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Brain Research 839 (1999) 74–84

BRAIN RESEARCH

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Research report

Psychological stress-induced enhancement of brain lipid peroxidation via nitric oxide systems and its modulation by anxiolytic and anxiogenic drugs in mice

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Accepted 8 June 1999

Abstract

We investigated the effect of psychological stress on lipid peroxidation activity in the mouse brain, the mechanism underlying the psychological stress-induced change in the activity, and the effects of anxiolytic and anxiogenic drugs on the activity in psychologically-stressed animals. Psychological stress exposure using a communication box paradigm for 2–16 h significantly increased the content of thiobarbituric acid reactive substance (TBARS), an index of lipid peroxidation activity, in the brain, and the effect was maximal after peaked by a 4-h stress exposure. In the animals stressed for over 4 h, the increased brain TBARS content lasted for 30 min after the stress exposure, while no significant increase of the TBARS content was observed in the liver or serum. Trolox (67.6 mg/kg, i.p.), an antioxidant drug, but not monoamine oxidase inhibitors, clorgyline (2.5–5 mg/kg, i.p.) or 5-(4-benzylphenyl)-3-(2-cyanoethyl)-(3*H*)-1,3,4-oxadiazol-2-one (1-5 mg/kg, i.p.), significantly suppressed the effect of psychological stress. The non-selective nitric oxide (NO) synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 10–100 mg/kg, i.p.) and the selective neuronal NOS inhibitor 7-nitroindazole (25 and 50 mg/kg, i.p.), but not the inducible NOS inhibitor aminoguanidine (1–100 mg/kg, i.p.), dose dependently suppressed the psychological stress-induced enhancement of lipid peroxidation in the brain. L-Arginine (300 mg/kg, i.p.), a substrate of NOS, antagonized the effect of L-NAME. Measurements of NO metabolites revealed a significant increase of NO production in the brains of stressed mice. The benzodiazepine (BZD) receptor agonist diazepam (0.05–0.5 mg/kg, i.p.), the 5-HT_{1A} receptor agonists (±)-8-hydroxy-di-propylaminotetralin and buspirone (0.1–1 mg/kg, i.p.), but not the 5-HT_3 receptor agonist MDL72222, dose-dependently suppressed the psychological stress-induced enhancement of brain lipid peroxidation. In contrast, the administration of anxiogenic drugs, FG7142 (an inverse BZD agonist: 1–10 mg/kg, i.p.) and 1-(3-chlorophenyl)piperazine (a mixed 5-$HT_{2A/2B/2C}$ agonist: 0.1–1 mg/kg, i.p.), potentiated it. The effects of diazepam and FG7142 were abolished by the BZD receptor antagonist flumazenil (10 mg/kg, i.p.). These results indicate that psychological stress causes oxidative damage to the brain lipid via enhancing constitutive NOS-mediated production of NO, and that drugs with a BZD or 5-HT_{1A} receptor agonist profile have a protective effect on oxidative brain membrane damage induced by psychological stress.

Keywords: Psychological stress; Lipid peroxidation; Brain; Nitric oxide; Anxiolytic drug

1. Introduction

Lipid peroxidation reaction induced by oxidative stress is a consequence of the production of excess free radicals, and this reaction has been proposed to produce marked damage to the structure and function of cell membranes [15]. The brain is particularly sensitive to free radical insults since it contains high concentrations of easily peroxidizable polyunsaturated fatty acid [2,6,8,10] and is not particularly enriched with protective antioxidant enzymes or other antioxidant compounds [22]. Thus, when ischemic or hypoxic brain is reperfused, the production of free radicals contributes to cerebral injury. Not only reperfusion of ischemic or hypoxic brain, but also a variety of physical stressors appear to affect lipid peroxidation activity in the brain [14,24,25,29,41]. For instance, immobilization stress causes oxidative damage to lipid in the brain of rats [29], although there are conflicting reports [24,41]. Clinical evi-

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0006-8993/99/$ - see front matter
PII: S0006-8993(99)01715-1

dence indicates a close relationship between the experience of psychological distress and the increase of lipid peroxidation activity in plasma [38]. These findings suggest that free radical generation is enhanced by stressful stimulations.

Nitric oxide (NO) is a free radical synthesized from L-arginine and oxygen by NO synthase (NOS) in the presence of NADPH. This enzyme exists as three distinct isoforms; two constitutive isoforms [the neuronal NOS (nNOS) and the endothelial NOS (eNOS)] and one inducible isoform (iNOS), and these isoforms are widely distributed in different brain areas [4,5]. Biochemical evidence indicates that the activation of nNOS and eNOS needs the elevation of intracellular Ca^{2+} [2]. Although NO plays an important role in many physiological processes in the central nervous system [40], a high concentration of NO produces a toxic effects via rapid interaction with superoxide anion (O_2^-) and forms peroxynitrite, which could be highly relevant to neurodegenerative diseases and neuronal membrane damage [2,3,47].

We previously reported that psychological stress caused gastric ulceration and a decrease in the duration of pentobarbital-induced sleep in mice, and that the $GABA_A$-benzodiazepine (BZD) receptor system is partly involved in these pathophysiological changes in psychologically-stressed animals [18,20]. However, very little information is available on the relationship between psychological stress and neurodegenerative diseases. Thus, in this study we attempted to investigate: (1) whether psychological stress produces oxidative damage to brain membrane lipid, (2) if it does, what is the role of NO in this damage, and (3) whether the psychological stress-induced oxidative brain membrane damage can be modulated by anxiolytic and anxiogenic drugs.

