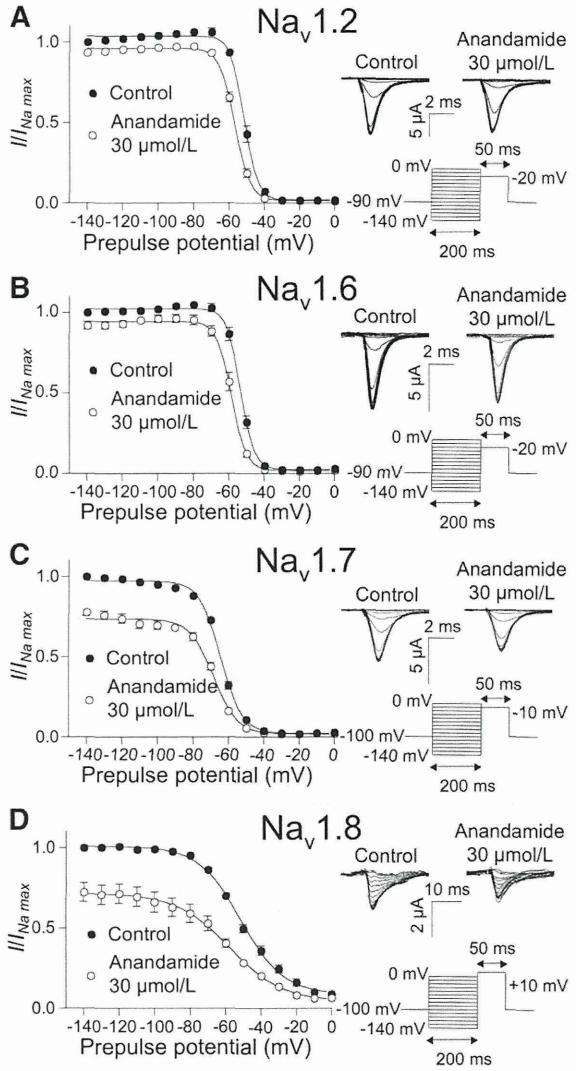

Figure 6. Effects of anandamide on inactivation curves in oocytes expressing $Na_v1.2$ (A), $Na_v1.6$ (B), $Na_v1.7$ (C), and $Na_v1.8$ (D) α subunits with β_1 subunits. Currents were elicited by a 50-millisecond test pulse to –20 mV for $Na_v1.2$ and $Na_v1.6$ or –10 mV for $Na_v1.7$ or +10 mV for $Na_v1.8$ after 200-millisecond (500-millisecond for only $Na_v1.8$) prepulses ranging from –140 mV to 0 mV in 10 mV increments from a holding potential of V_{max}; anandamide (30 μmol/L) was applied for 5 minutes; right panel, representative I_{Na} traces in both the absence and presence of anandamide; left panel, effects of anandamide on inactivation curves (closed circles, control; open circles, anandamide). Steady-state inactivation curves were fitted to the Boltzmann equation, and the $V_{1/2}$ values are shown in Table 1. Data are expressed as the mean ± SEM (n = 6–8).

carrageenan,[51,52] whereas $Na_v1.7$ protein decreased in the injured DRG after spared nerve injury in animals.[53] $Na_v1.8$ mRNA and protein increased in DRG neurons of rodents after injection of carrageenan into a hindpaw,[51,54,55] and yet peripheral nerve injury down-regulates $Na_v1.8$ mRNA and protein expression in the injured DRG.[29,53,56] Based on this evidence, suppression of sensory neuron sodium channel function by anandamide may be an important mechanism independent of the cannabinoid receptor. Because of the limitations of our experiments, further investigation is warranted to extrapolate our findings into clinical practice.

