Social Isolation Stress-Induced Oxidative Damage in Mouse Brain and Its Modulation by Majonoside-R2, a Vietnamese Ginseng Saponin

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Stressors with a physical factor such as immobilization, electric foot shock, cold swim, etc., have been shown to produce oxidative damage to membrane lipids in the brain. In this study, we investigated the effect of protracted social isolation stress on lipid peroxidation activity in the mouse brain and elucidated the protective effect of majonoside-R2, a major saponin component of Vietnamese ginseng, in mice exposed to social isolation stress.

Lipid peroxidation induced by oxidative stress is a consequence of the production of excess free radicals, such as reactive oxygen species, and provides marked damage to the structure and function of cell membranes in various tissues.1,2) Particularly, the brain is susceptible to free radical insults because it contains a high concentration of easily peroxidizable polyunsaturated fatty acids, and is not particularly enriched in protective antioxidant enzymes or other antioxidant compounds.3—5) Stressors with a physical factor such as immobilization, electric foot shock, cold swim, etc., have been demonstrated to produce oxidative damage to membrane lipid in the brain.6—9) In a previous study, mice exposed to psychological stress by a communication box paradigm also showed oxidative membrane damage due to enhanced lipid peroxidation in the brain.9) On the other hand, protracted social isolation of laboratory animals after weaning has been believed to be stressful9) because it produces a variety of physiological disturbances such as increases of corticosterone, ACTH and catecholamine levels in the serum10—12) and abnormal behaviors such as aggressiveness,13) hyperlocomotion,14) and anxiety-like behavior as the products of prolonged stress.15,16) Moreover, previous studies have demonstrated that protracted social isolation of male mice induces hyperactivity of central noradrenergic and corticotropin releasing factor systems3—7) and reduced function of γ-aminobutyric acid (GABA)-ergic system in the brain.16,18) However, no information is, so far, available if exposure of laboratory animals to protracted socially isolation stress, another kind of psychological stress produces oxidative damage in the brain.

Vietnamese ginseng (Panax vietnamensis HA et GRUSHV., Araliaceae) extract and total saponin have been shown to exert an inhibitory effect on lipid peroxidation reaction caused by the free radical generating system in the mouse brain and liver homogenates, while its major saponin constituent, majonoside-R2, failed to affect the reaction.19) However, it was found that when administered systemically, majonoside-R2 produced a protective effect on psychological stress-induced brain cell membrane damage by reducing an increase in thiobarbituric acid reactive substances (TBARS), NO, or glutathione levels in the brains of group-housed control mice but it significantly suppressed the increase in TBARS and NO levels and the decrease in glutathione levels caused by social isolation stress. These results suggest that mice subjected to 6—8 weeks of social isolation stress produces oxidative damage in the brain partly via enhancement of NO production, and that majonoside-R2 exerts a protective effect by modulating NO and glutathione systems in the brain.

Key words Social isolation stress; lipid peroxidation; glutathione; nitric oxide; majonoside-R2; Vietnamese ginseng

MATERIALS AND METHODS

Animals Male ICR mice (Japan SLC, Shizuoka, Japan) were obtained at the age of 4 weeks. They were housed in groups of 8—10 per cage (21×32×13 cm) or socially isolated by being housed individually for 2, 4, 6, or 8 weeks before the start of the experiments. Housing conditions were thermostatically maintained at 24±1°C with a constant humidity (65%) and a 12 h light : dark cycle (lights on 08:00—20:00). Food and water were given ad libitum The present studies were conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan and had the approval of the Institutional Animal Use and Care Com-
mittee in Toyama Medical and Pharmaceutical University.

**Chemicals** Majonoside-R2 (MR2) was isolated from five-year old Vietnamese ginseng root and rhizome (yield: 5.29%) as previously described.21 The other chemicals used in this study were obtained from the following commercial sources: albumin, Griess reagent, and glutathione reductase (Sigma Chem., St. Louis, MO, U.S.A.); 2-thiobarbituric acid, malondialdehyde, β-NADPH, EDTA, glutathione (GSH), 5,5’-dithiobis-(2-nitrobenzoic acid) (Wako Pure Chem., Osaka, Japan); Biuret solution (Nacalai Tesque., Kyoto, Japan).