2. Materials and methods

2.1. Animals

Male ICR mice (5–7-week-old, Japan SLC, Shizuoka, Japan) were used for the experiments. The animals were housed in groups of 12–20 per cage (35 × 30 × 16 cm) for at least 1 week before the start of the experiments. Housing condition was thermostatically maintained at 24 ± 1°C with a constant humidity (65%) and a 12:12-h light/dark cycle (lights on: 0700–1900 h). Food and water were given ad libitum. The present studies were conducted in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

2.2. Apparatus

The communication box paradigm originally described by Ogawa et al. [34,35] was used as previously reported [18–20]. Briefly, the communication box consists of two types of compartments; compartments A and B (10 × 10 cm each). These compartments (25 compartments in total) are arranged like a ''checkerboard'' and are separated by transparent Plexiglas walls. All compartments have stainless steel electric grid floors, but the floors of the B compartments are covered with Plexiglas plates. Animals were individually placed in each compartment and intermittent electric shocks (2 mA, 10-s duration, 110-s intershock interval) were delivered through the grid floor by a shock generator (CGS-002, Muromachi-Kikai, Tokyo, Japan). The animals in the A compartments (sender) received the electric footshock through the grids floor, while the animals in the B compartments (responder) were only exposed to psychological stress by seeing and hearing the struggle, jumping, and/or vocalization of the sender mice in the adjacent compartments. The animals were exposed to psychological stress for 4 h, except specially stated cases. The unstressed control mice were placed individually in the compartments of the control box (10 × 10 cm) without electric grid floor and without exposure to the senders for the same period as the stressed mice.

2.3. Measurement of lipid peroxidation activity

Lipid peroxidation in the brain, liver homogenate and serum was measured as previously described [17] by modifying the method of Ohkawa et al. [36]. The animals were decapitated 30 min after the termination of psychological stress exposure unless stated otherwise, and then the whole brain (excluding cerebellum) and liver were removed and homogenized in 10 vol. of ice-cold phosphate buffer (5 mM, pH 7.4) and 9 vol. of 1.15% KCl solution, respectively, using a Potter-Elvehjem homogenizer with a Teflon pestle. Whole blood samples were rapidly collected by decapitation and centrifuged at 3000 rpm at 4°C for 15 min to obtain clear serum. The tissue homogenate (0.5 ml) was supplemented with 0.5 ml of phosphate buffer (for the brain homogenate) or 1.15% KCl solution (for the liver homogenate), and then with 1 ml of 10% trichloroacetic acid. The mixture was centrifuged at 8000 × *g* at 4°C for 10 min. The serum samples and the supernatants of the tissue homogenates were incubated with 1 ml of 0.8% (w/v) 2-thiobarbituric acid at 100°C for 15 min. After a cooling period, TBARS concentration was spectrophotometrically determined at 532 nm (Beckman DU640 Spectrophotometer) using malondialdehyde (MDA) as a standard. The protein contents of tissue homogenates and serum were measured by the Biuret method and the Lowry method [26], respectively.

2.4. Measurement of total NO metabolite (NO_x^-) in the brain

To measure NO production in the brain, the contents of NO metabolites (i.e., NO_2^- and NO_3^-) in the brain ho-

mogenate were determined using an a HPLC-diazotization detecting method (HPLC-Griess). Briefly, the whole brain (excluding cerebellum) was homogenized in 4 vol. of methanol and centrifuged at 10,000 × *g* at 4°C for 10 min. The supernatant was mixed with the same volume of the mobile phase (0.15 M NaCl–NH_4Cl, 10% methyl alcohol containing 1.1 mM EDTA–4Na). NO_2^- and NO_3^- 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 polystyrene polymer; NO-PAK, 4.6 × 50 mm, Eicom, Kyoto, Japan) and a reduction column (5 × 5–6 mm; NO-RED, Eicom) packed with copperized cadmium to reduce separated NO_3^- to NO_2^-. The resultant NO_2^- was mixed with the Griess reagent (a solution containing 1.25% HCl, 5 g/l sulphanilamide, and 0.25 g/l *N*-naphthylethylenediamine) 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 Griess reagent were delivered by a pump at a rate of 0.33 and 0.1 ml/min, respectively. Total NO metabolite (NO_x^-) was calculated by summing NO_3^- and NO_2^- levels.

2.5. Drug treatment

Test drugs were administered i.p. just before the stress exposure except specially stated cases. Drugs were obtained from the following sources: Trolox (a water-soluble vitamin E, Aldrich Chemical, Milwaukee); N^G-nitro-L-arginine methyl ester (L-NAME) and L-arginine (Nacalai tesque, Kyoto); 7-nitroindazole, clorgyline HCl, aminoguanidine hemisulfate (AG) and FG7142 (Research Bio-

Table 1
Effects of Trolox and monoamine oxidase inhibitors on psychological stress-induced increase in TBARS content in the brain
Trolox, an antioxidant, clorgyline, a selective MAO_A inhibitor, or 5-(4-benzylphenyl)-3-(2-cyanoethyl)-(3*H*)-1,3,4-oxadiazol-2-one [BPCEO], a selective MAO_B inhibitor, was administered i.p. just before starting 4 h of psychological stress exposure. Thirty minutes after terminating stress exposure, the animals were decapitated and the brain homogenate was prepared. TBARS in the homogenate was determined using malondialdehyde as a standard. The mean of the brain TBARS content in each unstressed vehicle group is expressed as 100%. Each value is the mean ± S.E.M. ($n = 6–7$).

