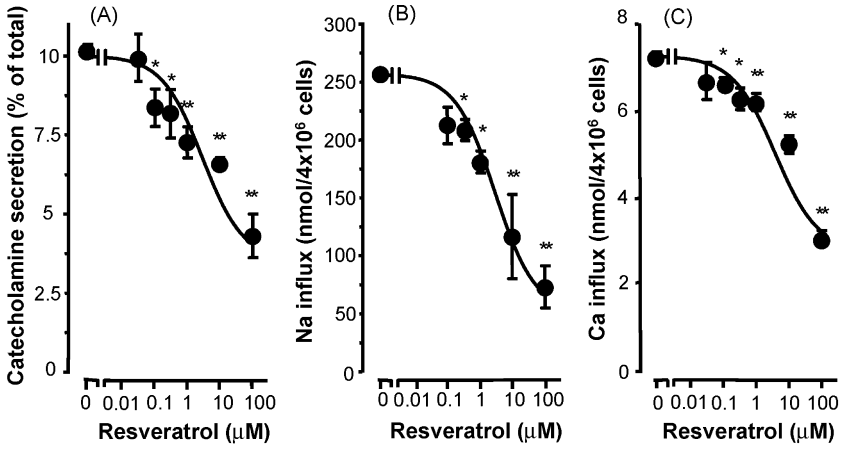

Fig. 2 – Effects of resveratrol on acetylcholine-induced catecholamine secretion (A), $^{22}Na^+$ influx (B) and $^{45}Ca^{2+}$ influx (C). (A) After preincubation with resveratrol (0–100 μM) for 10 min, cells were stimulated with or without acetylcholine (300 μM) in the presence or absence of resveratrol (0.03–100 μM) for 10 min at 37 °C. Catecholamines secreted into the medium were measured, and expressed as a percentage of the total catecholamines in the cells. (B, C) Cells were incubated with acetylcholine (300 μM) and 1.5 μCi of $^{22}NaCl$ (B) or $^{45}CaCl_2$ (C) for 10 min at 37 °C in the presence or absence of resveratrol (0.1 or 0.03–100 μM). The influx of $^{22}Na^+$ and $^{45}Ca^{2+}$ measured are expressed as nmol/4 × 10^6 cells. Data are means ± S.E.M. from three to four separate experiments carried out in triplicate. *P < 0.01 and **P < 0.001, compared with acetylcholine alone.

induced by veratridine (Fig. 3A–C). Furthermore, resveratrol suppressed catecholamine secretion (IC_{50} = 35.9 μM) and $^{45}Ca^{2+}$ influx (IC_{50} = 184 μM) induced by 56 mM K^+ in a concentration-dependent manner (Fig. 4A and B).

3.3. *Inhibitory mode of resveratrol on catecholamine secretion induced by acetylcholine*

We attempted to determine whether resveratrol was competing with acetylcholine for the binding sites on the nicotinic acetylcholine receptors. When the concentrations of acetylcholine in the incubation medium increased, resveratrol-induced inhibition of catecholamine secretion was not overcome by these concentrations (3–300 μM) (Fig. 5A). Double-reciprocal plot analysis showed that resveratrol exerts a noncompetitive type of inhibition with respect to acetylcholine (Fig. 5B), indicating that resveratrol does not compete with acetylcholine for the binding site on nicotinic acetylcholine receptors.

3.4. *Effects of resveratrol on acetylcholine responses in Xenopus oocytes expressing nicotinic acetylcholine receptors*

The direct effects of resveratrol on acetylcholine responses in *Xenopus* oocytes expressing rat α3β4 nicotinic acetylcholine receptors were examined. As shown in Fig. 6, resveratrol reversibly inhibited acetylcholine-induced currents in a concentration-dependent manner, and a significant inhibition was observed at resveratrol concentrations of 3–100 μM (IC_{50} = 25.9 μM).

3.5. *Inhibitory mode of resveratrol on catecholamine secretion induced by veratridine or 56 mM K^+*

The voltage-dependent Na^+ channels consist of the principal α-subunit, which is associated with a noncovalently attached $β_1$-subunits, and a disulfide-linked $β_2$-subunit [22]. The α-subunits issued from a large multigene contain the ion-pore and the toxin binding sites, i.e., site 1 for tetrodotoxin, site 2 for veratridine, site 3 for α-Scorpion toxin (α-ScTx), site 4 for β-Scorpion toxin (β-ScTx), and site 5 for *P. brevis* toxin-3 (PbTx-3) [22]. To characterize the pharmacological action of resveratrol on voltage-dependent Na^+ channels, we examined the effect of resveratrol on catecholamine secretion induced by various concentrations of veratridine. When the concentration of veratridine increased, resveratrol-induced inhibition of catecholamine secretion was not overcome by these concentrations (1–500 μM) of veratridine (Fig. 7A). We further examined the effect of resveratrol on catecholamine secretion induced by veratridine and three toxins such as α-ScTx, β-ScTx and PbTx-3. As shown in Fig. 7B, veratridine (100 μM) caused catecholamine secretion corresponding to 20% of the total catecholamines, which was inhibited by resveratrol (100 μM) to 54%. In the presence of veratridine (100 μM), α-ScTx, β-ScTx and PbTx-3 further enhanced catecholamine secretion corresponding to 35, 27 and 32% of the total catecholamines. Resveratrol completely inhibited the stimulatory effect of β-ScTx on veratridine-induced catecholamine secretion, whereas α-ScTx and PbTx-3 stimulated catecholamine secretion induced by veratridine even in the presence of resveratrol.