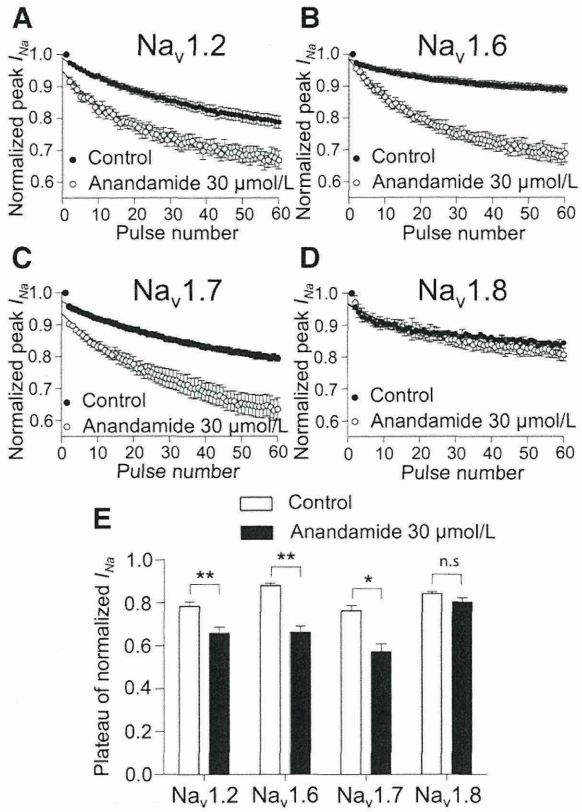

Figure 7. Use-dependent block of sodium channel on $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$ α subunits with β_1 subunits of anandamide. Currents were elicited at 10 Hz by a 20-millisecond depolarizing pulse of –20 mV for $Na_v1.2$ and $Na_v1.6$, or –10 mV for $Na_v1.7$, or +10 mV for $Na_v1.8$ from a $V_{1/2}$ holding potential in both the absence and presence of 30 μmol/L anandamide; anandamide was applied for 5 minutes. Peak currents were measured and normalized to the first pulse and plotted against the pulse number (A, $Na_v1.2$; B, $Na_v1.6$; C, $Na_v1.7$; D, $Na_v1.8$). Closed circles represent control; open circles indicate the effect of anandamide. Data were fitted to the monoexponential equation, and values for fractional block of the plateau of normalized I_{Na} are shown in (E). Data are expressed as the mean ± SEM (n = 5–6). *P < 0.05 and **P < 0.01, compared with the control (paired t test).

In conclusion, anandamide at pharmacologically relevant concentrations inhibited sodium currents of $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$ α subunits expressed in the *Xenopus* oocytes with differences in the effects on sodium channel gating. These results provide a better understanding of the mechanisms underlying the analgesic effects of anandamide, but further studies are needed to clarify the relevance of sodium channel inhibition by anandamide to analgesia.

DISCLOSURES

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Contribution: This author helped data collection, data analysis, and manuscript preparation.

Attestation: Dan Okura approved the final manuscript and attests to the integrity of the original data and the analysis reported in this manuscript.

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Current Perspective

New Insights Into the Pharmacological Potential of Plant Flavonoids in the Catecholamine System

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Abstract. Flavonoids are biologically active polyphenolic compounds widely distributed in plants. Recent research has focused on high dietary intake of flavonoids because of their potential to reduce the risks of diseases such as cardiovascular diseases, diabetes, and cancers. We report here the effects of plant flavonoids on catecholamine signaling in cultured bovine adrenal medullary cells used as a model of central and peripheral sympathetic neurons. Daidzein (0.01 – 1.0 μM), a soy isoflavone, stimulated ^{14}C-catecholamine synthesis through plasma membrane estrogen receptors. Nobiletin (1.0 – 100 μM), a citrus polymethoxy flavone, enhanced ^{14}C-catecholamine synthesis through the phosphorylation of Ser19 and Ser40 of tyrosine hydroxylase, which was associated with $^{45}Ca^{2+}$ influx and catecholamine secretion. Treatment with genistein (0.01 – 10 μM), another isoflavone, but not daidzein, enhanced [^{3}H]noradrenaline uptake by SK-N-SH cells, a human noradrenergic neuroblastoma cell line. Daidzein as well as nobiletin ($\geq$ 1.0 μM) inhibited catecholamine synthesis and secretion induced by acetylcholine, a physiological secretagogue. The present review shows that plant flavonoids have various pharmacological potentials on the catecholamine system in adrenal medullary cells, and probably also in sympathetic neurons.