Drugs	Dose (mg/kg, i.p.)	TBARS content in the brain (% of control)	
		Unstressed	Stressed
Antioxidant			
Trolox	0	100.0 ± 0.8	137.2 ± 3.6*
	67.6	100.3 ± 1.1	110.5 ± 2.7#
MAO_A inhibitor			
Clorgyline	0	100.0 ± 4.1	145.1 ± 5.5*
	2.5	101.9 ± 5.2	142.7 ± 5.3
	5.0	97.9 ± 5.0	140.7 ± 7.8
MAO_B inhibitor			
BPCEO	0	100.0 ± 6.7	147.6 ± 3.3*
	1	108.9 ± 4.4	144.7 ± 4.7
	5	106.9 ± 6.3	143.3 ± 6.4

* $p < 0.05$ compared with the unstressed vehicle group.
# $p < 0.05$ compared with the stressed vehicle group (the Student–Newman–Keuls test).

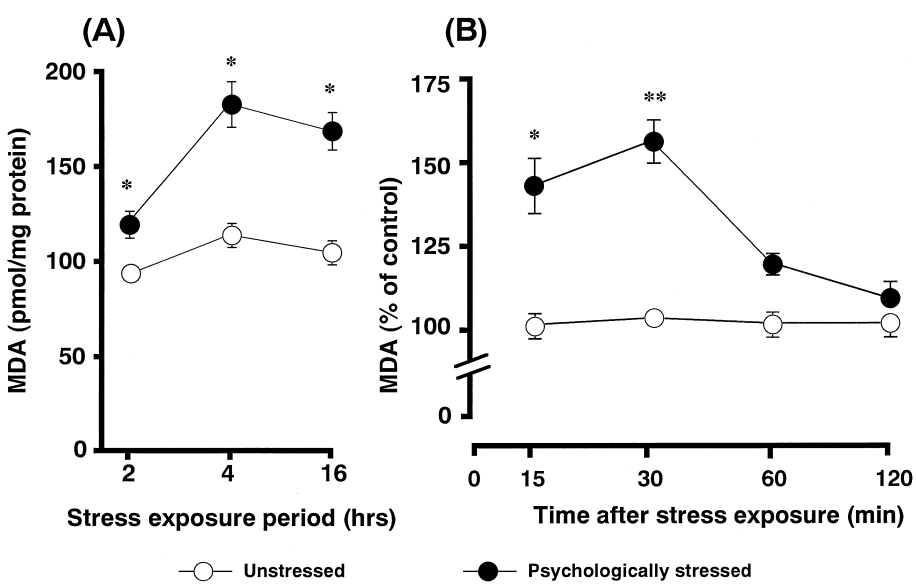

Fig. 1. Psychological stress-induced increase in TBARS in the mouse brain. (A) Mice were exposed to psychological stress for 2, 4 or 16 h. Two hours after stress exposure, TBARS, the product of lipid peroxidation, in the brain homogenate was determined using malondialdehyde (MDA) as a standard. Each data point is the mean ± S.E.M. ($n = 6$). * $p < 0.05$ vs. respective unstressed groups. (B) After psychological stress exposure for 4 h, the animals were decapitated at 15–120 min after stress exposure and then TBARS in the brain homogenate was determined. The mean of the brain TBARS content in each unstressed vehicle control group is expressed as 100%. Each data point is the mean ± S.E.M. ($n = 5$). * $p < 0.05$, ** $p < 0.01$ compared with the respective control.

chemicals, Natick, MA); diazepam (Cercine® injection, Takeda Chemical Industries, Osaka); flumazenil (Yamanouchi Pharm., Tokyo); 5-(4-benzylphenyl)-3-(2-cyanoethyl)-(3*H*)-1,3,4-oxadiazol-2-one (BPCEO), (±)-8-hydroxy-di-propylaminotetralin HBr (8-OH-DPAT), buspirone HCl, MDL72222, and 1-(3-chlorophenyl)piperazine (mCPP: TOCRIS, Bristol). Diazepam and Trolox were dissolved in saline containing 40% propylene glycol. 7-Nitroindazole and flumazenil were suspended in saline containing 0.1% Tween 80. BPCEO, FG7142 and MDL72222 were suspended in saline containing 0.5% sodium carboxymethyl cellulose. Other test drugs were