It is reported that at least three types of voltage-dependent Ca^{2+} channels (L-, N- and P-type) are involved in the secretion of catecholamines induced by 56 mM K^+ in bovine adrenal medullary cells [23]. Using three specific inhibitors of voltage-dependent Ca^{2+} channels such as nitrendipine (for L-type), ω-agatoxin-IVA (for P-type) and ω-conotoxin-GVIA (for N-type) at the maximally effective concentration, we examined the effect of combination of resveratrol with various inhibitors of voltage-dependent Ca^{2+} channels on catecholamine secretion induced by 56 mM K^+. Fig. 8 showed that the degree of inhibition observed with combination of resveratrol (100 μM)

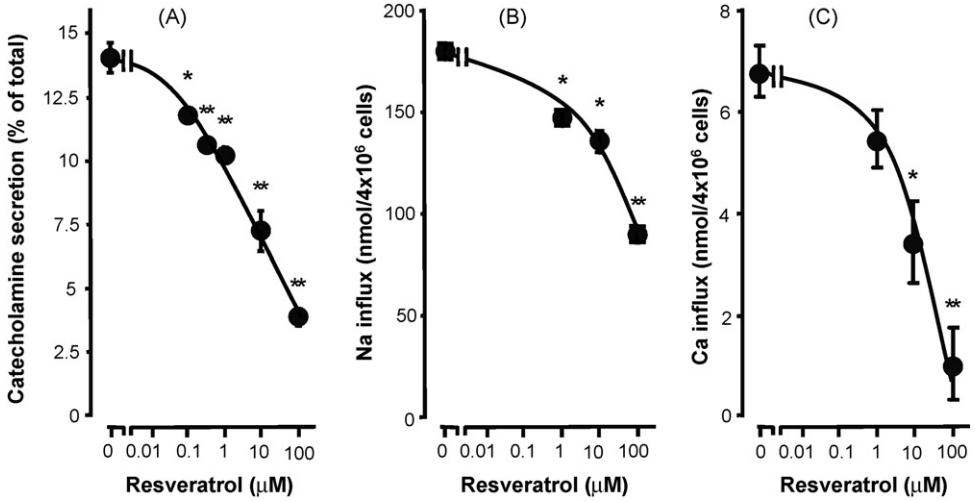

Fig. 3 – Effects of resveratrol on veratridine-induced catecholamine secretion (A), $^{22}Na^{+}$ influx (B) and $^{45}Ca^{2+}$ influx (C). (A) After preincubation with resveratrol (0–100 μM) for 10 min, cells were stimulated with veratridine (0.1 mM) in the presence or absence of resveratrol (0.1–100 μM) for 10 min at 37 °C. Catecholamines secreted into the medium were measured, and expressed as a percentage of the total catecholamines in the cells. (B, C) Cells were incubated with veratridine (0.1 mM) and 1.5 μCi of $^{22}NaCl$ (B) or $^{45}CaCl_2$ (C) for 10 min at 37 °C in the presence or absence of resveratrol (1–100 μM). The influx of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ measured is expressed as nmol/4 × 10^6 cells. Data are means ± S.E.M. from three separate experiments carried out in triplicate. *P < 0.01 and **P < 0.001, compared with veratridine alone.

with nitrendipine (10 μM) or ω-agatoxin-IVA (100 nM) on 56 mM K^{+}-induced secretion of catecholamines was significantly larger than that of resveratrol alone. In contrast, combination of resveratrol (100 μM) with ω-conotoxin-GVIA (10 μM) did not produce any further inhibition, compared to that caused by resveratrol alone.

3.6. *Effects of resveratrol on catecholamine secretion induced by histamine*

In order to investigate the effect of resveratrol on the function of another type of receptor involved in the mobilization of intracellular Ca^{2+}, we used histamine [24]

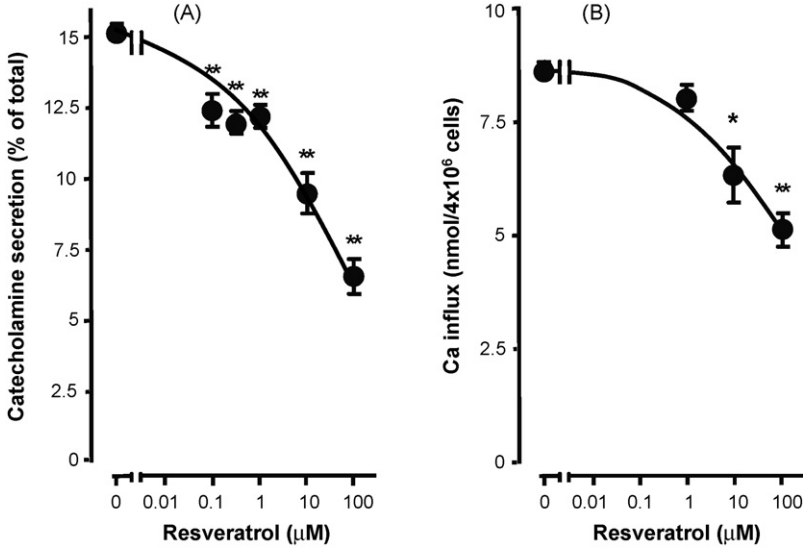

Fig. 4 – Effects of resveratrol on high K^{+}-induced catecholamine secretion (A) and $^{45}Ca^{2+}$ influx (B). (A) Cells were pretreated with resveratrol (0–100 μM) for 10 min, and then stimulated with high K^{+} (56 mM) in the presence or absence of resveratrol (0.1–100 μM) for 10 min at 37 °C. Catecholamines secreted into the medium were measured, and expressed as a percentage of the total catecholamines in the cells. (B) Cells were incubated with high K^{+} (56 mM) and 1.5 μCi of $^{45}CaCl_2$ for 10 min at 37 °C in the presence of resveratrol (1–100 μM). The influx of $^{45}Ca^{2+}$ measured is expressed as nmol/4 × 10^6 cells. Data are means ± S.E.M. from three separate experiments carried out in triplicate. *P < 0.01 and **P < 0.001, compared with high K^{+} alone.

