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Rapid Communication

Protective Activity of Antioxidants in the Hypothalamic Paraventricular Nucleus of Chronic Restraint-Stressed Mice

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1 Introduction

Antioxidants are a group of phytochemicals, vitamins, and other nutrients that protect cells from free radical-induced damage.[1] They are believed to play a role in preventing the development of such chronic diseases as cancer, heart disease, hypertension, ataxia, and cataracts.[2-6] Cells that use oxygen naturally produce free radicals, and these can cause severe damage. Antioxidants act as "free radical scavengers" and prevent or repair free-radical-induced damage.

Astaxanthin (ASX) and proanthocyanidin (PAC) are both classified as antioxidants. ASX is a carotenoid that is commonly found in crustaceans such as shrimp and crab, as well as marine organisms including salmon, salmon roe, krill, and algae. On the other hand, PAC, a flavonoid, is mainly extracted from maritime pine bark. A number of *in vitro* and *in vivo* studies recently demonstrated the antioxidant and neuroprotective effects of ASX and PAC.[7-10] However, their neuroprotective action against chronic stress in the brain, especially in the hypothalamic paraventricular nucleus, has yet to be investigated.

Stress can be positive, causing the release of adrenaline in humans, which can enhance decision-making and problem-solving abilities. However, chronic stress, which is constant and persists over an extended period of time, can be debilitating and overwhelming. Chronic stress can affect both the physical and psychological well-being by causing various problems including anxiety, insomnia, muscle pain, high blood pressure, and a weakened immune system. Previous studies demonstrated that stress contributed to the development of mental disorders such as depression, agoraphobia, and anxiety.[11-14] Therefore, although the consequences of chronic stress can be very serious, many people under prolonged stress do not make the necessary lifestyle changes to reduce stress and ultimately prevent health problems. In addition, the neural substrates underlying stress resilience remain unknown. The expression of c-Fos has generally been used as a marker to detect stress-induced neural activation in the brain.[15,16] Particular stressors have been shown to increase the immunoreactivity of c-Fos in a regionally-specific manner. For example, limbic areas are activated in response to neurogenic, but not systemic stressors, while the hypothalamic paraventricular nucleus is activated regardless of the stress type. BDNF is also an important mediator of activity-dependent functions in the nervous system and its overexpression induces learning and memory impairments.[17] Blood corticosterone levels have been used as a peripheral stress marker.[18] Previous studies reported that the expression of c-Fos and BDNF in the hypothalamic paraventricular nucleus and blood corticosterone levels in restraint-stressed mice were significantly higher than those in non-restraint control mice.[15,19] Therefore, the purpose of the present study was to clarify immunohistologically the protective effects of antioxidants against chronic stress in the hypothalamic paraventricular nucleus of mice.

2 Materials and Methods

2.1 Animals

Six male ICR mice (over 8 weeks old) were purchased from SLC Inc. (Hamamatsu, Japan). They were individually housed under standard conditions (23−25°C and 50±5% humidity), with a 12-h light/dark cycle (lights on at 0800 and off at 2000). Food and water were available *ad libitum*. An-

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imals were handled in accordance with the guidelines established by the Institutional Animal Care and Ethical Committee at Osaka Prefecture University.

2.2 Food and restraint stress procedure

Mice were randomly divided into three experimental groups (i.e., control, ASX, and PAC). Each group was maintained on their individual diets for 4 weeks before the restraint stress procedure. Standard laboratory food (Oriental Yeast Co., Ltd., Tokyo, Japan) was provided to the control group. Standard laboratory food containing 1% astaxanthin ([ASX] AstaREAL, Fuji Chemical Industry Co., Ltd., Toyama, Japan) and 1% proanthocyanidin ([PAC] Pycnogenol, Horphag Research, Geneva, Switzerland), respectively, was provided to the ASX and PAC groups. The doses of these antioxidants were considered appropriate and adequate amounts based on the findings of a previous study.[18] Chronic restraint stress was applied for 10 consecutive days. A stainless mesh was used to allow for a close fit to the mice (4 h/day between 0800 and 2000).[20]

2.3 Immunohistochemistry

After stress, mice were deeply anesthetized with diethyl ether, and transcardially perfused with ice-cold saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The brain was post-fixed in the same fixative and cryoprotected in 0.1 M PBS (30% sucrose). The brain was embedded in Tissue-Tek O.C.T. compound (Sakura Finetech Japan Co., Ltd., Tokyo, Japan). Coronal sections (20 μm) were made by a cryostat (HM550E, MICROM, Walldorf, Germany), and all sections between stereotaxic coordinates 2.2 and 3.0 anterior to the bregma, according to the brain atlas of Paxinos and Franklin (2004), were collected. Standard avidin–biotin immunohistochemistry (ABC Elite System Vector Laboratories; Burlingame, CA, USA) was used to reveal c-Fos, a proto-oncogene, (1:1500; Oncogene, Cambridge, MA, USA) or BDNF, a brain-derived neurotrophic factor, (1:1500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Free-floating sections were incubated in 0.1% hydrogen peroxide in 0.1 M PBS for 30 min, rinsed 5 times, and then blocked for 1 hr in 0.1M PBS containing 10% normal donkey serum and 1% albumin. Sections were then incubated overnight at 4°C in 0.1 M PBS containing the primary rabbit polyclonal c-Fos or BDNF antibody. After rinses in PBS, sections were incubated for 3 hr in biotinylated goat anti-rabbit secondary IgG. They were then rinsed 5 times, and incubated overnight in the avidin-biotin complex. Sections were then processed in 0.02% diaminobenzidine tetrahydrochloride and 0.005% hydrogen peroxide to yield a black product in c-Fos- or BDNF-containing structures. Immunostained cells were observed using a light microscope.

