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Research article

Single oral administration of flavan 3-ols induces stress responses monitored with stress hormone elevations in the plasma and paraventricular nucleus

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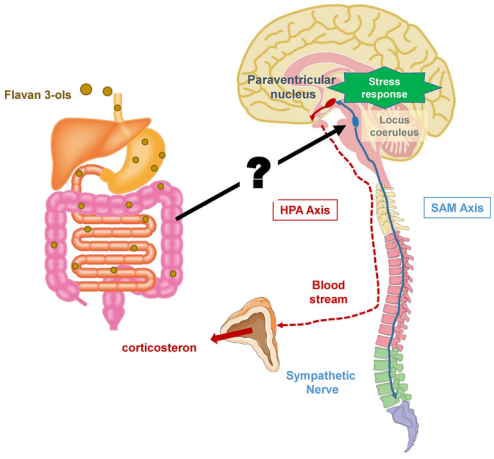
GRAPHICAL ABSTRACT



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ABSTRACT

We previously confirmed that postprandial alterations in the circulation and metabolism after a single oral dose of flavan 3-ols (mixture of catechin and catechin oligomers) were involved in an increase in sympathetic nervous activity. However, it is well known that, in response to various stresses, activation of the hypothalamic-pituitary-adrenal (HPA) axis occurs together with sympathetic nerve activity, which is associated with activation of the sympathetic-adrenal-medullary (SAM) axis. In this study, we examined whether the HPA axis was activated after a single dose of flavan 3-ols. We administered an oral dose of 10 or 50 mg/kg flavan 3-ols to male ICR mice, removed the brains, and fixed them in paraformaldehyde-phosphate buffer. Other animals that were treated similarly were decapitated, and blood was collected. In the paraventricular nucleus (PVN), c-fos mRNA expression increased significantly at 15 min after administration of either 10 or 50 mg/kg flavan 3-ols. Corticotropin-releasing hormone (CRH) mRNA expression levels significantly increased at 240 min after administration of 10 mg/kg flavan 3-ols, and at 60 min after administration of 50 mg/kg flavan 3-ols. Plasma corticosterone levels were also significantly increased at 240 min after ingestion of 50 mg/kg flavan 3-ols. In this

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experiment, we confirmed that the ingestion of flavan 3-ols acted as a stressor in mammals with activation both the SAM and HPA axes.

1. Introduction

It is well established that flavan 3-ol monomers and oligomers, such as catechins and B-type procyanidins, which are linked by C4-C8 bonds, are present as astringent substances in chocolate, red wine, immature apples, pine bark, and some other foods [1–3]. Consequently, foods rich in flavan 3-ols, such as tea, cocoa, red wine, grapes, and apples, have significant potential for managing cardiovascular health [4–6]. Recent meta-analyses suggested that flavan 3-ols consumption reduced the risk of cardiovascular diseases, including coronary heart disease, myocardial infarction, and stroke [7–9]. In addition, numerous randomized, controlled trials confirmed that dark chocolate, which contains large amounts of flavan 3-ols, improves several conditions that contribute to metabolic syndrome, including hypertension [10,11], dyslipidemia [12,13], and glucose intolerance [14,15]. Subsequent meta-analyses confirmed that dark chocolate could reduce the risk of cardiovascular disease [16–20]. A recent comprehensive review on polyphenol and human health suggested that the mechanism underlying the beneficial effect of flavan 3-ols has not been fully elucidated, due to their poor bioavailability [21]. Both (+) catechin and (-) epicatechin are readily absorbed from the gastrointestinal tract, and they are almost completely metabolized in the circulation; therefore, unchanged forms are nearly absent in blood samples [22]. In contrast, B-type oligomeric catechins are rarely absorbed from the gut into the blood [23–25].

On the other hand, we have observed transient elevations of heart rate and blood pressure, immediately after flavan 3-ols administration [26]. It was also found that flavan 3-ols administration was associated with elevations in thermogenesis. Flavan 3-ols increased uncoupling protein-1 mRNA expression in brown adipose and caused elevations in plasma catecholamine concentrations [27]. In addition, we confirmed that these changes were eliminated by pretreatment with adrenergic blockers [28,29]. Those results suggested that administration of flavan 3-ols induced sympathetic nerve stimulation. Generally, when an organism is exposed to stress, the body causes an adaptive response by activating the sympathetic-adrenal-medullary (SAM) axis and the hypothalamic- pituitary-adrenal (HPA) axis [30]. According to previous meta-analyses, a close association was indicated between SAM and HPA responses to a variety of stressors [31]. The enhancement of sympathetic nerve activity seen after administration of flavan 3-ols was thought to be due to activation of SAM axis. In the present study, we investigated whether flavan 3-ols caused a stress response in the HPA axis. After a single administration of flavan 3-ols, we performed assays to detect changes in c-fos, a neural activity marker, CRH, a stress hormone in the hypothalamus, and plasma corticosterone.