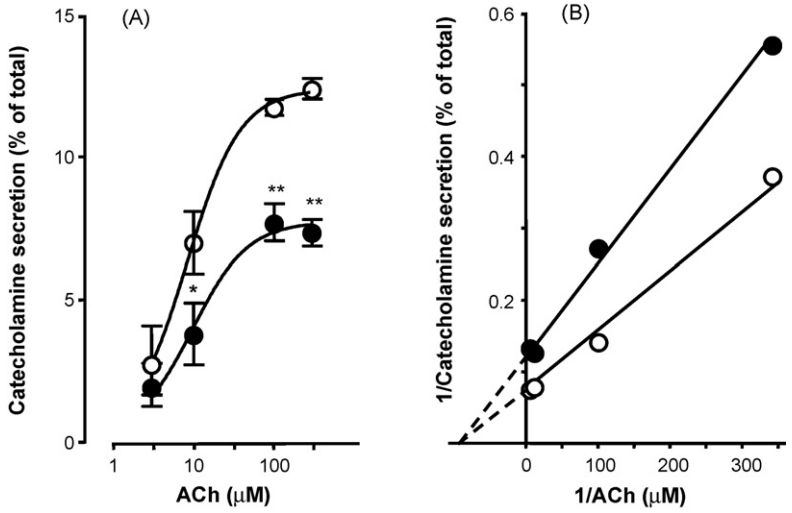

Fig. 5 – Characterization of the inhibitory effect of resveratrol on catecholamine secretion induced by acetylcholine. (A) After preincubation for 10 min with or without resveratrol (100 μM), cells were incubated with (●) or without (○) resveratrol (100 μM) in the presence of acetylcholine (ACh) (3–300 μM). Catecholamines secreted into the medium were measured, and expressed as a percentage of the total catecholamines in the cells. Data are means ± S.E.M. from three separate experiments carried out in triplicate. *P < 0.01 and **P < 0.001, compared with ACh alone. (B) Double-reciprocal plot analysis of the data in (A).

as a secretagogue and examined the effect of resveratrol on histamine-induced catecholamine secretion. Resveratrol (100 μM) significantly suppressed catecholamine secretion induced by histamine in the presence of extracellular Ca^{2+}, whereas resveratrol failed to inhibit catecholamine secretion induced by histamine in a Ca^{2+}-free medium (Fig. 9).

3.7. *Effects of resveratrol on basal and acetylcholine-induced ^{14}C-catecholamine synthesis from [^{14}C]tyrosine in the cells*

The adrenal medullary cells were incubated with 20 μM [^{14}C]tyrosine in KRP buffer in the presence or absence of various concentrations of resveratrol at 37 °C for 20 min.

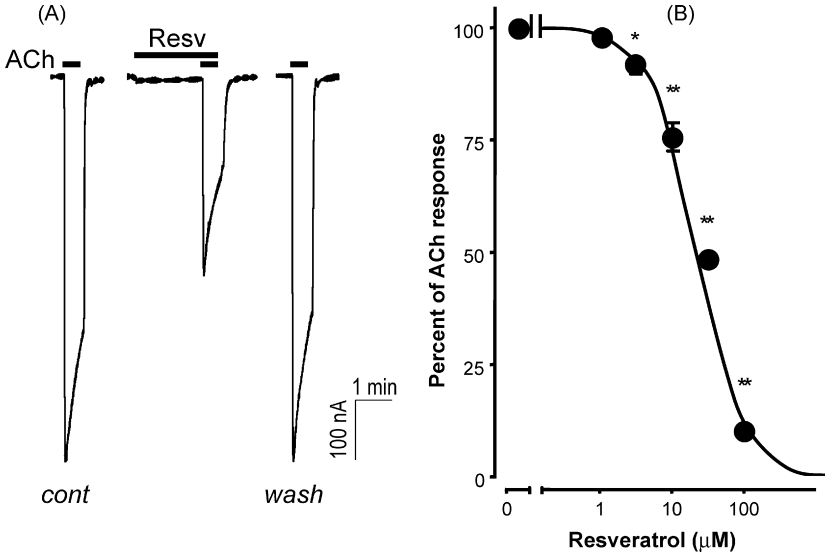

Fig. 6 – Effects of resveratrol on acetylcholine response in nicotinic acetylcholine receptors expressed in *Xenopus* oocytes (A) and its concentration-inhibition curve (B). (A) Representative tracings obtained from a single *Xenopus* oocyte expressing rat α3β4 nicotinic acetylcholine receptors demonstrate an inhibitory effect of resveratrol (Resv, 30 μM) on the current induced by the EC_{50} of acetylcholine (ACh). (B) The effect of resveratrol on acetylcholine-evoked currents is significant (*P < 0.01 and **P < 0.001, using one-way ANOVA with Dunnett's multiple comparison *post hoc* test) at concentrations of 3 μM and higher. Nonlinear regression analysis was performed and the mean value of IC_{50} for resveratrol is 25.9 μM. All values are represented as mean ± S.E.M. from five to seven oocytes. In some cases, the error bars are smaller than the points.