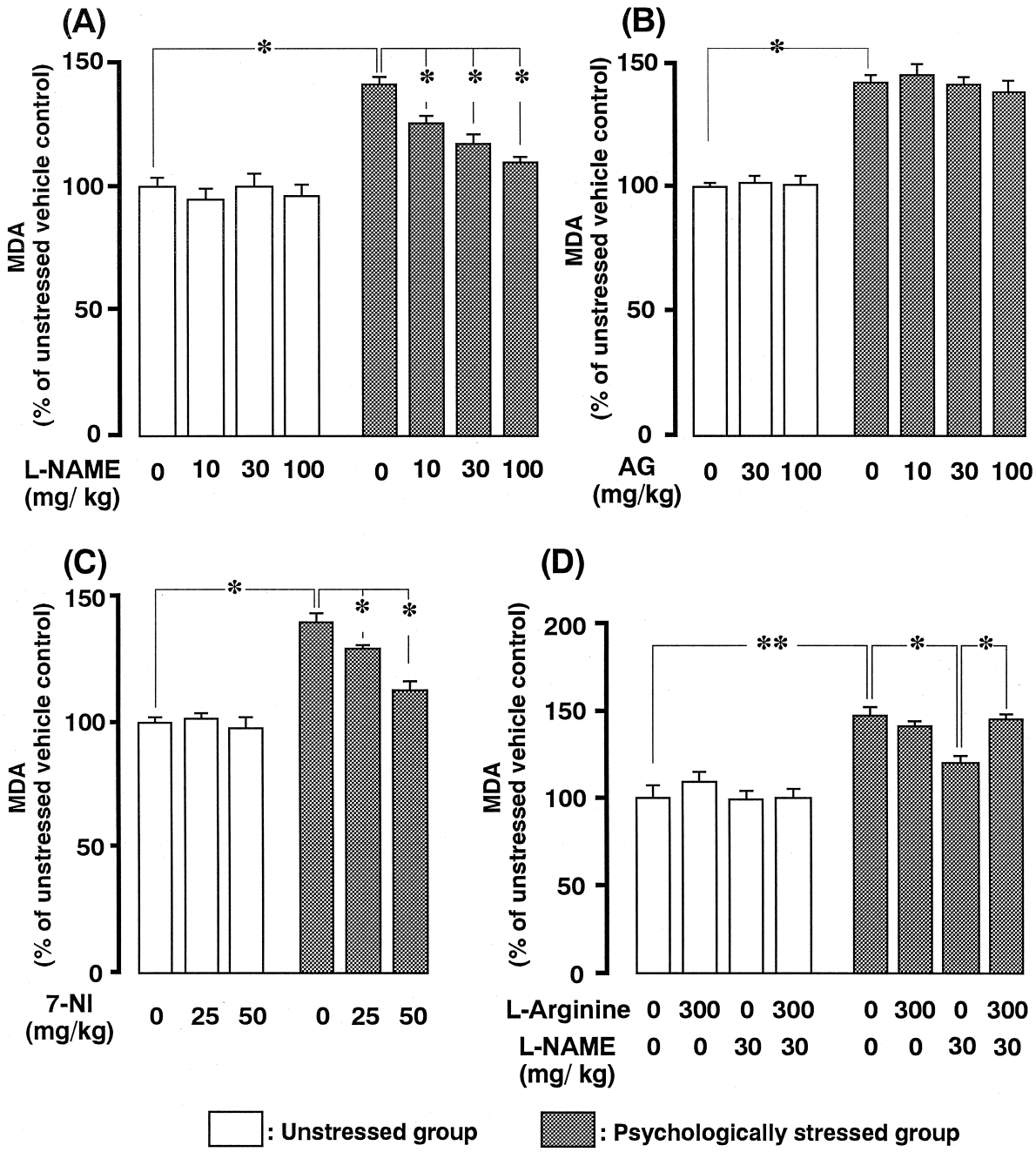

Fig. 2. Effects of NO synthase inhibitors, L-NAME, aminoguanidine (AG) and 7-nitroindazole (7-NI), and a NO donor, L-arginine, on psychological stress-induced enhancement of TBARS production in the brain homogenate. A–C: The non-selective NOS inhibitor L-NAME (A: 10–100 mg/kg), the selective iNOS inhibitor aminoguanidine (B: 10–100 mg/kg), and the selective nNOS inhibitor 7-nitroindazole (C: 25–50 mg/kg) were administered i.p. immediately before psychological stress exposure for 4 h. D: L-arginine and L-NAME were coadministered immediately before the stress exposure for 4 h. Thirty minutes later, the animals were decapitated and the brain homogenate was prepared. TBARS in the homogenate was determined using malondialdehyde (MDA) as a standard. TBARS in the homogenate was determined as described in the text. The mean of the brain TBARS content in each unstressed vehicle control group is expressed as 100%. Each value is the mean ± S.E.M. ($n = 6$). * $p < 0.05$, ** $p < 0.001$ (the Student–Newman–Keuls test).

dissolved in saline. Drug solutions were prepared just before the start of the experiments and injected i.p. at a constant volume of 0.1 ml/10 g body weight.

2.6. Statistics

Data were 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.

3. Results

3.1. Psychological stress-induced enhancement of lipid peroxidation in the whole brain

When mice were exposed to psychological stress, the TBARS content, an index of lipid peroxidation activity, in the brain was significantly increased in an exposure period-dependent manner and it was maximally increased by a 4-h stress exposure (Fig. 1A). Therefore, the animals were exposed to the stress for 4 h in the other experiments. The increased brain TBARS content in psychologically-stressed mice lasted for over 30 min after termination of stress exposure and then gradually returned to the same level as that in unstressed animals (Fig. 1B). On the other hand, psychological stress exposure for 4 h had no significant effect on TBARS contents in the liver homogenate or serum: TBARS contents in the liver homogenates prepared from unstressed control and psychologically-stressed mice were 162.9 ± 8.9 and 186.2 ± 17.0 pmol/mg protein (mean $\pm$ S.E.M., $n = 8$), respectively, and TBARS contents in serum were 1379.9 ± 132.2 ($n = 5$) and 1296.2 ± 188.2 pmol/mg protein ($n = 6$), respectively.

As summarized in Table 1, pretreatment of the animals with the antioxidant drug Trolox significantly antagonized the psychological stress-induced increase in the brain TBARS content, but it had no effect on the brain TBARS content in unstressed animals. In contrast, the selective monoamine oxidase (MAO)-A inhibitor (MAO_A) clorgyline and the selective MAO_B inhibitor BPCEO, did not change the brain TBARS content in either animal group.

3.2. Involvement of NO in psychological stress-induced enhancement of lipid peroxidation activity in the brain

To clarify the possible involvement of the NO radical in the psychological stress-induced increase in the brain TBARS content, animals were pretreated with various NOS inhibitors before stress exposure. As shown in Fig. 2, the non-selective NOS inhibitor L-NAME (10–100 mg/kg, i.p.) and the selective neuronal NOS inhibitor 7-nitroindazole (25 and 50 mg/kg, i.p.) dose dependently attenuated the increase of TBARS production in the brain of psychologically stressed animals without having any effect on the brain TBARS content in unstressed animals. However, AG (10–100 mg/kg, i.p.), a selective inhibitor of inducible NOS, had exhibited no effect on the increased production of TBARS in the brain. Coadministration of L-arginine (300 mg/kg, i.p.) with L-NAME (30 mg/kg, i.p.) completely reversed the suppressive action of L-NAME in psychologically-stressed animals without affecting the basal level of brain TBARS in unstressed or psychologically stressed animals (Fig. 2C). As shown in Fig. 3, when measured immediately after a 4-h stress exposure, the total NO_x^- level in the brain was significantly higher in psychologically stressed animals than in unstressed control animals. However, no significant difference in the total NO_x^- level between unstressed and psychologically stressed mice was observed at 30 min after termination of stress exposure.