Keywords: adrenal medulla, catecholamine, flavonoid, membrane estrogen receptor, tyrosine hydroxylase

Introduction

Flavonoids are a group of plant secondary metabolites with variable phenolic structures and are found in plants fruits, vegetables, roots, stems, flowers, wine, tea, and traditional Chinese herbs (1, 2). More than 5,000 individual flavonoids have been identified, which are classified into at least 10 subgroups according to their chemical structure (3). In these flavonoids, 6 principal subgroups (flavones, flavonols, flavanones, flavanols, isoflavones, and anthocyanidins) are relatively common in human diets (Fig. 1) (4). The different flavonoids have diverse biological functions, including protection against ultraviolet radiation and phytopathogens, auxin transport, the coloration of flowers, and visual signals (1, 3). Furthermore, recent research has focused on high dietary intake of plant flavonoids because flavonoids may have potential pharmacological benefits associated with reduced risks of age and life style–related diseases such as cardiovascular diseases, diabetes, and cancers (4).

Adrenal medullary cells derived from embryonic neural crests are functionally homologous to sympathetic ganglionic neurons. Our previous studies, using cultured bovine adrenal medullary cells, demonstrated that acetylcholine (ACh)-induced $^{22}Na^+$ influx via nicotinic

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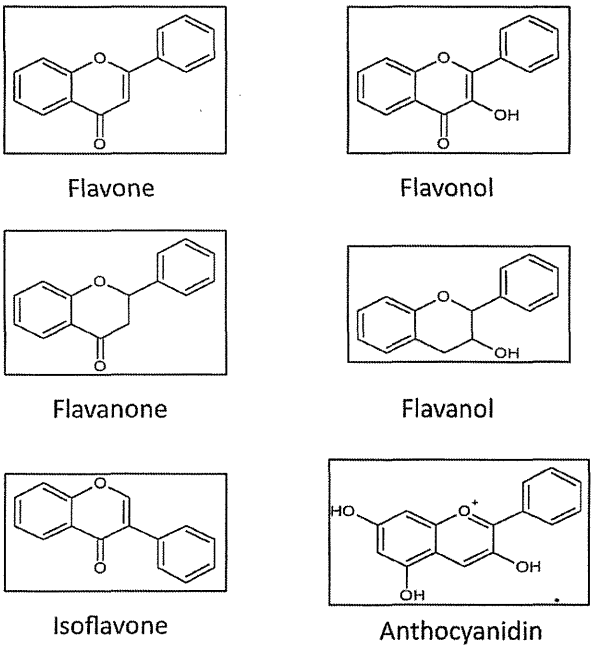

Fig. 1. Chemical structures of the main class of diet flavonoids.

acetylcholine receptor (nAChR)-ion channels increases $^{45}Ca^{2+}$ influx via voltage-dependent Ca^{2+} channels and that the enhanced Ca^{2+} influx is a prerequisite for the secretion of catecholamines (5). Furthermore, stimulation of catecholamine synthesis induced by ACh is associated with the $^{45}Ca^{2+}$ influx and the activation of tyrosine hydroxylase (6). Tyrosine hydroxylase is acutely regulated by its phosphorylation at Ser19, Ser31, and Ser40 via the activation of protein kinases, including Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II), extracellular signal-regulated protein kinase (ERK), and cAMP-dependent protein kinase (protein kinase A), respectively (7). Catecholamine secretion mediated by stimulation of these ion channels, and the mechanism underlying the stimulation of catecholamine synthesis in adrenal medullary cells, are both thought to be similar to those of noradrenaline in sympathetic neurons and brain noradrenergic neurons. Thus, adrenal medullary cells have provided a good model for the detailed analysis of cardiovascular (6) and analgesic (8) drugs that act on catecholamine synthesis, secretion, and reuptake.