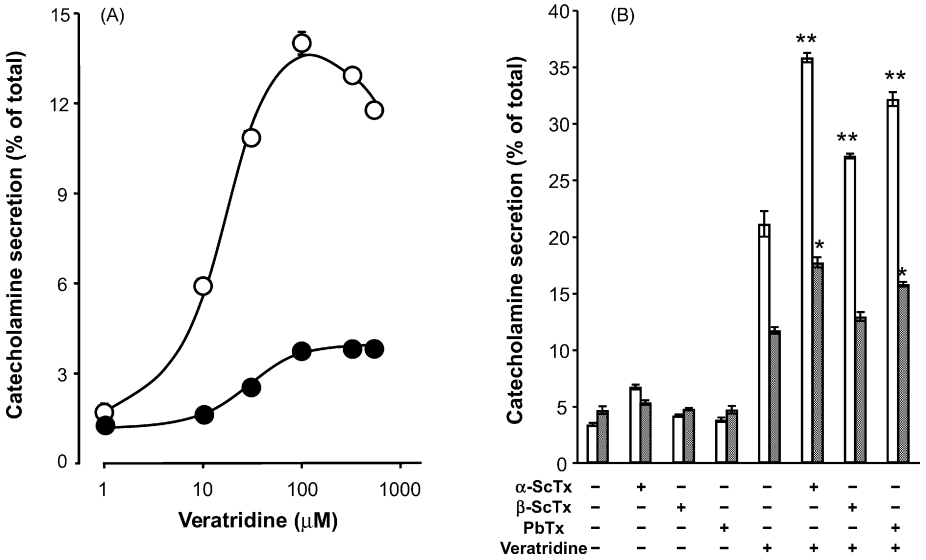

Fig. 7 – Effects of resveratrol on catecholamine secretion induced by veratridine, α-ScTx β-ScTx and PbTx-3. (A) After preincubation for 10 min with or without resveratrol (100 μM), cells were incubated with (●) or without (○) resveratrol (100 μM) in the presence of veratridine (1–500 μM). (B) After preincubation for 10 min with (■) or without (□) resveratrol (100 μM), cells were incubated with or without resveratrol (100 μM), α-scorpion toxin (α-ScTx) (0.5 μg/ml), β-scorpion toxin (β-ScTx) (0.5 μg/ml), *Ptychodiscus brevis* toxin-3 (PbTx-3) (1 μM), and/or veratridine (100 μM). Catecholamines secreted into the medium were measured, and expressed as a percentage of the total catecholamines in the cells. Data are means ± S.E.M. from three separate experiments carried out in triplicate. *P < 0.01 compared with veratridine plus resveratrol and **P < 0.001, compared with veratridine alone.

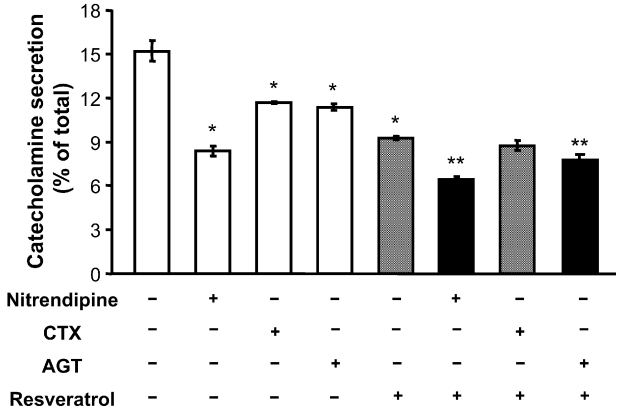

Fig. 8 – Effects of combinations of resveratrol and various inhibitors of Ca^{2+} channels on catecholamine secretion induced by 56 mM K^+.
After pretreatment with or without resveratrol (100 μM) for 10 min, cells were incubated with 56 mM K^+ for 10 min at 37 °C in the presence or absence of nitrendipine (10 μM), ω-conotoxin-GVIA (CTX) (10 μM), ω-agatoxin-IVA (AGT) (100 nM) and/or resveratrol (100 μM). The effects of resveratrol plus nitrendipine, ω-conotoxin-GVIA or ω-agatoxin-IVA were compared with those of resveratrol alone. Catecholamines secreted into the medium were measured. Data are means ± S.E.M. from three separate experiments carried out in duplicate. *P < 0.001, compared with 56 mM K^+ alone and **P < 0.01, compared with 56 mM K^+ plus resveratrol.

Resveratrol at 0.1 and 1.0 μM caused a small but significant ($P < 0.05$) increase (10–15% over the control) in basal ^{14}C-catecholamine synthesis from [^{14}C]tyrosine, while it (100 μM) inhibited basal synthesis (Fig. 10). Acetylcholine (0.3 mM) increased ^{14}C-catecholamine synthesis by 496% over the control, which was significantly suppressed by resveratrol (1–100 μM) in a concentration-dependent manner (IC_{50} = 5.32 μM).

4. Discussion

4.1. *Inhibition by resveratrol of various ion channel-mediated catecholamine secretion*

The present study demonstrated that resveratrol at concentrations of 0.1–100 μM inhibits catecholamine secretion induced by acetylcholine and veratridine in adrenal medullary cells. We have previously reported that Na^+ influx through nicotinic acetylcholine receptor-ion channels and voltage-dependent Na^+ channels stimulates Ca^{2+} influx through voltage-dependent Ca^{2+} channels and evokes the secretion of catecholamines in adrenal medullary cells [9,12]. Resveratrol inhibited $^{22}Na^+$ influx, $^{45}Ca^{2+}$ influx, and catecholamine secretion induced by acetylcholine and veratridine in a concentration-dependent manner (Figs. 2 and 3). These findings suggest that resveratrol reduces acetylcholine- and veratridine-evoked secretion of catecholamines, primarily by inhibiting the Na^+ influx through nicotinic acetylcholine receptor-ion channels and voltage-dependent Na^+ channels, respectively. Resveratrol inhibited $^{45}Ca^{2+}$ influx and catecho-