3.3. Effects of anxiolytic and anxiogenic drugs on psychological stress-induced enhancement of lipid peroxidation activity in the brain

3.3.1. BZD receptor-related drugs

Diazepam (0.05–0.5 mg/kg, i.p.), a BZD receptor agonist, dose dependently suppressed the psychological

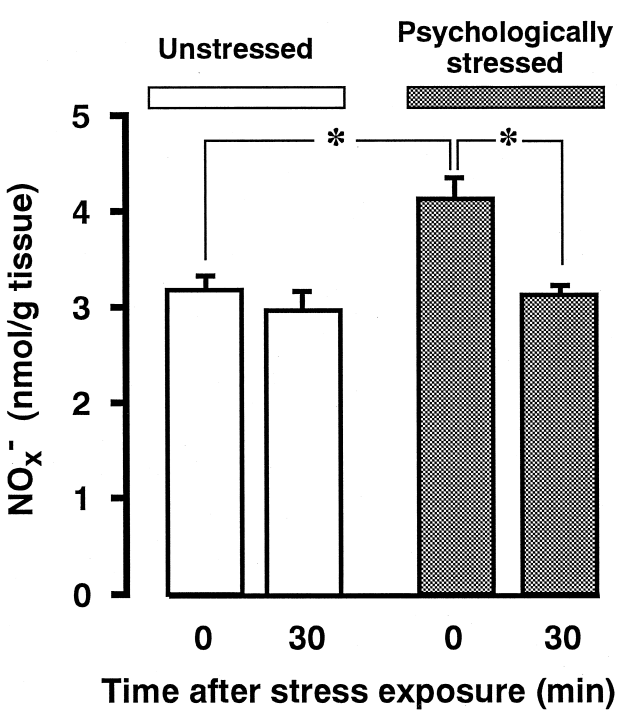

Fig. 3. Psychological stress-induced elevation of NO_x^- level in the brain. Mice were exposed to psychological stress for 4 h. Immediately or 30 min after stress exposure, the animals were decapitated and the brain homogenate was prepared as described in the text. NO_3^- and NO_2^- in the homogenate were determined using HPLC, and the content of $NO_3^- + NO_2^-$ in the brain was expressed as NO_x. Each value is the mean $\pm$ S.E.M. of 7–8 ICR mice. * $p < 0.05$ (the Student–Newman–Keuls test).

