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A HUMORAL LIPID INDUCING STRESS-COPING BEHAVIOR MOTIONLESSNESS ON MOUSE GIVEN FORCED SWIMMING STRESS

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Key words : adaptogenic activity, forced swimming stress, humoral lipid, mouse age, stress duration

Abstract

Forced swimming in narrow space gives mice mental and physical stress. Their stress-coping behavior is recognized as hyperactivity climbing for escaping from the stressful situation, or motionlessness for keeping their physical strength. Mice given the stress would produce adaptogenic substances inducing the stress-coping behaviors. In the present study, conditions inducing motionlessness and humoral lipid inducing the behavior are investigated. The motionlessness of 9 weeks-old DDY mouse was increased in proportion to the given stress duration one day before. The motionlessness of mice with different age given the 10 minutes-stress one day before was increased corresponding to the age. Injection of lipid-fraction eluted with 100 mM NaCl obtained from sera of 9 weeks-old mice given the 15 minutes-stress increased motionlessness of mice without the previous stress. Fraction eluted with 100 mM NaCl of cerebroside sulfates also increased the motionlessness with a dose-dependent manner. These suggest that the humoral lipid with adaptogenic activity might be a cerebroside sulfate.

Key words : adaptogenic activity, forced swimming stress, humoral lipid, mouse age, stress duration

Introduction

Living things produce internal substances against stresses for keeping their adaptation. Some of the botanical substances, which are neither proteins nor peptides, have stress-coping activities on animals. They have been called adaptogen[1,2]. Animals could also produce their adaptogenic substances after given stress. Stress-coping behaviors of animals are recognized as hyperactivity for escaping from the stressful situation and motionlessness to avoid wasting the physical and mental strength. These behaviors have been also found in mice given forced swimming stress. An adaptogenic humoral lipid inducing the hyperactivity was previously reported[3]. In the present study, the author examines style of the motionlessness in mouse, and investigates humoral lipid inducing the motionlessness.

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Materials and Methods

1. Animals

Nine, 13 or 17 weeks-old female DDY mice (Japan SLC., Shizuoka) were used. They were housed in a group of five in a plastic cage (338×140×225 mm) with free access to food (F_2 : Funabashi Farm Co. Tokyo) and water. The animal room was kept at 21-25°C with 50-60% humidity and illuminated from 7 : 00 to 19 : 00. The experiments were performed from 13 : 00 to 17 : 00. All of the experiments were carried out under the control of the Ethics Committee for Animal Experiments of Akita University School of Medicine, Japan.

2. Apparatus in the present investigation

The apparatus used to give forced swimming stress and to investigate mouse behaviors in the forced swimming test was prepared according to previous report (cylinder ; 10 cm diameter, 20 cm height, containing water of 25°C at a depth of 10 cm)[4].

3. Measure of motionlessness duration

Mouse in the forced swimming situation is given continuous stress. The stress-coping behaviors are climbing for escaping from the situation, and floating and stereotyped behaviors for keeping the physical and mental strength[5]. Duration of the former is easily measured, but that of the latter is not[6]. Therefore, the duration of motionlessness was measured as no climbing in the present study.

4. Statistical analysis

The Kruskal-Wallis rank test was used to find significant differences among clusters. When significant differences were found (*P*-value of less than 0.01), the Mann-Whitney U test was used.

5. Examination on mice given different stress duration

① *Mice*

Nine weeks-old mice were used.

② *Duration of the motionlessness*

Five mice were individually given forced swimming stress for 5, 10 or 15 min in the apparatus described above. One day after the treatment, duration of the motionlessness of these mice was measured for 3 min in the apparatus. As control, the duration of other 5 mice, which were left in the apparatus without water for 15 min one day before, was also measured for 3 min in the apparatus with water.

6. Examinations on mice with different age

① *Animals*

Nine, 13 or 17 weeks-old mice were used.

② *Duration of the motionlessness*

Each 5 mice with the different age were individually given 10 min-forced swimming stress in the apparatus. Duration of the motionlessness of these mice was measured for 3 min one day after given the stress in the apparatus. As control, other 5 of mice with the different age were left in the apparatus without water for 10 min, and duration of the motionlessness of these mice was also measured for 3 min in the apparatus with water one day after the pretreatment.