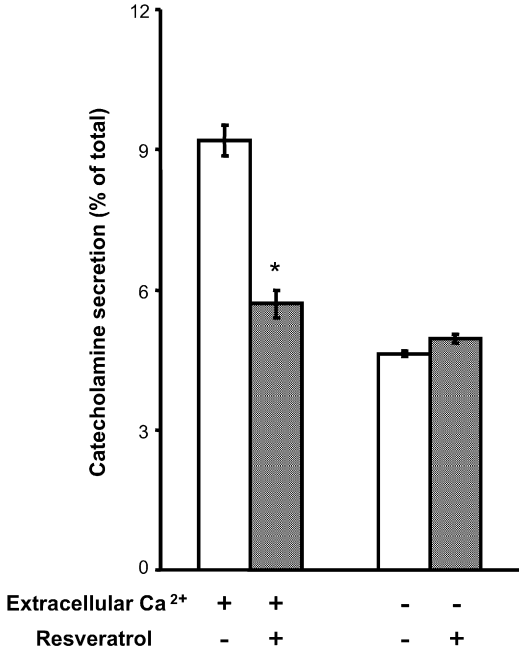

Fig. 9 – Effects of resveratrol on catecholamine secretion induced by histamine. After pretreatment with or without resveratrol (100 μM) for 10 min, cells were incubated with or without histamine (100 μM) and/or resveratrol (100 μM) for 20 min at 37 °C in the presence or absence of extracellular Ca^{2+}. Catecholamines secreted into the medium were measured. Data are means ± S.E.M. from three separate experiments carried out in duplicate. *P < 0.001, compared with histamine alone.

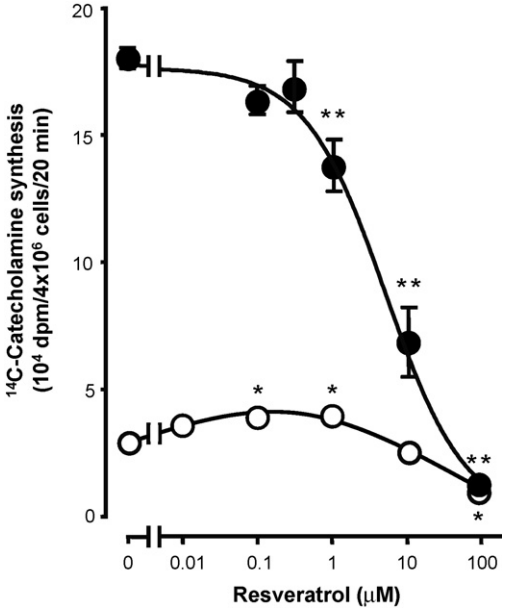

Fig. 10 – Effects of resveratrol on basal and acetylcholine-induced ^{14}C-catecholamine synthesis from [^{14}C] tyrosine. After preincubation for 10 min with or without resveratrol (0.01–100 μM), cells were incubated with various concentrations of resveratrol and 20 μM L-[U-^{14}C] tyrosine (1 μCi) in KRP buffer in the presence (●) or absence (○) of 0.3 mM acetylcholine at 37 °C for 20 min. After removing the incubation medium by aspiration, cells were harvested in 0.4 M PCA and centrifuged at 1600 × *g* for 10 min. ^{14}C-Labelled catechol compounds were separated further by ion exchange chromatography on Duolite C-25 columns (H^+-type, 0.4 × 7.0 cm) and counted for radioactivity [19]. ^{14}C-Catecholamine synthesis was expressed as the sum of the ^{14}C-catecholamines (epinephrine, norepinephrine and dopamine). Data are means ± S.E.M. from three to six separate experiments carried out in duplicate. *P < 0.01, compared to control and **P < 0.01 compared to ACh alone.

lamine secretion induced by 56 mM K^+ (Fig. 4), an activator of voltage-dependent Ca^{2+} channels, suggesting that resveratrol also inhibits 56 mM K^+-evoked catecholamine secretion and Ca^{2+} influx through voltage-dependent Ca^{2+} channels. To the best of our knowledge, this is the first evidence that resveratrol inhibits all three ion channels to suppress catecholamine secretion in cultured bovine adrenal medullary cells.

4.2. *Inhibitory modes of resveratrol on catecholamine secretion by secretagogues*

We investigated the mechanism or site of action of the effects of resveratrol on nicotinic acetylcholine receptor-ion channels. Even when the concentrations of acetylcholine were increased, the inhibitory effects of resveratrol on acetylcholine-induced secretion of catecholamines were not overcome. These findings suggest that resveratrol does not compete with acetylcholine at nicotinic acetylcholine receptors and acts at a site different from that for acetylcholine binding on nicotinic acetylcholine receptors. In the *Xenopus* oocytes expressing α3β4 nicotinic acetylcholine receptors, resveratrol inhibited acetylcholine-induced Na^+ currents in a concentration-dependent manner similar to that observed in adrenal medullary cells. From these results, it seems that resveratrol interferes with nicotinic acetylcholine receptor-ion channels and inhibits catecholamine secretion from adrenal medullary cells.

Several previous studies reported that resveratrol inhibits voltage-dependent Na^+ channels in tsA201 cells expressed with recombinant human heart Na_v 1.5 channels [15] and in rat dorsal root ganglion neurons [25]. In the present study, resveratrol-induced inhibition of catecholamine secretion was not overcome by increasing concentrations (1–500 μM) of veratridine, suggesting that resveratrol does not compete with veratridine for the site 2 of α-subunit of Na^+ channels. Resveratrol completely abolished the stimulatory effect of β-ScTx on veratridine-induced catecholamine secretion, whereas even in the presence of resveratrol, α-ScTx and PbTx-3 still increased the secretion induced by veratridine. This result gives rise to the possibility that resveratrol inhibits veratridine-induced catecholamine secretion, at least in part, via acting on the site 4 of the channel α-subunits. To confirm this possibility, further studies of resveratrol on site 4 of the channels will be required in future.