**Majonoside-R2 Treatment** When examined the effect of majonoside-R2, mice were group-housed or socially isolated for 6 weeks prior to the experiments. Majonoside-R2 dissolved in saline was administered intraperitoneally (i.p.) to the animals once daily (at around 10 a.m.) for totally 3 d before the experiments. Saline administration group was used as a control.

**Measurement of Lipid Peroxidation Activity** Lipid peroxidation in the brain homogenate was measured as previously described.20,22 Briefly, the whole brain (excluding cerebellum) was homogenized in 10 vol. of ice-cold phosphate buffer (5 mM, pH 7.4) using a Potter–Elvehjem homogenizer with a Teflon pestle. The brain homogenates were mixed with the same volume of 10% (w/v) trichloroacetic acid and then centrifuged at 8000×g for 10 min. The supernatant was incubated with 0.8% (w/v) 2-thiobarbituric acid at 100°C for 15 min. After a cooling period, the content of thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation activity, was spectrophotometrically determined at 532 nm (Beckman DU640 Spectrophotometer) using malondialdehyde (MDA) as a standard. The results were expressed as pmol of MDA/mg protein. The protein contents of tissue homogenates were measured by the Biuret method.23

**Measurement of Total Nitric Oxide (NO) Metabolites in the Brain** To measure NO production in the brain, the contents of NO metabolites (i.e., NO2 and NO3) in the brain homogenate were determined using a HPLC-diazotization detecting method (HPLC-Griess) as previously described.23 The whole brain (excluding cerebellum) was homogenized in 4 vol. of methanol and centrifuged at 10000×g at 4°C for 10 min. The supernatant was mixed with the same volume of the mobile phase (0.15 M NaCl–NH4Cl, 10% methanol containing 1.1 mM EDTA–4Na). NO2 and NO3 in each sample were determined using an automated NO-detector HPLC system (ENO-20, Eicom, Kyoto, Japan) equipped with a reverse-phase separation column (10 μm polysyrene polymer; 4.6×50 mm, NO-PAK, Eicom, Kyoto, Japan) and a reduction column (5×5—6 mm; NO-RED, Eicom, Kyoto, Japan) packed with copperized cadmium to reduce separated NO3 to NO2. The resultant NO2 was mixed with the Griess reagent (a solution containing 1.25% HCl, 5 g/l sulphanilamide, and 0.25 g/l N-naphthylenediamine) to form a purple azo dye in a reaction coil placed in a column oven (35°C), and the absorbance of the color of the product dye at 540 nm was measured. The mobile phase solution and the Griess reagent were delivered by a pump at a rate of 0.33 and 0.1 ml/min, respectively. Total NO metabolites, NO2 and NO3, were calculated by summing NO2 and NO3 levels and expressed as nmol NOx/g tissue.

**Measurement of Total GSH Level in the Brain** Total GSH (GSH+GSH disulfide) content in the brain was measured using a modified spectrophotometric technique of Sedlack and Lindsay.24,25 The mouse brain was homogenized in a 0.1 M phosphate buffer (pH 7.4) and 10% trichloroacetic acid solution (2:1) to yield a 10% w/v homogenate. The homogenate was centrifuged at 3000×g for 15 min and the supernatant was mixed with 6% metaphosphoric acid (1:4). After re-centrifugation at 8000×g for 10 min, the supernatant was combined with 10 mM 5,5’-dithiobis-(2-nitrobenzoic acid) in the presence of 5 mM NADPH. After a 3-min incubation period at 25°C, the reaction was started by adding GSH reductase (1 unit/ml). The rate of change in absorbance was measured at 412 nm using a spectrophotometer (DU640, Beckman Instruments, Fullerton, CA, U.S.A.). The total amount of GSH in each sample was determined from a standard curve and expressed as nmol of GSH/mg protein.

**Statistical Analysis** Data are expressed as the mean±S.E.M. and analyzed by one- or two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons among different groups. Differences with p<0.05 were considered significant.

**RESULTS**

**Social Isolation Stress-Induced Enhancement of Lipid Peroxidation in the Whole Brain** In mice subjected to social isolation stress, the TBARS content in the brain was increased in an exposure period-dependent manner. The increase of the content reached a significant level at 6 and 8 weeks after starting social isolation stress exposure [F(3, 23)=3.24, p<0.05] (Fig. 1A). Moreover, the content of total NO metabolites NO2 and NO3 (NOx) in the brain, an index of NO production, was significantly higher in socially isolated mice than in unstressed control mice at 6 weeks after the start of social isolation stress exposure [F(7, 44)=5.58, p<0.01] (Fig. 1B). The NO production returned to the control level after 8 weeks of social isolation. On the other hand, the brain content of GSH was significantly decreased in socially isolated mice than in unstressed control mice at 6 weeks after the start of social isolation stress exposure [F(7, 45)=5.65, p<0.01]. The content was maximally decreased by a 6-week stress exposure (Fig. 1C).