In our previous studies, treatment of bovine adrenal medullary cells with environmental estrogenic pollutants such as *p*-nonylphenol and bisphenol A stimulated catecholamine synthesis and tyrosine hydroxylase activity, probably through plasma membrane estrogen receptors (9). We further demonstrated the occurrence and functional roles of unique estrogen receptors in the plasma membranes isolated from bovine adrenal medullary cells (10). Daidzein, a flavonoid, stimulated catecholamine synthesis via the activation of extracellular signal-regulated protein kinases (ERKs) through the plasma membrane estrogen receptors (11). In the present review, we discuss our recent studies of plant flavonoids on catecholamine synthesis, secretion, and uptake in bovine adrenal medullary cells.

Regulation of catecholamine synthesis, secretion, and uptake by soy isoflavones, daidzein, and genistein

Natural estrogens induce a wide array of biological effects on cell differentiation and proliferation, homeostasis, and the female reproductive system through classical nuclear estrogen receptors (ERs), including ER-α and ER-β (12). In addition to these established mechanisms of action, a growing body of evidence suggests that estrogens have non-genomic actions via the activation of estrogen receptors in the plasma membrane. Incubation of the cells with 17β-estradiol (E_2) and daidzein for 20 min resulted in a small (15% – 25%) but significant increase in ^{14}C-catecholamine synthesis from [^{14}C]tyrosine in a concentration-dependent manner (Fig. 2A) (10, 11). Significant ($P < 0.01$) increases in ^{14}C-catecholamine synthesis induced by E_2 and daidzein were observed at 0.3 and 10 nM, respectively, and the maximum effect occurred at approximately 10 – 100 nM and 100 – 1000 nM, respectively. Tyrosine hydroxylase was also activated after incubation with E_2 or membrane-impermeable E_2–bovine serum albumin at 100 nM and daidzein as well as daidzein plus ICI182,780, an inhibitor of nuclear estrogen receptors. These findings suggest that E_2 and daidzein each activates tyrosine hydroxylase activity and then stimulates catecholamine synthesis, likely via plasma membrane estrogen receptors distinct from the more extensively investigated classical cytoplasmic/nuclear receptors.

We examined the specific binding of [^3H]E_2 to plasma membranes isolated from bovine adrenal medulla. When the plasma membranes were incubated with increasing concentrations (0.25 – 300 nM) of [^3H]E_2, specific binding was observed (10). Scatchard analysis revealed the presence of at least two classes of [^3H]E_2 binding sites. The specific binding of [^3H]E_2 (5 nM) was most strongly inhibited by E_2 and to a lesser extent by daidzein and other steroid hormones such as testosterone, corticosterone, and 17α-estradiol, the natural stereoisomer of E_2. When plasma membranes isolated from the adrenal medulla were incubated with various concentrations of daidzein and [^3H]E_2 (5 nM), the specific binding of [^3H]E_2 was competitively inhibited by daidzein in a concentration-dependent manner (10 – 1000 nM) (Fig. 2B) (11). These findings suggest that E_2 and daid-