On the other hand, using three specific inhibitors of voltage-dependent Ca^{2+} channels such as nitrendipine (for

L-type), ω-agatoxin-IVA (for P-type) and ω-conotoxin-GVIA (for N-type), we demonstrated that the degree of inhibition observed with combination of resveratrol (100 μM) with nitrendipine (10 μM) or ω-agatoxin-IVA (100 nM) on 56 mM K^+-induced secretion of catecholamines was significantly larger than that of resveratrol alone, whereas combination of resveratrol (100 μM) with ω-conotoxin-GVIA (10 μM) did not produce any further inhibition. It suggests that resveratrol and ω-conotoxin-GVIA inhibit the same Ca^{2+} channels, that is, resveratrol attenuates 56 mM K^+-induced secretion of catecholamines preferentially by inhibiting N-type Ca^{2+} channels in the cells. This is, however, incompatible with a recent report showing an inhibitory effect of resveratrol on L-type Ca^{2+} current in rat ventricular myocytes [14]. The discrepancy of the data between them might be due to several reasons such as tissue or species differences, although the reason is not clear at present.

To examine the effect of resveratrol on the mobilization of intracellular Ca^{2+}, we used histamine, which is known to increase Ca^{2+} influx from the extracellular medium [26] and the formation of inositol triphosphate in bovine adrenal medullary cells [24], the latter of which induces Ca^{2+} mobilization from the intracellular Ca^{2+} stores. In the present study, we observed that resveratrol suppressed catecholamine secretion induced by histamine in the presence of extracellular Ca^{2+}, but not in Ca^{2+}-free medium. This suggests that resveratrol interferes with Ca^{2+} influx from the extracellular medium, but not Ca^{2+} mobilization from the intracellular Ca^{2+} stores.

4.3. *Dual effects of resveratrol on catecholamine synthesis*

We recently reported that treatment of cultured bovine adrenal medullary cells with environmental estrogenic pollutants increases catecholamine synthesis probably through plasma membrane estrogen receptors [20]. Furthermore, we showed the occurrence and pharmacological characterization of plasma membrane estrogen receptors in the bovine adrenal medullary cells [27]. Since the chemical structure of resveratrol, a stilbene, is similar to that of the synthetic estrogen diethylstilbestrol, resveratrol has been characterized as a phytoestrogen to activate nuclear estrogen receptors [2]. In the present study, we observed that resveratrol at low concentrations of 0.1–1.0 μM slightly enhances basal ^{14}C-catecholamine synthesis, which might be due to activation of plasma membrane estrogen receptors by resveratrol, as shown by 17β-estradiol [27]. On the other hand, at high concentrations (≥100 μM and ≥1 μM) resveratrol inhibits basal and acetylcholine-induced catecholamine synthesis, respectively. The inhibition of acetylcholine-induced catecholamine synthesis by resveratrol seems to be caused primarily by the suppression of Na^+ and subsequent Ca^{2+} influx through nicotinic acetylcholine receptor-ion channels and voltage-dependent Ca^{2+} channels.

In our recent study, we reported that capsaicin inhibited catecholamine secretion induced by carbachol, veratridine and 56 mM K^+ through a vanilloid receptor-independent pathway [28]. Capsaicin also suppressed both basal and carbachol-stimulated ^{14}C-catecholamine synthesis in the cells. These observations induced by capsaicin seem to be similar to that of resveratrol. In our previous report, because of very high concentrations used and the lipophilic property of capsaicin, we considered that the mechanism by which capsaicin can block various ion channels underlies the promiscuous regulation of membrane protein function through the changes in elasticity of the membrane bilayer [28]. Furthermore, a previous review proposed that in addition to specific receptor-mediated actions, steroid hormones such as estrogens at high concentrations (≥10 μM) could be inserted into bilayers of cellular membranes and direct steroid-membrane interactions alter the physicochemical membrane properties such as the fluidity and the microenvironment of membrane receptors or ion channels [29]. Therefore, it is possible that resveratrol, one of phytoestrogens, interacts with three ion channels via changing the membrane properties in adrenal medullary cells. In the present study, however, we found that (i) resveratrol selectively inhibits catecholamine secretion induced by veratridine plus β-ScTx, at least in part, via acting on the site 4 of voltage-dependent Na^+ channel α-subunits, (ii) resveratrol preferentially suppresses N-type voltage-dependent Ca^{2+} channels and (iii) resveratrol at low concentrations (0.1–1.0 μM) stimulates basal ^{14}C-catecholamine synthesis probably through activation of plasma membrane estrogen receptors. From these results, resveratrol may not exert its effects on catecholamine synthesis and secretion merely by a nonspecific action on the membrane properties, although its precise mechanism remains to be determined.

4.4. *Pharmacological significance of resveratrol-induced suppression of catecholamine synthesis and secretion*

Resveratrol is found in the skin of grapes and is relatively abundant in red wines with concentrations estimated to range from 1 to 10 mg/l [30] or 0.05–8.5 mg/l [31], although higher or lower values are frequently found. The oral absorption of 25 mg of resveratrol/70 kg subject in white wine, grape juice, and vegetable juice was studied in healthy men. The peak concentration of resveratrol in the serum was ≃40 nM at 30 min after consumption [32,33], suggesting that a few glasses of red wine could be a source of measurable resveratrol concentrations (≃10 nM) in the serum. In the present study, we found that resveratrol (≥0.1 and ≥1 μM) significantly inhibits acetylcholine-induced catecholamine secretion and synthesis, respectively. Therefore, the concentrations of resveratrol in the plasma of individuals who consume red wine may be lower than those required to suppress catecholamine synthesis and secretion suggested by the present study. Dietary polyphenols, however, may accumulate in tissues, resulting in higher local concentrations of resveratrol. For example, resveratrol concentrations have been reported to be 2.4-fold higher in mouse liver and heart than the plasma concentrations [34]. Therefore, it is possible that prolonged consumption of red wine in the diet could lead to increased concentrations of resveratrol, which may affect the synthesis and secretion of catecholamines induced by acetylcholine.