**The Effects of Majonoside-R2 Treatment on the Contents of TBARS, NO, and GSH in the Brain of Socially Isolated Mice** We examined whether majonoside-R2 administration affects the social isolation stress-induced oxidative damage in the brain caused by a 6-week social isolation stress exposure. The systemic administration of majonoside-R2 (10—50 mg/kg, i.p.) for 3 d prior to the experiments had no effect on the contents of TBARS, NO, and GSH in the brain of unstressed control mice. However, it significantly suppressed the increase in the brain contents of TBARS and NO and the decrease in the brain content of GSH caused by social isolation stress [For MDA: Fstress×MR2(2, 41)=6.04, p<0.01; for NO: Fstress×MR2(2, 41)=6.7598, p<0.01; for GSH: Fstress×MR2(2, 41)=13.569, p<0.01] (Figs. 2A—C).

**DISCUSSION**

In this study, we have demonstrated that social isolation stress causes oxidative damage to the brain via enhanced...
lipid peroxidation and NO production accompanied with depletion of brain GSH content, one of the endogenous antioxidants in the brain tissue. Moreover, we found that majonoside-R2, a major saponin component of Vietnamese ginseng, exerted a protective effect on social isolation stress-induced oxidative damage by modulating NO and GSH systems in the brain.

Previous behavioral and pharmacological studies demonstrated that social isolation stress for 4—10 weeks caused a decreased susceptibility to barbiturates and other \( \gamma \)-aminobutyric acid (GABA) mimetic drugs and enhancement of aggressive behavior. In the present study, it was found that social isolation of mice produced oxidative brain membrane damage in a manner related to the duration of social isolation stress and that a significant increase in the brain content of TBARS occurred in the mice exposed to social isolation stress for 6—8 weeks. Reactive free radicals including reactive oxygen species are implicated in the pathogenesis of central nervous system injury, through a mechanism known as oxidative stress. Lipid peroxidation results from reactions between reactive oxygen species and polyunsaturated fatty acids of cell membrane and the amount of lipid peroxidation products is frequently used as an index of oxidative stress. There are lines of evidence indicating that one of the mechanisms underlying pathological consequences of stress in the brain is membrane damage caused by enhancement of lipid peroxidation. These results suggest that social isolation stress causes oxidative damage to brain membrane lipids via enhancement of free radical generation.

Besides the increase of TBARS, this study revealed an increase in NO production and a decrease of GSH content in the brain of socially isolated animals. These neurochemical alterations were significantly revealed after 6—8 weeks of stress exposure, while no change was observed in 2-week or 4-week isolation stressed mice. These findings suggested that social isolation stress is a model of chronic oxidative stress that leads to formation of free radicals such as superoxide radical, hydroxyl radical, and peroxynitrite and thereby causes neuronal damage. In fact, superoxide radicals play an important role in the toxic events, elicit GSH depletion, and react rapidly with NO to form peroxynitrite, a reactive and highly toxic free radical, hydroxyl radical, and peroxynitrite and thereby causes neuronal damage. In fact, superoxide radicals play an important role in the toxic events, elicit GSH depletion, and react rapidly with NO to form peroxynitrite, a reactive and highly toxic free radical.

NO, a free radical synthesized from \( \text{l} \)-arginine and oxygen by NO synthase (NOS), plays an important role in many physiological processes in the central nervous system. However, NO at a high concentration produces toxic effects after being converted to peroxynitrite, a reactive and highly toxic radical species, which initiates lipid peroxidation. In this study, we measured total NO\textsuperscript{−} content as an index of NO production, and demonstrated an increased level of NO in the brain of mice that had been socially isolated for 6 weeks. Moreover, the increase occurred partly in association with the change in the brain TBARS production. We previously reported that psychological stress exposure by a communica-
tion box paradigm elevated TBARS level in the brain and suggested an involvement of constitutive NOS-mediated production of NO in the stress-induced elevation of TBARS in the brain. Therefore, one may infer that the elevation of NO production also plays an important role in social isolation stress-induced oxidative damage via conversion to peroxynitrite. However, the mice socially isolated for 8 weeks showed a significantly increased level of TBARS but not NO in the brain. These findings suggest that enhancement of NO production is not a major factor involved in social isolation-stress induced brain membrane damage.