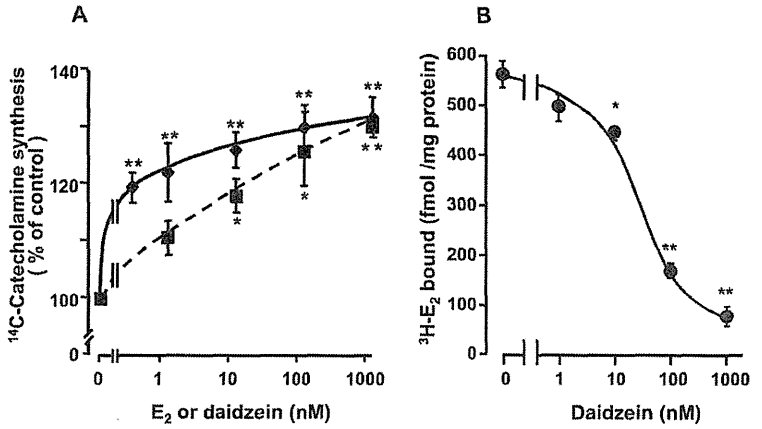

Fig. 2. Concentration–response curves of E_2 and daidzein for ^{14}C-catecholamine synthesis from [^{14}C]tyrosine (A) and concentration–inhibition curve of daidzein for [^{3}H]E_2 specific binding (B). A) Cultured cells (4×10^6/dish) were incubated with E_2 (closed diamonds) and daidzein (closed squares) at the indicated concentrations for 20 min at 37°C in 1.0 ml KRP buffer containing L-[U-^{14}C]tyrosine (20 μM, 1 μCi). The ^{14}C-labeled catecholamines formed are shown as the total ^{14}C-catecholamines (adrenaline, noradrenaline, and dopamine). Data are expressed as % of the control. B) Plasma membranes isolated form bovine adrenal medulla were incubated at 4°C for 30 min with various concentrations of daidzein in the presence of [^{3}H]E_2 (5 nM, 0.1 μCi). Non-specific binding was determined in the presence 1 μM of E_2 and specific binding was obtained by subtracting non-specific binding from total binding. Values shown are expressed as the mean ± S.E.M. of 4 experiments carried out in duplicate. $^*P < 0.05$ and $^{**}P < 0.01$, compared with the control. Data modified from Yanagihara et al. (10) and Liu et al. (11).

zein act on the same site of membrane estrogen receptors.

Recently, several types of estrogen receptor have been reported in plasma membranes, including classical nuclear estrogen receptors such as ER-α (13) as well as ER-X, a novel member of the estrogen receptor family (14), and GPR30, which has high homology with the G protein–coupled receptor superfamily in breast cancers (15). To determine whether the membrane estrogen receptors we observed are identical to, or distinct from, previously reported plasma membrane estrogen receptors, it will be necessary to precisely identify the plasma membrane estrogen receptors in future studies.

Genistein, another isoflavone, is also a major natural phytoestrogen found in soybeans. Treatment with genistein, but not daidzein, at 0.01 – 10 μM for 20 min stimulated [^{3}H]noradrenaline uptake by SK-N-SH cells, the human noradrenergic neuroblastoma cell line expressing noradrenaline transporter (16). Genistein is well-known to be a broad-spectrum inhibitor of protein tyrosine kinases, whereas daidzein is a structural analogue of genistein that lacks activity towards tyrosine kinase and is often used as a negative control of genistein in this respect (17). Since tyrophostin 25, an inhibitor of receptor-type protein tyrosine kinases, also enhanced uptake of [^{3}H]noradrenaline by cells, it seems that genistein stimulates noradrenaline transporter activity probably via the inhibition of receptor-type tyrosine kinases but not by the activation of plasma membrane estrogen receptors in the cells.

Stimulatory effects of nobiletin, a citrus flavonoid, on catecholamine synthesis and secretion

Nobiletin is a major component of polymethoxylated flavones found in the peels of citrus fruits and is used in a traditional Chinese herbal medicine. Nobiletin has attracted great interest by virtue of its broad spectrum of pharmacological activities, including antitumor, anti-oxidative, and anti-inflammatory properties (18). Furthermore, several lines of evidence have shown that nobiletin has beneficial cardiovascular effects, as well as neurotrophic and anti-dementia effects (19). In our previous study, nobiletin (1.0 – 100 μM) induced $^{45}Ca^{2+}$ influx and catecholamine secretion without $^{22}Na^+$ influx via the activation of voltage-dependent Ca^{2+} channels or Na^+/Ca^{2+} exchangers (20). Furthermore, nobiletin also simulated ^{14}C-catecholamine synthesis from [^{14}C]tyrosine and tyrosine hydroxylase activity in a concentration-dependent manner, similar to the case with $^{45}Ca^{2+}$ influx and catecholamine secretion (21).

The stimulatory effects of nobiletin on catecholamine synthesis and tyrosine hydroxylase activity were suppressed by H-89 and KN-93, inhibitors of protein kinase A and CaM kinase II, respectively, which are considered to phosphorylate tyrosine hydroxylase at Ser40 and Ser19, respectively. Indeed, nobiletin enhanced the phosphorylation of tyrosine hydroxylase at the same sites. Based on these findings, it is likely that nobiletin enhances the activity of tyrosine hydroxylase via the activation of CaM kinase II and protein kinase A,

which in turn, stimulates catecholamine synthesis in the cells. A previous report (22) showed that 4′-demethylnobiletin, a major metabolite of nobiletin in the urine of mice enhances cyclic AMP response element–mediated transcription by activating a protein kinase A/ERK pathway in cultured hippocampal neurons of mice. Therefore, it is interesting to examine the effect of its metabolites on the catecholamine synthesis.