To date, resveratrol has been considered to be one of the candidate molecules to explain the French Paradox of low mortality from cardiovascular diseases [3,4]. Indeed, resveratrol is thought to protect against atherosclerosis and

coronary heart diseases [35], through various mechanisms that may include vasorelaxation [5,6] and anti-platelet effects [7,8]. Furthermore, resveratrol is a well-characterized grape polyphenol that has exhibited diverse pharmacological effects such as neuroprotection [36], anti-inflammation [37], anticancer properties [38], and antioxidant properties [39]. In addition to these pharmacological effects of resveratrol, in the present study, we showed that resveratrol inhibits catecholamine synthesis and secretion induced by acetylcholine, suggesting that resveratrol attenuates the catecholamine synthesis and secretion induced by stress or emotional excitation, thus causing the stimulation of sympathetic nerves and the adrenal medulla. Although catecholamines play a pivotal role in the regulation of normal functions in cardiovascular systems, stress-induced over expression of catecholamines would contribute to the involvement and augmentation of cardiovascular diseases such as heart failures, atherosclerosis, coronary heart disease and hypertension. Indeed, chronic heart failure is associated with activation of the sympathetic nervous system as manifested by increased circulating level of norepinephrine and increased regional activity of the sympathetic nervous system [17,18,40,41]. Thus, our findings may explain, at least in part, the cardiovascular protective effects of resveratrol. To confirm this point, further in vivo administration of resveratrol to animals or humans will be required in future studies.

In summary, we have demonstrated that resveratrol inhibits catecholamine synthesis and secretion induced by acetylcholine by inhibiting nicotinic acetylcholine receptor-ion channels in the adrenal medulla and probably sympathetic neurons. The present findings may add new pharmacological actions of resveratrol on cardiovascular systems to our understanding of the French Paradox.

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REFERENCES

[1] Wu JM, Wang ZR, Hsieh TC, Bruder JL, Zou JG, Huang YZ. Mechanism of cardioprotection by resveratrol, a phenolic antioxidant present in red wine (review). Int J Mol Med 2001;8:3–17.
[2] Gehm BD, McAndrews JM, Chien P-Y, Jameson JL. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci USA 1997;94:14138–43.
[3] St Leger AS, Cochrane AL, Moore F. Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. Lancet 1979;1:1017–20.
[4] Renaud S, de Lorgeril M. Wine, alcohol, platelets, and the French Paradox for coronary heart disease. Lancet 1992;339:1523–6.
[5] Duarte J, Perez Vizcaino F, Utrilla P, Jimenez J, Tamargo J, Zarzuelo A. Vasodilatory effects of flavonoids in rat aortic smooth muscle. Structure–activity relationships. Gen Pharmacol 1993;24:857–62.
[6] Andriambeloson E, Kleschyov LA, Muller B, Beretz A, Stoclet JC, Andriantsitohaina R. Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. Br J Pharmacol 1997;120:1053–8.
[7] Pace-Asciak CR, Rounova O, Hahn SE, Diamandis EP, Goldberg DM. Wines and grape juices as modulators of platelet aggregation in healthy human subjects. Clin Chim Acta 1996;246:163–82.
[8] Bertelli AA, Giovannini L, Giannessi D, Migliori M, Bernini W, Fregoni M, et al. Antiplatelet activity of synthetic and natural resveratrol in red wine. Int J Tissue React 1995;17:1–3.
[9] Wada A, Takara H, Izumi F, Kobayashi H, Yanagihara N. Influx of ^{22}Na through acetylcholine receptor-associated Na channels: relationship between ^{22}Na influx, ^{45}Ca influx and secretion of catecholamines in cultured bovine adrenal medulla cells. Neuroscience 1985;15:283–92.
[10] Yanagihara N, Wada A, Izumi F. Effects of α_2-adrenergic agonists on carbachol-stimulated catecholamine synthesis in cultured bovine adrenal medullary cells. Biochem Pharmacol 1987;36:3823–8.
[11] Yanagihara N, Okazaki M, Terao T, Uezono Y, Wada A, Izumi F. Stimulatory effects of brain natriuretic peptide on cyclic GMP accumulation and tyrosine hydroxylase activity in cultured bovine adrenal medullary cells. Naunyn-Schmiedeberg's Arch Pharmacol 1991;343:289–95.
[12] Kajiwara K, Yanagita T, Nakashima Y, Wada A, Izumi F, Yanagihara N. Differential effects of short and prolonged exposure to carvedilol on voltage-dependent Na^+ channels in cultured bovine adrenal medullary cells. J Pharmacol Exp Ther 2002;302:212–8.
[13] Toyohira Y, Kubo T, Watanabe M, Uezono Y, Ueno S, Shinkai K, et al. Selective blockade of nicotinic acetylcholine receptors by pimobendan, a drug for the treatment of heart failure: reduction of catecholamine secretion and synthesis in adrenal medullary cells. Naunyn-Schmiedeberg's Arch Pharmacol 2005;371:107–13.
[14] Zhang LP, Yin JX, Liu Z, Zhang Y, Wang QS, Zhao J. Effect of resveratrol on L-type calcium current in rat ventricular myocytes. Acta Pharmacol Sin 2006;27:179–83.
[15] Wallace CH, Baczko I, Jones L, Fercho M, Light PE. Inhibition of cardiac voltage-gated sodium channels by grape polyphenols. Br J Pharmacol 2006;149:657–65.
[16] Gao ZB, Hu GY. Trans-resveratrol, a red wine ingredient, inhibits voltage-activated potassium currents in rat hippocampal neurons. Brain Res 2005;1056:68–75.
[17] Kaye DM, Lefkowits J, Jennings GL, Bergin P, Broughton A, Esler MD. Adverse consequences of high sympathetic nervous activity in the failing human heart. J Am Coll Cardiol 1995;26:1257–63.
[18] Lymperopoulos A, Rengo G, Funakoshi H, Eckhart AD, Koch WJ. Adrenal GRK2 upregulation mediates sympathetic overdrive in heart failure. Nat Med 2007;13:315–23.
[19] Yanagihara N, Oishi Y, Yamamoto H, Tsutsui M, Kondoh J, Sugiura T, et al. Phosphorylation of chromogranin A and catecholamine secretion stimulated by elevation of intracellular Ca^{2+} in cultured bovine adrenal medullary cells. J Biol Chem 1996;271:17463–8.
[20] Yanagihara N, Toyohira Y, Ueno S, Tsutsui M, Utsunomiya K, Liu M, et al. Stimulation of catecholamine synthesis by environmental estrogenic pollutants. Endocrinology 2005;146:265–72.