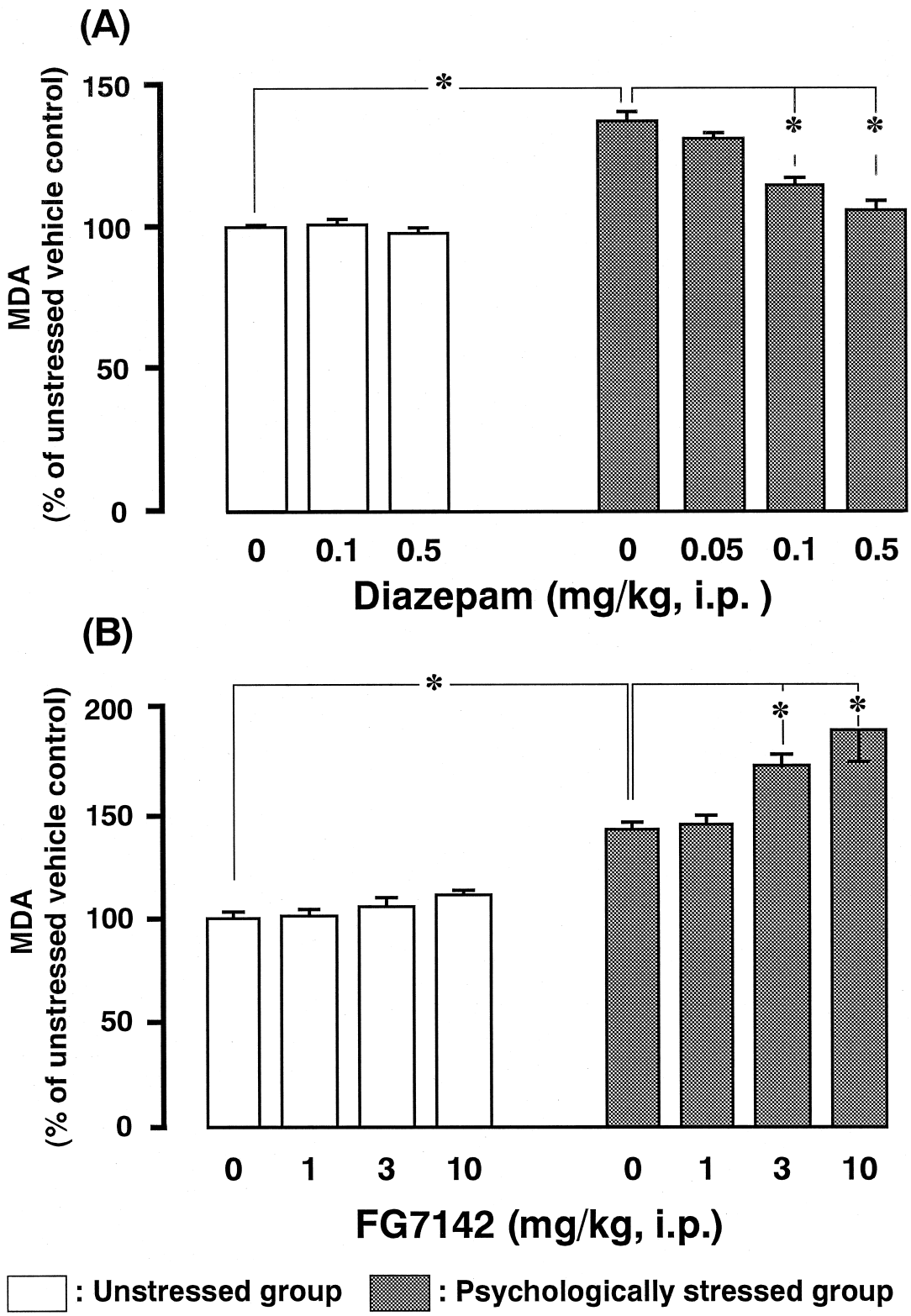

Fig. 4. Effects of diazepam and FG7142 on psychological stress-induced enhancement of TBARS production in the brain. Diazepam (A: 0.05–0.5 mg/kg) or FG7142 (B: 1–10 mg/kg) was administrated i.p. just before the start of stress exposure. The mice were decapitated 30 min after stress exposure and the brain homogenate was prepared. TBARS in the homogenate was determined using malondialdehyde (MDA) as a standard. TBARS in the homogenate was determined as described in the text. The mean of the brain TBARS content in each unstressed vehicle control group is expressed as 100%. Each value is the mean ± S.E.M. ($n = 5–8$). $^{*}p < 0.05$ (the Student–Newman–Kuels test).

stress-induced enhancement of TBARS generation in the brain, while FG7142 (1–10 mg/kg, i.p.), an inverse BZD agonist, potentiated the effect of psychological stress in a dose-dependent fashion (Fig. 4). These drugs had no effect