Inhibitory effects of flavonoids on catecholamine secretion and synthesis induced by ACh, a natural secretagogue

We previously reported that ACh activates nAChR-ion channels, and this activation in turn induces Na^+ influx and subsequent Ca^{2+} influx and catecholamine secretion. K^+ (56 mM), an activator of voltage-dependent Ca^{2+} channels, directly gates voltage-dependent Ca^{2+} channels to increase Ca^{2+} influx and catecholamine secretion (5). In the present study, daidzein (1.0 – 100 μM and 100 μM) and nobiletin (0.1 – 100 μM and 1.0 – 100 μM) were found to inhibit catecholamine secretion induced by ACh (0.3 mM) and 56 mM K^+, respectively, although daidzein by itself did not affect basal catecholamine secretion and Ca^{2+} influx. These results suggest that both flavonoids attenuate catecholamine secretion induced by ACh and 56 mM K^+ through the inhibition of nAChR-ion channels and voltage-dependent Ca^{2+} channels.

To investigate the mechanism by which flavonoids inhibit ACh-induced catecholamine secretion, we examined whether or not the inhibitory effect of nobiletin on catecholamine secretion is overcome when the concentration of ACh is increased. However, they did not overcome the inhibitory effect of nobiletin and the double-reciprocal plot analysis showed a non-competitive type of inhibition. A previous review proposed that at high concentrations ($\geq$ 10 μM), steroid hormones such as estrogens could be inserted into the bilayers of cellular membranes and that direct steroid-membrane interactions alter physicochemical membrane properties, such as the fluidity and microenvironment of membrane receptors and/or ion channels, in addition to specific receptor-mediated effects (23). It is possible that daidzein and nobiletin at high concentrations may interact with these ion channels via the alteration of the membrane properties of adrenal medullary cells. However, it remains to be clarified whether or not these flavonoids may exert their effects on catecholamine secretion merely by nonspecific effects on the membrane properties.

Pharmacological significance of flavonoids' effects on the catecholamine system

The serum concentrations of daidzein have been reported to be around 200 – 350 nM in Japanese people older than 40 years (24). Furthermore, the serum concentrations of daidzein in humans consuming 3 meals per day that contained soy milk or a single soy meal can reach as high as 4.0 – 5.0 μM (25). Therefore, it seems that the concentrations used in our studies are relevant in people's daily lives because these concentrations partially overlap with those in the plasma of individuals who consume soy products.

Nobiletin is rich in the peels of citrus fruits, and the dried peels are used in a traditional Chinese herbal medicine. Nogata et al. (26) reported the contents of nobiletin in various citrus fruits: total tissue, 0.4 – 8.1 (3.93 ± 0.87) mg / 100 g; peel tissue, 1.5 – 18.5 (11.5 ± 2.2) mg / 100 g; juice vesicle tissue, 0 – 0.9 (0.25 ± 0.13) mg / 100 g. When we used 60 kg for the body weight of a man, 4.5 L of the total volume of human blood, and 0.1 of the nobiletin bioavailability (27), the calculated plasma concentrations of nobiletin might be 0.02 – 0.45 (0.22 ± 0.05) μM, 0.08 – 1.0 (0.63 ± 0.22) μM, and 0 – 0.05 (0.014 ± 0.01) μM, respectively. Indeed, the previous report (27) showed that the maximal concentrations of nobiletin in the serum and brain of mice were 0.94 mg/L (2.3 μM) and 9.27 mg/L (23 μM) or 3.6 mg/L (8.9 μM) and 22 mg/L (55 μM) after the p.o. or i.p. administration of 50 mg/kg nobiletin, respectively. Based on the previous documents, the concentrations of nobiletin (0.1 – 10 μM) used in our experiment may be appropriate, but relatively high compared to the blood concentrations of nobiletin calculated from juice vesicle tissue.