2. Materials and methods

2.1. Animals

The study was approved by the Animal Care and Use Committee of the Shibaura Institute of Technology (Permit Number: 27–2956). All mice received humane care under the guidelines of this institution. Male ICR mice (8 weeks old) were purchased from Charles River Laboratories, Japan, Inc. (Tokyo, Japan). All animals were housed in a room maintained under standard conditions of light (12 /12 h light/dark cycles), temperature (23–25 °C), and humidity (50 ± 5%), with *ad libitum* water and food.

2.2. Materials

Flavan 3-ols were extracted from soybeans, according to the method of Ito et al [32]. Briefly, black soybean seed coats were soaked in 70% acetone/0.5% acetic acid solution for 3 h at room temperature. The solution was filtered and dried with rotary evaporation. The dried residue was dissolved in 50% methanol, and applied to a Sepabead SP700 column (Mitsubishi Chemical Co., Japan). After rinsing the column with distilled water, the flavan 3-ol fraction was eluted with 60% ethanol. Fractions were dried with rotary evaporation. The concentrations of catechins and procyanidins were measured with high-performance liquid chromatography (HPLC). The compositions of the recovered flavan 3-ols are shown in Table 1. The chemical structures of major components, including the catechin monomer, dimer, trimer, and tetramer procyanidin, are shown in Fig. 1.

2.3. Experimental procedures

Mice were handled cup to avoid inducing anxiety behavior for 2 weeks of acclimatization period [33]. Fig. 2a showed an experiment procedure for *in situ* hybridization analysis. Mice were divided into three treatment groups, as follows: before (no treatment, n = 6), 10 mg/kg flavan 3-ols (n = 4–7), and 50 mg/kg flavan 3-ols (n = 6–10). Flavan 3-ols were suspended in purified water. At 15, 30, 60, 120, 240 min after administration of flavan 3-ols, mice were anesthetized with pentobarbital (50 mg/kg body weight, administered i.p.; Tokyo Chemical Industry, Tokyo, Japan). We used a gastric tube with round edges, an infusion rate of 1.0 ml/min and maintain temperature of administration solution at 37 °C to avoid any stress induced by this procedure. Then, mice were perfused through the left ventricle with 25 mL phosphate buffered saline (PBS) and then with 25 mL 4% paraformaldehyde (PFA) in PBS for fixation. Brains were immediately removed and placed in a post-fix solution of 4% PFA at room temperature. Fig.2b showed an experiment procedure for plasma corticosterone measurement. Similarly treated mice (n = 6–9) were decapitated, and blood was collected and stored with ethylenediamine tetraacetic acid (EDTA).

2.4. Determinations of c-fos and CRH mRNA expression with in situ hybridization

Whole brains were dehydrated with ethanol, immersed in xylene, and subsequently embedded in paraffin. Paraffin coronal sections (8-μm thick) of the paraventricular nucleus (PVN) were prepared from -0.3 mm bregma with a microtome (Erma, Tokyo, Japan).

In situ hybridization was carried out to evaluate c-fos mRNA and CRH mRNA expression with the RNAscope® 2.5HD Duplex Assay (Advanced Cell Diagnostics, CA, USA). Briefly, the samples were incubated at 60 °C for 1 h to deparaffinize. Then, they were immersed twice in xylene for 5 min and ethanol for 1 min, and dried completely at room temperature. An ImmEdge™ pen (H-4000, Vector Laboratories

Table 1
Composition of flavan 3-ols derived from black soy beans seed coat.

	Concentration (w/w %)
Catechins[a]	6.22
procyanidin B2	6.35
procyanidin C1	2.69
cinnamtannin A2	1.25
total polyphenol[b]	77.1

[a] Total amount of (+)-catecihn and (-)-epicatechin.
[b] The value was measured by the method of vanillin-sulfuric acid.