7. Humoral lipid fraction inducing motionlessness

① *Humoral lipid fraction*

Twenty five of 9 weeks-old mice were individually given forced swimming stress for 5, 10 or 15 min. These mice were scarified one day after the stress-treatment, and the sera were collected and pooled. Humoral lipid fractioning was initially performed using methanol-chloroform method previously described[7]. Briefly, each 10 ml of these sera was added 12.5 ml of chloroform and 25 ml of methanol. The solution was agitatedly shaken for 3 min, and left for 10 min at room temperature (RT). Then, 12.5 ml of chloroform was added to the solution, and was agitatedly shaken for 30 s. Furthermore, 10 ml of water was added to the solution, and agitatedly shaken for 30 s. The solution was centrifuged with 150 x g for 5 min at RT. The lower chloroform layer was collected and evaporated. Then, the extracted lipids were dissolved in 10 ml of water. The solution was applied on 2 ml of an ion-exchanger DE-52 (Whatman Co., Maidstone, UK), which had been saturated with a buffer of 10 mM $NaHCO_3$ pH 8.3 and washed with water, and eluted with 6 ml of 50, 100, 150, 200, 250 and 300 mM NaCl. These

fractions were prepared to 10 ml with water.

② *Fraction increasing duration of the motionlessness*

New 5 of 9 weeks-old mice were intraperitoneally injected with 400 ml of the fraction described above or 400 ml of physiological saline (PS) as control. Ten min after the injection, duration of the motionlessness of these mice was measured for 3 min.

③ *Dose-dependent effect of the fraction*

New 5 of the mice were intraperitoneally injected with 500, 300 or 100 ml of the fraction eluted with 100 mM NaCl of sera of mice given the 15 min-stress. As control, other new 5 mice were intraperitoneally injected with 500 ml of PS. Ten min after the injection, duration of the motionlessness of these mice was measured for 3 min.

④ *Lipid-glycosylation in the fraction*

Lipid-glycosylation in the fraction eluted with 100 mM NaCl of 9 weeks-old mice given the 15 min-stress was detected using 50% ethanol lectin ELISA[8]. Briefly, 50 ml of the fraction and 50 ml of ethanol was poured into a well of a 96 holes plastic plate (Sumitomo Bakelite Co. Osaka) and left for two h at RT. Each well was blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) for 30 min at RT. After 5 times washing with PS, the well was reacted with biotinized lectins : AAL recognizing Fuca1-3GlcNAc, DBA recognizing GalNAca1-3GalNAc, MAM recognizing Siala2-3Gal, PNA recognizing Galb1-3GalNAc and SBA recognizing GalNAca1-3Gal (Seikagaku Co. Tokyo) for 45 min at RT. After 5 times washing with PS, horseradish peroxidase-conjugated avidin (Seikagaku Co.) was poured into each well and was left for 5 min at RT. The degree of color change obtained by a peroxidase-coloring kit (Sumitomo Bakelite Co.) was measured at dual wavelength 455/600 nm.

⑧ *Effect of fraction eluted with 100 mM NaCl of cerebroside sulfates on motionlessness*

Ten mg of cerebrosides sulfates (Avanti Polar Lipids Inc., Alabama, USA) was dissolved in 1 ml of water and was fractioned and prepared to 1 ml with the method described in 7-①. New 5 of 9 weeks-old mice were intraperitoneally injected with 100, 50 or 25 ml of the fraction eluted with 100 mM NaCl, or 100 ml of PS as control. Ten min after the injection, duration of the motionlessness of these mice was measured for 3 min.

Results

1. *Findings in 9 weeks-old mice given different stress duration*

Duration of the motionlessness was increased in proportion to the given forced swimming duration. The duration of mice given the 15 min-stress was longer than that of mice given the 5 or 10 min-stress, furthermore than that of the control mice (Table 1).

2. *Findings in mice of different age*

The motionlessness-duration of mice one day after given 10 min-forced swimming stress was increased in proportion to the age. Although the duration of mice previously given no swimming stress was not different in the age (Table 2).

Table 1. Motionlessness-duration (sec) of 9 weeks-old mice one day after given the different forced swimming stress-duration (min)

The duration (sec)			
Stress duration (min) given mice			Control
15	10	5	0
178.2±2.2*	131.4±2.2*	127.4±3.3*	143.2±6.4

The value in this table is the mean±SD of the duration of the 5 mice.
*$P<0.05$ compared to control, Mann-Whitney U test.

Table 2. Motionlessness-duration of mice with different age one day after given 10 min-forced swimming stress

The duration (sec)			
	Mouse age (weeks-old)		
	17	13	9
Given the stress	173.6±4.5	164.0±2.6	130.0±4.4
Control	150.6±6.1	150.0±6.7	146.0±2.4

The value in this table is the mean±SD of the duration of the 5 mice.
Control : 5 mice no given the forced swimming stress one day before.

3. *Humoral lipid fraction increasing motionlessness-duration*

Mice injected with lipid fraction eluted with 100 mM NaCl of mice given the 10 or 15 min-stress showed increase of the motionlessness-duration (Table 3).

Lipid-fraction eluted with 100 mM NaCl of mice given the 15 min-stress dose-dependently increased motionlessness-duration of mice without previous stress (Table 4).

Lipid in the fraction eluted with 100 mM NaCl of 9 weeks-old mice given the 15 min-stress did not show affinity to lectin which interacts with Fucα_{1-3}GlcNAc, GalNAcα_{1-3}GalNAc, Sialα_{2-3}Gal, Galβ_{1-3}GalNAc or GalNAcα_{1-3}Gal (Table 5).