It is well documented that catecholamines play pivotal roles in the regulation of normal functions, not only in central and peripheral noradrenergic neurons as a neurotransmitter but also in adrenal medulla as an endocrine hormone. Flavonoids, including daidzein and nobiletin, by themselves induce a small but significant increase in catecholamine synthesis and/or secretion, suggesting that these flavonoids strengthen or enhance the sympatho-adrenal system.

On the other hand, several lines of evidence have shown that prolonged stress-induced over-expression of catecholamines contributes to the involvement and augmentation of cardiovascular diseases such as heart failure, atherosclerosis, coronary heart failure, and hypertension. Indeed, chronic heart failure is associated with the activation of the sympathetic nervous system as manifested by increased circulating catecholamines and increased regional activity of the sympathetic nervous system (28). Chronic stress responses can be associated with disease symptoms such as peptic ulcers or cardiovascular disorders (29). Recently, Hara et al. (30) reported that the stress hormone adrenaline stimu-

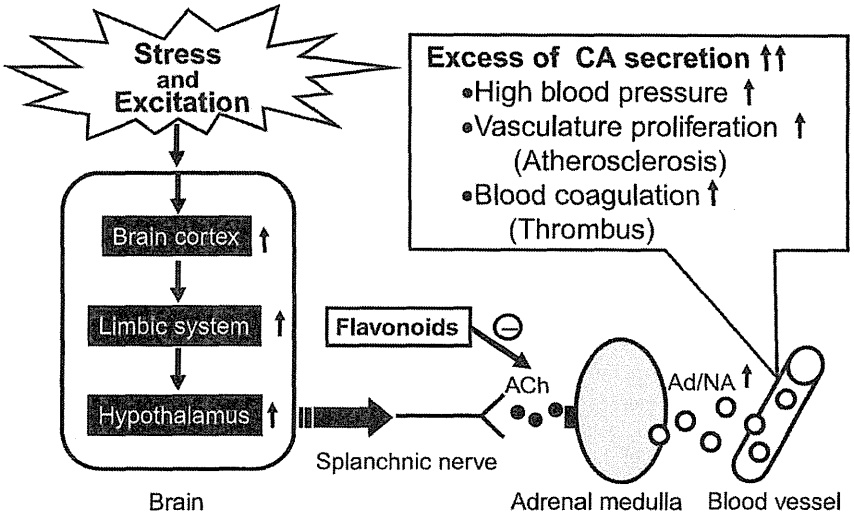

Fig. 3. Inhibitory mechanism of flavonoids on stress or excitation-induced excess of catecholamine secretion. Prolonged and strong stress or excitation stimulates the brain cortex, limbic system, and hypothalamus, which evoke acetylcholine release from the splanchnic sympathetic nerves. Released acetylcholine induces a massive secretion of adrenaline/noradrenaline from the adrenal medulla, which may cause various deleterious symptoms or diseases such as high blood pressure (hypertension), vasculature proliferation (atherosclerosis), and blood coagulation (thrombus).

Table 1. Summary of flavonoids' effects on catecholamine synthesis, secretion, and uptake

Flavonoids	CA synthesis		CA secretion		NA uptake	[^{3}H] E_2 binding
	basal	ACh	basal	ACh		
Daidzein	↑	↓	→	↓	→	↓
Genistein	N.D.	N.D.	N.D.	N.D.	↑	↓
Nobiletin	↑	↓	↑	↓	N.D.	→

CA, catecholamine; NA, noradrenaline; E_2, 17β-estradiol; ACh, ACh-stimulated; N.D., not determined; →, no effect; ↑, stimulation; ↓, inhibition.

lates β_2-adrenoceptors, which in turn induces the Gs-protein–dependent activation of protein kinase A and the β-arrestin-mediated signaling pathway, and then suppresses p53 levels and triggers DNA damage. From these previous and present results, it gives rise to the possibility that flavonoids suppress the hyperactive catecholamine system induced by prolonged stress or emotional excitation which evokes the secretion of ACh from the splanchnic nerves and stimulates a massive secretion of catecholamines from the adrenal medulla (Fig. 3).