In this study, the brain NO content in mice housed in group or socially isolated for 2 weeks was apparently higher than those in the animal housed for 4—8 weeks. The reason for this difference in the brain NO content is unclear, but it may be due to developmental changes in NO production in the brain since constitutive NO synthase (NOS) activity and neuronal NOS containing neuron activity peak at around postnatal day 14—15.

In this study, the reduction of brain GSH content in 6-week or 8-week socially isolated animals was observed in association with a rise in lipid peroxidation. GSH is a potent endogenous antioxidant that reacts directly with radicals in non-enzymatic reactions and donates electrons for reduction of peroxides in the GSH peroxide-catalyzed reaction, and thereby protects cells from a number of noxious stimuli including oxygen-derived free radicals. Many studies have shown that animals subjected to various stressors such as cold restraint, water-immersion restraint and exercise have diminished GSH levels in various tissues. At least three possibilities seem to explain the social isolation stress-induced decrease in GSH content in the brain: increased consumption of GSH as an antioxidant, down-regulation of GSH synthesis, and increased metabolism of GSH secondary to stress. To clarify the exact mechanism(s) underlying the decrease in brain GSH levels resulting from social isolation stress requires further investigation.

It is of interest to note that systemic administration of majonoside-R2 exhibited a suppressive effect on lipid peroxidation in the brain caused by social isolation stress. The in vivo antioxidant effect of majonoside-R2 found in this study is consistent with previous reports demonstrating that administration of majonoside-R2 attenuated the increase of lipid peroxidation induced by psychological stress. Moreover, the present study revealed that majonoside-R2 reversed both the increase in NO content and the decrease in GSH level caused by social isolation stress. Taken together, it is possible that the antioxidant effect of majonoside-R2 is due to suppression of radical production systems such as NOS or due to facilitation of endogenous antioxidative mechanisms, and or both.

Stress causes enhancement of the release of the excitatory neurotransmitter glutamate in the brain, and thereby stimulates NO production via increasing an influx of Ca2+ into neuronal cells through the N-methyl-D-aspartate receptor- gated ion channel. A previous study demonstrated that psychological stress-induced increases in TBARS content in the brain was prevented by pretreatment with diazepam, an anxiolytic GABA_A receptor agonist. These results suggested that the enhancement of GABA_A neurotransmission by diazepam might reduce the activity of glutamatergic neurons and thereby suppress the effect of psychological stress on lipid peroxidation in the brain. Moreover, we reported that systemic administration of majonoside-R2 also attenuated psychological stress-induced increase in TBARS content in the brain and that the effect was blocked by pregnenolone sulfate, a negative allosteric modulator of GABA_A receptor.

In addition, in our previous studies, majonoside-R2, as well as a neuroactive steroid allotetrahydroxycorticosterone, reversed social isolation stress-induced decrease in responsiveness to GABA_A mimetic drugs such as pentobarbital and the effect was abolished by pregnenolone sulfate, suggesting that the effect of majonoside-R2 is mediated by the neurosteroid site on the GABA_A receptor complex in socially isolated mice. Thus, a speculative explanation for the antioxidative effect of majonoside-R2 in socially isolated animals is that majonoside-R2 may facilitate the reduced GABAergic function in socially isolated mice and thereby induces a protective effect on oxidative brain damage caused by social isolation stress. Nevertheless, further investigations will be required to elucidate the role of GABAergic systems in the antioxidant effect of majonoside-R2.

We cannot exclude the possibility that oxidative metabolism of catecholamines, which can be released excessively by stress, is involved in social isolation stress-induced oxidative damages in the mouse brain. It has been reported that extracellular dopamine levels in the cortex are higher in socially isolated mice compared to group-housed mice and that monoamines and related metabolites are capable of scavenging free radicals, chelating metal ions and inhibiting lipid peroxidation. Thus, it will be interesting to investigate further if catecholamine metabolism contributes to social isolation stress-induced oxidative damage in the brain, and whether majonoside-R2 exerts antioxidant activity via catecholamine pathway. Such investigations are now in progress in this laboratory.

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