Table 3. Motionlessness-duration (sec) of 9-weeks-old mice treated with humoral lipid-fraction of mice given the different stress-duration (min)

The duration (sec)				
Fraction eluted with NaCl (mM)	Stress duration (min) given mice 15	10	5	Control (400 µl of PS)
50	144.0±5.8	145.7± 6.2	147.0± 6.0	
100	165.4±3.5*	162.6± 2.4*	140.3± 5.3	141.2±12.3
150	147.0±6.4	125.0±12.3	132.1± 6.6	
200	147.3±4.5	145.0± 4.2	146.3± 3.8	
250	142.6±9.4	143.6± 5.2	146.8± 8.6	
300	142.6±3.2	143.8± 8.4	141.6±13.4	

Mice were treated with 400 µl of the fraction.
The value in this table is the mean±SD of the duration of the 5 mice.
*$P<0.05$ compared to control, Mann-Whitney U test in the 100 mM fraction.
PS : physiological saline.

Table 4. Dose-dependent effect of the lipid-fraction eluted with 100 mM NaCl of sera of 9 weeks-old mice given the 15 min-stress on the motionlessness-duration

The duration (sec)			
Dose (µl) 500	300	100	Control (500 µl of PS)
172.2±1.7*	168.4±1.2*	150.6±3.4*	142.3±1.8

The value in this table is the mean±SD of the duration of the 5 mice.
*$P<0.05$ compared to control, Mann-Whitney U test.
PS : physiological saline.

4. *Effect of fraction eluted with 100 mM NaCl of cerebroside sulfates*

The fraction dose-dependently increased motionlessness-duration of mice without previous stress (Table 6).

Discussion

Motionlessness-duration of DDY mice was increased in proportion to the previously given stress duration and corresponding to their age. When the motionlessness is considered as defensive stress-coping behavior for keeping physical and mental strength, this stress-coping style

Table 5. Lectin binding affinity in lipid-fraction eluted with 100 mM NaCl of sera of 9 weeks-old mice given the 15 min-stress

Light absorbance					
	Lectin AAL	DBA	MAM	PNA	SBA
Subject	0.038	0.038	0.070	0.010	0.005
Blank (PS)	0.036	0.034	0.006	0.015	0.010

Light absorbance (dual-wave length 455/600 nm)
AAL recognizes Fucα_{1-3}GlcNAc, DBA does GalNAcα_{1-3}GalNAc, MAM does Sialα_{2-3}Gal, PNA does Galβ_{1-3}GalNAc and SBA does GalNAcα_{1-3}Gal.
PS : physiological saline.

Table 6. Motionlessness-duration of 9 weeks-old mice treated with fraction eluted with 100 mM NaCl of cerebroside sulfates

The duration (sec)			
Dose (µl) 100	50	25	Control (100 µl of PS)
166.2±2.2*	158.4±5.5*	146.4±3.5	147.6±3.1

The value in this table is the mean±SD of the 5 mice.
*$P<0.05$ compared to control, Mann-Whitney U test.
PS : physiological saline.

would be found in other animals.

Behavior of animals is controlled by neurological system of the brain. Neurological system inducing the motionlessness would relate to production of the adaptogenic humoral lipid. The lipid would enter brain through blood-brain barrier and affect the neurological system. In fact, the author and the colleagues previously reported that humoral lipid inducing offensive stress-coping behavior climbing was globopentaosylceramide, Forssman antigen pentose[3]. They also reported that the glycolipid production was related to alpha2-adrenoceptor activity in the central neurological system[9]. On the other hand, although it has been known that humoral glycolipids have a sugar chain structure, Fuc$\alpha_{1\text{-}3}$GlcNAc, GalNAc$\alpha_{1\text{-}3}$GalNAc, Sial$\alpha_{2\text{-}3}$Gal, Gal$\beta_{1\text{-}3}$GalNAc or GalNAc$\alpha_{1\text{-}3}$Gal[10], these activities were not found in the fraction increasing motionlessness. Furthermore, fraction eluted with 100 mM NaCl of cerebroside sulfates increased motionlessness-duration. These suggest that the effective humoral lipid might be a cerebroside sulfate. But the author could not identify the humoral lipid, and the neurological mechanism inducing motionlessness has not yet been clarified.

Nevertheless, it was previously reported that forced swimming stress increased anabolic hormone[11], that anabolic hormone increased beta-adrenoceptor activity[12], and that beta-adrenoceptor activity was closely related to relaxation[13]. When the motionlessness is considered to a behavioral mode of relaxation, the present findings might suggest that the adaptogenic humoral lipid inducing motionlessness affects neurological system relating with relaxation to keep the metabolic adaptation via the beta-adrenoceptor activity.

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