Future perspectives

What major pending problems or questions does the present study reveal? While the in vitro effects of plant flavonoids have been well clarified using cultured bovine adrenal medullary cells or SK-N-SH cells, the in vivo effects are not as clear. Therefore, to confirm the effects of these flavonoids on the catecholamine system, further in vivo studies on the effects of the administration of daidzein, genistein, and nobiletin to animals or humans will be needed in the near future. Furthermore, the question arises as to how best to demonstrate the protective effects of flavonoids on stress-induced catecholamine synthesis and secretion. The protective effects of flavonoids against stress should be examined using laboratory animals under various stress conditions. Analysis with in vivo studies will provide more conclusive information and add to our knowledge about the pharmacological actions of plant flavonoids on the catecholamine system.

Concluding remarks

Flavonoids are major natural products in plants. In the present review, we have demonstrated that plant flavonoids such as daidzein, genistein, and nobiletin exert a variety of effects on catecholamine signaling, including catecholamine synthesis, secretion, and uptake in the adrenal medulla (Table 1). These findings may provide new insight into the pharmacological potentials of plant flavonoids on the catecholamine system.

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Conflicts of Interest

The authors have no conflicts of interest to report.

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Full Paper

Effects of Selective Estrogen Receptor Modulators on Plasma Membrane Estrogen Receptors and Catecholamine Synthesis and Secretion in Cultured Bovine Adrenal Medullary Cells

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Abstract. We previously reported the occurrence and function of plasma membrane estrogen receptors in cultured bovine adrenal medullary cells. Here we report the effects of raloxifene and tamoxifen, selective estrogen receptor modulators, on plasma membrane estrogen receptors and catecholamine synthesis and secretion in these cells. Raloxifene caused dual effects on the specific binding of [^{3}H]17β-estradiol to the plasma membranes isolated from bovine adrenal medulla; that is, it had a stimulatory effect at 1.0 – 10 nM but an inhibitory effect at 1.0 – 10 μM, whereas tamoxifen (1.0 nM – 10 μM) increased binding at all concentrations (except for 100 nM). Tamoxifen at 100 nM caused a significant increase in basal ^{14}C-catecholamine synthesis from [^{14}C]tyrosine, whereas tamoxifen and raloxifene at higher concentrations attenuated basal and acetylcholine-induced ^{14}C-catecholamine synthesis. Raloxifene (0.3, 1.0, and 3 – 100 μM) and tamoxifen (10 – 100 μM) also suppressed catecholamine secretion and $^{45}Ca^{2+}$ and $^{22}Na^{+}$ influx, respectively, induced by acetylcholine. Raloxifene (1.0 μM) inhibited Na^{+} current evoked by acetylcholine in *Xenopus* oocytes expressing $\alpha4\beta2$ neuronal nicotinic acetylcholine receptors. The present findings suggest that raloxifene and tamoxifen at low concentrations allosterically modulate plasma membrane estrogen receptors and at high concentrations inhibit acetylcholine-induced catecholamine synthesis and secretion by inhibiting Na^{+} and Ca^{2+} influx in bovine adrenal medulla.

Keywords: adrenal medulla, catecholamine synthesis and secretion, plasma membrane estrogen receptor, raloxifene, selective estrogen receptor modulator

Introduction

Selective estrogen receptor modulators (SERMs) are compounds that bind to nuclear or classical estrogen receptors (ERs) and exert either estrogenic or anti-estrogenic effects depending on the specific organs (1, 2). At present, at least two SERMs, tamoxifen for the treatment and prevention of breast cancer and raloxifene for the prevention of osteoporosis, are clinically available in Japan (3). Although the precise molecular mechanisms by which SERMs exert their clinical effects are unknown, their estrogenic or anti-estrogenic actions at target tissues are mediated through two ERs, ERα, and ERβ (4). In addition to the genomic ER actions, several lines of evidence have shown that SERMs acutely modulate ionic current through neuronal nicotinic acetylcholine receptors (nAChRs)-ion channels (5, 6) and also modulate functions of the cardiovascular systems (3). Furthermore, estrogens and raloxifene are reported to inhibit catecholamine secretion from rat and bovine

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