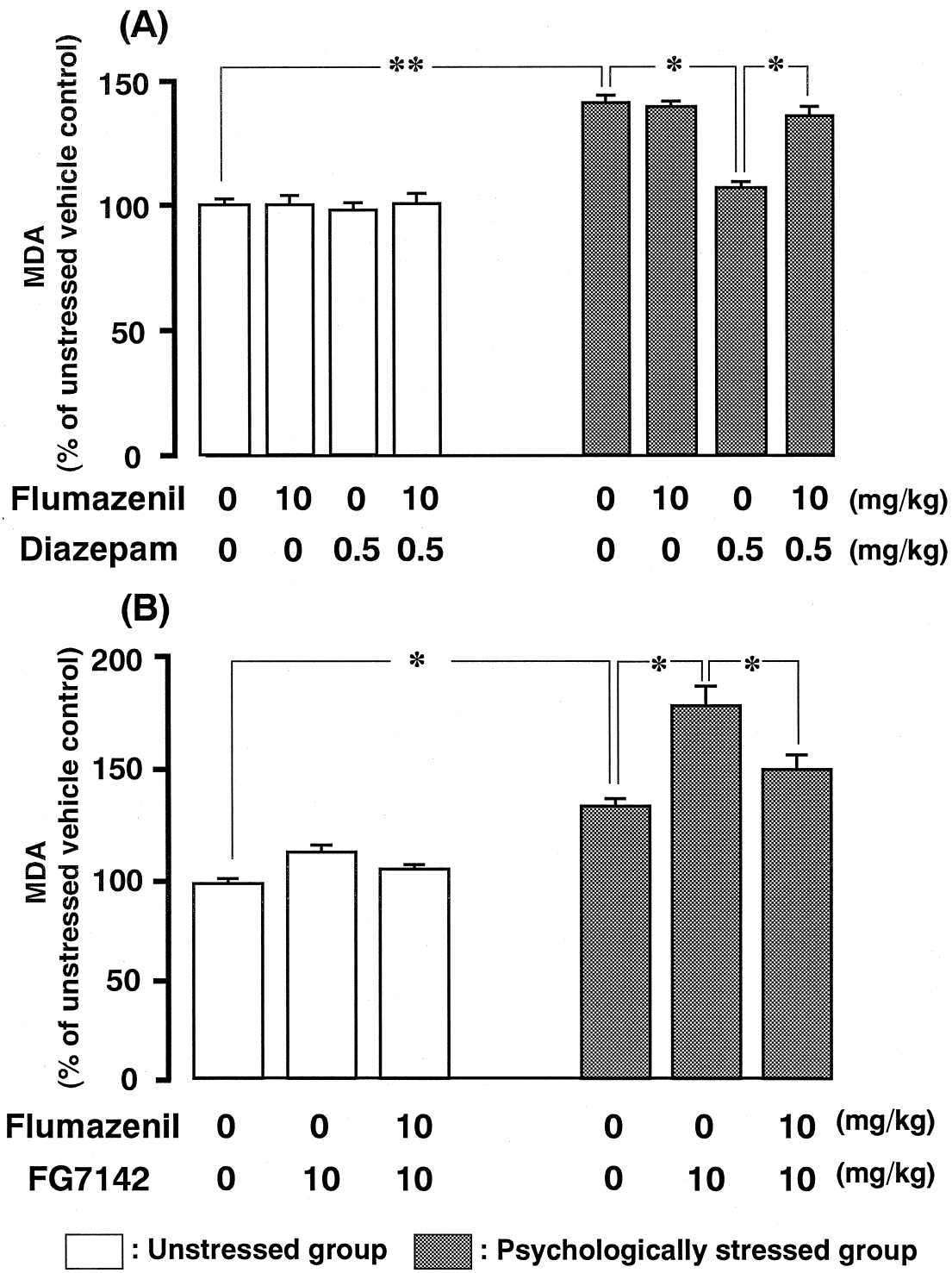

Fig. 5. Effects of flumazenil on diazepam-induced suppression (A) and FG7142-induced potentiation (B) of TBARS production in the psychologically-stressed mouse brain. Flumazenil (10 mg/kg) was administered i.p. 15 min before stress exposure. Diazepam (0.5 mg/kg) or FG7142 (10 mg/kg) was administered i.p. 15 min before stress exposure. The animals were decapitated 30 min after stress exposure and then the brain homogenate was prepared. TBARS in the homogenate was determined using malondialdehyde (MDA) as a standard. TBARS in the homogenate was determined as described in the text. The mean of the brain TBARS content in each unstressed vehicle control group is expressed as 100%. Each value is the mean ± S.E.M. ($n = 6$). ** $p < 0.01$, * $p < 0.05$ (the Student–Newman–Kuels test).

on the TBARS content in the brain of unstressed animals. As shown in Fig. 5, the effects of diazepam (0.5 mg/kg, i.p.) and FG7142 (10 mg/kg, i.p.) in psychologically stressed animals were significantly attenuated by pretreatment with flumazenil (10 mg/kg, i.p.), a selective BZD receptor antagonist.

3.3.2. Serotonin receptor-related drugs

As summarized in Table 2, buspirone (0.1–1 mg/kg, i.p.), a partial 5-HT_{1A} receptor agonist, and 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist, both dose dependently attenuated the enhancement of TBARS generation caused by psychological stress without affecting the TBARS generation in unstressed animals. In contrast, the mixed 5-$HT_{2A/2B/2C}$ receptor agonist mCPP (0.1–1 mg/kg, i.p.) dose dependently potentiated the psychological stress-induced enhancement of TBARS production in the brain, but it had no effect on the brain TBARS content in unstressed animals. The selective 5-HT_3 receptor antagonist MDL72222 (0.1–1 mg/kg, i.p.) had no modulatory effect on the TBARS generation in unstressed or psychologically stressed animals.

Table 2
Effects of serotonergic drugs on psychological stress-induced increase in TBARS content in the brain
The 5-HT_{1A} agonists buspirone and 8-OH-DPAT, the 5$HT_{2A/2B/2C}$ agonist mCPP, or the 5-HT_3 antagonist MDL72222 was administered i.p. just before stress exposure. Animals were either exposed to psychological stress for 4 h or unstressed. Thirty minutes after terminating stress exposure, the animals were decapitated and the brain homogenate was prepared. TBARS in the homogenate was determined using malondialdehyde as a standard. The mean of the brain TBARS content in each unstressed vehicle group is expressed as 100%. Each value is the mean ± S.E.M. ($n = 6$).

Drugs	Dose (mg/kg, i.p.)	TBARS content in the brain (% of control)	
		Unstressed	Stressed
Buspirone	0	100.0 ± 1.5	133.3 ± 4.3*
	0.1	n.d.	129.4 ± 2.8
	0.3	97.5 ± 3.3	122.6 ± 3.0#
	1.0	99.2 ± 3.4	108.4 ± 3.4#
8-OH-DPAT	0	100.0 ± 2.5	137.5 ± 4.6*
	0.1	99.5 ± 5.4	132.4 ± 4.9
	0.3	98.4 ± 3.6	113.8 ± 2.6#
	1.0	91.0 ± 5.1	106.7 ± 1.7#
mCPP	0	100.0 ± 6.0	137.5 ± 2.9*
	0.1	n.d.	143.5 ± 7.4
	0.3	107.4 ± 5.9	147.8 ± 11.2
	1.0	104.5 ± 7.0	167.1 ± 16.7#
MDL72222	0	100.0 ± 2.4	134.7 ± 3.6*
	0.1	n.d.	135.1 ± 4.6
	0.3	98.1 ± 3.4	128.5 ± 5.8
	1.0	99.8 ± 5.1	128.6 ± 3.7

* $p < 0.05$ compared with the unstressed vehicle group.
# $p < 0.05$ compared with the stressed vehicle group (the Student–Newman–Keuls test).

4. Discussion

A number of studies have demonstrated stress-induced lipid peroxidation not only in peripheral tissues [16,28,49] but also in the brains [29,44] of experimental animals. Most of these findings, however, are derived from the experimental paradigms based on physical stress or stress with a physical stimulatory factor, so that psychological stress-induced oxidative damage to brain tissue is less well understood. In this study, we examined the effect of psychological stress on oxidative damage to brain lipid by using the communication box system, which has been shown to expose experimental animals to conditioned emotional stimuli which is more psychologically conditioned stress than the commonly used stressors such as immobilization, electric footshock, water-immersion, etc. [1,34]. The present results demonstrated that psychological stress given by this paradigm causes a significant enhancement of lipid peroxidation in the mouse brain, and that this enhancement is due to an increase in NO production mediated by constitutive NOS in the brain.

Psychological stress exposure increased the brain TBARS content in a manner that depended on the time of stress exposure. This increase was maximal after a 4 h of psychological stress exposure, and lasted for 30 min after relief from the stress. It should be noted that under the same conditions (4-h stress exposure), no significant changes in the TBARS content were observed in the liver homogenate or serum prepared from psychologically stressed animals. These results suggest that the brain tissue can be more easily damaged by psychological stress than other tissues, such as the liver. In addition, it can be considered that the psychological stress-induced increase in the TBARS content is mediated by enhanced generation of free radicals in the brain because Trolox, a radical-scavenging antioxidant, totally suppressed the increase [52].