2.4 Measurement of corticosterone levels

Corticosterone levels were measured with an Assay-Max Corticosterone ELISA kit (Assaypro, St. Charles, MO, USA) according to the manufacturer's manual, with minor modifications. Briefly, mice were sacrificed and 1 mL of blood was collected with 30 μL of 100 mM EDTA immediately after the final restraint stress. Samples were centrifuged (5 min, 4000 rpm, 4°C). Each supernatant was added to a well in a 96-well plate at a dilution of 1:100 in duplicate and subjected to the immunoassay. The optical density of the enzyme products was read at 420 nm (Model 680XR Microplate Reader, Bio-Rad Labs. Inc., Hercules, CA, USA). A 4-parameter logistic equation was used for the quantitative analysis of corticosterone levels.

3 Results

The expression of c-Fos and BDNF in the hypothalamic paraventricular nucleus of mice is shown in Figure 1. The expression of both c-Fos and BDNF was lower in both the ASX and PAC groups than in the control group. Especially in the ASX group, the expressions were lower than those in the control group.

Blood corticosterone levels after chronic restraint stress were markedly lower in the ASX group than in both the control and PAC groups. The levels of corticosterone level in the ASX group were approximately one-tenth that in the control group, and less than that in the PAC group (Data not shown).

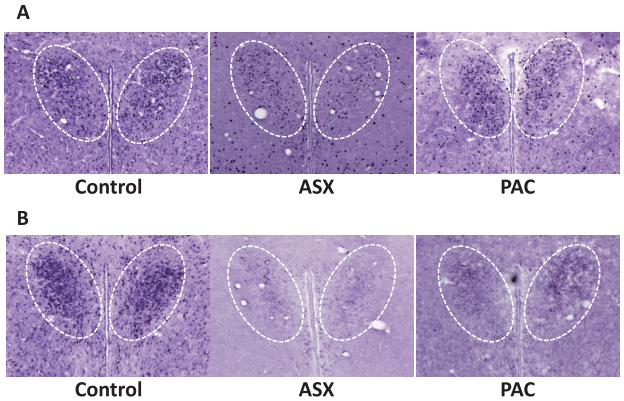

Fig. 1 The expression of c-Fos (A) and BDNF (B) in the hypothalamic paraventricular nucleus (circled with white dot-lines) of each group.

4 Discussion

The relationship between corticosteroids (endogenous and exogenous) and stress is well known.[21-23] The hypothalamic-pituitary-adrenal (HPA) axis exhibits a circadian rhythm, and is activated by stress and inhibited by corticosteroids. The stress-induced facilitation of HPA axis activity may be mediated by a parallel stress-induced (corticotropin-releasing hormone [CRH]-dependent) increase in the ca-

pacity of brain noradrenergic cell groups to respond to stress.[24-26] In addition, free radicals are known to be a naturally occurring byproduct of normal metabolic processes and are normally regulated by the body's immune system. However, a marked increase in the level of free radicals has been observed with chronic stress in humans, and this overwhelms the body's natural defenses, resulting in severe damage to the body. Increases in corticosterone levels and the expression of c-Fos and BDNF have also been reported in the blood and the brain, respectively, under this condition.

We focused on the hypothalamic paraventricular nucleus in the brain in the present study. Two antioxidants (i.e., ASX and PAC) were used to assess the effects of antioxidants on mental and physical stress. Both of these antioxidants reduced the expression of c-Fos and BDNF, which indicated that they may protect the hypothalamic paraventricular nucleus from chronic stress-induced damage. The expression of c-Fos and BDNF was significantly lower in the ASX group than in the PAC group, which indicates that ASX is more effective against stress than PAC. The notable difference between these antioxidants is that ASX is lipid soluble, whereas the majority of ASX ingested is also egested. Even though low levels of ASX are absorbed, it may still penetrate into the cell membrane and prevent cell degeneration due to oxidative damage. On the other hand, water-soluble PAC does not cross the cell membrane easily and is not very effective in the hypothalamic paraventricular nucleus.

The levels of corticosterone observed in the blood were consistent with the results obtained for immunohistological expression. These results showed that the restraint stress used in the present study caused appropriate damage both centrally and peripherally. In other words, mechanical chronic stress activated the HPA axis and changed blood corticosterone levels. Therefore, the blood corticosterone levels were highest in the control group and lower in the ASX and PAC groups. This result suggests that these antioxidants also reduced the effects of chronic stress peripherally. Thus, antioxidants such as ASX and PAC may be beneficial for both the nervous system and vasculature and protect the brain and body from stress-induced damage. However, whether antioxidants initially act to produce an effect centrally or peripherally has yet to be clarified. Further studies are needed to address this issue.

5 Conclusion

When ASX and PAC were systematically administered by ingestion before and during chronic restraint stress, they markedly reduced the expression of c-Fos and BDNF in the hypothalamic paraventricular nucleus, which should have been increased by the chronic restraint stress protocol used. These results suggest that antioxidants such as ASX and PAC may protect the brain against chronic stress.

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