[21] Ueno S, Tsutsui M, Toyohira Y, Minami K, Yanagihara N. Sites of positive allosteric modulation by neurosteroids on ionotropic γ-aminobutyric acid receptor subunits. FEBS Lett 2004;566:213–7.
[22] Catterall WA. From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. Neuron 2000;26:13–25.
[23] Artalejo CR, Mougul DJ, Perlman RL, Fox AP. Three types of bovine chromaffin cell Ca^{2+} channels: facilitation increases the opening probability of A27ps channel. J Pysiol (Lond) 1991;444:213–40.
[24] Stauderman KA, Pruss RM. Different patterns of agonist-stimulated increases of ^{3}H-inositol phosphate isomers and cytosolic Ca^{2+} in bovine adrenal chromaffin cells: comparison of the effects histamine and angiotensin II. J Neurochem 1990;54:946–53.
[25] Kim HI, Kim TH, Song JH. Resveratrol inhibits Na^{+} currents in rat dorsal root ganglion neurons. Brain Res 2005;1045:134–41.
[26] Cheek TR, Morgan A, O'Sullivan AJ, Moreton RB, Berridge MJ, Burgoyne RD. Spatial localization of agonist-induced Ca^{2+} entry in bovine adrenal chromaffin cells. Different patterns induced by histamine and angiotensin II, and relationship to catecholamine release. J Cell Sci 1993;105:913–21.
[27] Yanagihara N, Liu M, Toyohira Y, Tsutsui M, Ueno S, Shinohara Y, et al. Stimulation of catecholamine synthesis through unique estrogen receptors in the bovine adrenomedullary plasma membrane by 17β-estradiol. Biochem Biophys Res Commun 2006;339:548–53.
[28] Takahashi K, Toyohira Y, Ueno S, Tsutsui M, Yanagihara N. Capsaicin inhibits catecholamine secretion and synthesis by blocking Na+ and Ca2+ influx through a vanilloid receptor-independent pathway in bovine adrenal medullary cells. Naunyn-Schmiedeberg's Arch Pharmacol 2006;374:107–16.
[29] Falkenstein E, Tillmann H-C, Christ M, Feuring M, Wehling M. Multiple actions of steroid hormones – a focus on rapid, nongenomic effects. Pharmacol Rev 2000;52:513–55.
[30] Goldberg DM, Tsang E, Karumanchiri A, Diamandis E, Soleas G, Ng E. Method to assay the concentrations of phenolic constituents of biological interest in wines. Anal Chem 1996;68:1688–94.
[31] Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727–47.
[32] Soleas GJ, Yan J, Goldberg DM. Measurement of trans-resveratrol, (+)-catechin, and quercetin in rat and human blood and urine by gas chromatography with mass selective detection. Methods Enzymol 2001;335:130–45.
[33] Goldberg DM, Yan J, Soleas GJ. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem 2003;36:79–87.
[34] Sale S, Verschoyle RD, Boocock D, Jones DJ, Wilsher N, Ruparelia KC, et al. Pharmacokinetics in mice and growth-inhibitory properties of the putative cancer chemopreventive agent resveratrol and the synthetic analogue trans 3,4,5,4′-tetramethoxystilbene. Br J Cancer 2004;90:736–44.
[35] Hung LM, Chen JK, Huang SS, Lee RS, Su MJ. Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. Cardiovasc Res 2000;47:549–55.
[36] Bastianetto S, Zheng WH, Quirion R. Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. Br J Pharmacol 2000;131:711–20.
[37] Jang DS, Kang BS, Ryu SY, Chang IM, Min KR, Kim Y. Inhibitory effects of resveratrol analogs on unopsonized zymosan-induced oxygen radical production. Biochem Pharmacol 1999;57:705–12.
[38] Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 1997;275:218–20.
[39] Leonard SS, Xia C, Jiang BH, Stinefelt B, Klandorf H, Harris GK, et al. Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. Biochem Biophys Res Commun 2003;309:1017–26.
[40] Freedman NJ, Lefkowitz RJ. Anti-β_1-adrenergic receptor antibodies and heart failure: causation, not just correlation. J Clin Invest 2004;113:1379–82.
[41] Westfall TC, Westfall DP. Adrenergic agonists and antagonists. In: Brunton LL, Lazo JS, Parker KL, editors. Goodman & Gilman the pharmacological basis of therapeutics. 11th ed., New York: McGraw-Hill; 2005. p. 237–95.