Neurochemical evidence demonstrated the enhancement of catecholamine release from various brain regions evoked by stressful stimulation [9,13,21,46] and it has been previously reported that monoamines and related metabolites are capable of scavenging free radicals, chelating metal ions and thereby inhibiting lipid peroxidation [27]. Considering these findings, it is likely that the increase in the brain TBARS content caused by psychological stress is underestimated. However, the present results indicate that such underestimation is likely to be very small because clorgyline, a selective MAO-A inhibitor, and BPCEO, a selective MAO-B inhibitor, had no effect on the brain TBARS level in psychologically stressed animals.

It should be noted that the non-selective NOS inhibitor L-NAME and the selective nNOS inhibitor 7-nitroindazole, but not the selective iNOS inhibitor AG, antagonized the psychological stress-induced enhancement of lipid peroxidation in the brain and that L-arginine almost completely abolished the effect of L-NAME without changing the lipid peroxidation activity in unstressed or psychologi-

cally stressed animals. These findings strongly suggest that the nNOS-mediated elevation of NO production is at least implicated in the enhancement of lipid peroxidation in psychologically stressed mice. This idea is further supported by the present determination of total NO_x, which revealed a significant increase of the brain NO level in psychologically stressed mice, although the time course of the change in the brain NO_x content differed from that of the change in the brain TBARS production in psychologically stressed animals. Thus, it is possible that the elevated NO may trigger a lipid peroxidation reaction in the brain, provably via being converted to peroxynitrite. The reason for the discrepancy in the time course between NO and TBARS productions in psychologically stressed mice remains unclear, but it may be due to a difference in the kinetics of disappearance from the brain tissue between NO metabolites and TBARS.

Biochemical evidence indicates that NO production by constitutive NOS needs L-arginine, oxygen, NADPH and elevation of cytosolic Ca^{2+} [2] and that NO reacts with O_2^-, which is likely produced by circulating xanthine oxidase, mitochondria and/or other sources, and thereby produces peroxynitrite, a reactive species which is highly toxic and initiates lipid peroxidation [2,37,50]. Moreover, it has been demonstrated that the excitatory neurotransmitter glutamate stimulates NO production by causing an influx of Ca^{2+} into the neuronal cells via activation of the *N*-methyl-D-aspartate (NMDA) receptor-gated ion channel [33]. Thus, the most plausible explanation for the psychological stress-induced enhancement of brain lipid peroxidation is that this stress may over-activate glutamatergic neurotransmission in the brain, resulting in excessive elevation of intracellular Ca^{2+} concentration and an increased production of NO. This idea seems to be partly supported by the previous findings that stress causes enhancement of glutamate release in the brain [30,32,39].

We previously demonstrated that psychological stress exposure caused a decrease in the duration of pentobarbital sleep and an increase in the threshold to show a nociceptive response in mice, and suggested that GABAergic systems are at least partly involved in these behavioral changes following psychological stress exposure [19,20]. In the present study, diazepam, an anxiolytic BZD receptor agonist, and FG7142, an anxiogenic BZD receptor inverse agonist, exerted opposite effects on lipid peroxidation activity in the brains of psychologically stressed animals, i.e., suppression and exacerbation of the activity, respectively, in a manner sensitive to flumazenil, a selective BZD receptor antagonist. The antagonism by flumazenil gives evidence that scavenging of radicals is not involved in the suppressive action of diazepam, and suggests a modulatory role for the $GABA_A$/BZD receptor/chloride ionophore complex in the psychological stress-induced enhancement of brain lipid peroxidation. The exact mechanism by which BZD drugs affect the enhancement of brain lipid peroxidation caused by psychological stress remains unclear. Brain microdialysis studies have demonstrated that FG7142 enhances glutamate release from glutamatergic nerve terminals in the prefrontal cortex, while anxiolytic BZD drugs reduces it in the hippocampus [23,43]. Thus, it is likely that the enhancement of $GABA_A$ receptor function should reduce the activity of glutamatergic neurons and thereby suppress the effect of psychological stress on lipid peroxidation activity in the brain. It is also possible that enhancement of GABAergic neurotransmission by diazepam produces inhibitory effects on postsynaptic Ca^{2+} channels coupled with NMDA receptors [31].

In this study, we also found that the 5-HT_{1A} receptor full agonist 8-OH-DPAT and the 5-HT_{1A} receptor partial agonist buspirone attenuated the psychological stress-induced enhancement of lipid peroxidation in the brain, whereas mCPP, a mixed $5HT_{2A/2B/2C}$ receptor agonist, exacerbated it. The previous reports from this and other laboratories demonstrated that systemic administrations of 8-OH-DPAT or buspirone exerts an anxiolytic effects in experimental animals [11,42,45,51] although there is a conflicting report [7]. On the other hand, mCPP reportedly produces an anxiogenic effects in experimental animals [48]. Considering these findings, the different effects of 5-HT_{1A} and $5HT_{2A/2B/2C}$ receptor agonists observed in this study may be closely related to the difference in the effects of these drugs on the anxiety state of psychologically stressed animals. This idea seems further supported by the finding that MDL72222, a 5-HT_3 receptor antagonist, failed to affect lipid peroxidation enhanced by stress, since, in contrast to 5-HT_{1A} receptor agonists, the 5-HT_3 antagonists ICS205-930 and MDL72222 did not produce an anxiolytic effect-like action in previous studies [12,42].

In conclusion, the present study demonstrated that psychological stress exposure causes an increase in lipid peroxidation activity in the brain via facilitating NO production mediated by constitutive NOS, and that the psychological stress-induced membrane damage in the brain can be prevented by diazepam and 5-HT_{1A} receptor agonists with anxiolytic activity.

Acknowledgements

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan.

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