Specific Alternation of Rhythm in Temperature (SART) Stress-Induced Irritable Bowel Syndrome-Like Changes in Mice and Effects of Drugs

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Stress is closely associated with the manifestation and progress of irritable bowel syndrome (IBS). For the purpose of establishing experimentally the relationship between IBS and stress, the transportation capacity of the small intestine in specific alternation of rhythm in temperature (SART)-stressed animals was studied using charcoal transportation method. The charcoal suspension was administered orally into the stomach of fasting mice. Mice were sacrificed after a certain time and %charcoal transit (%CT) of the small intestine was measured. The %CTs in SART-stressed mice were greater than those in unstressed or continuously cold-stressed mice. This increase in %CT remained for 1 week after discontinuation of SART stress loading. Cholinergic blockers decreased %CTs in SART-stressed mice. Increases in %CT by a cholinesterase inhibitor were less in SART-stressed mice than in unstressed mice. Increases of %CT in SART-stressed mice were suppressed by Neurontine. These results suggested that the parasympathetic hypertonicity, not just cold, played a role in the increases in the transportation capacity in SART-stressed mice and that these animals can be a useful tool for elucidation of the mechanism of IBS.

Key words stress; irritable bowel syndrome; cholinergic nerve; intestine; charcoal transit

In recent years, the number of patients with gastrointestinal (GI) motility disorders such as irritable bowel syndrome (IBS) has been increasing rapidly. IBS is one of the functional disorders of the GI tract and is classified into C1 of the Rome III criteria. IBS is diagnosed when patients have no organic diseases of the intestine, but have diarrhea or constipation, or both, accompanied by abdominal pain and often exacerbation of symptoms and psychosocial factor (stress) is regarded as one of the criteria for IBS. However, there still is no good model of IBS at the experimental level. The objectives of the present study were to examine intestinal motility of stressed animal.

Specific alternation of rhythm in temperature (SART) stress is loaded upon animals by exposure to cold temperature of short period repeatedly for 7 days. Animals under this stress are in the state of autonomic imbalance with systemic symptoms of parasympathetic hypertonicity and sympathetic hypotonicity, which were displayed in mecholyl test and Ashner’s ocular compression test. In the GI system, the level of acetycholine (ACh) elevates and the activity of ACh esterase decreased in the small intestine. In the duodenum of SART-stressed animals, contractile response to ACh and number of muscarinic cholinergic receptors were decreased.

In the present study, the transportation capacity of the small intestine of SART-stressed mice was investigated using charcoal transportation method. SART stress-induced motility abnormalities of the small intestine were studied to elucidate partly the relationship between IBS and stress.

MATERIALS AND METHODS

Experimental Animals ddY strain male mice, 4- to 5-weeks old, weighing approximately 25 g (Japan SLC, Hamamatsu, Japan) in the normal state were used in accordance with ethical procedures following the guidelines for the care and use of laboratory animals issued by the Japanese government and Japanese Pharmacological Society. Animals were reared at room temperature (24±1 °C) and 12 h-light and dark cycle (lights on at 8:00 a.m., lights off at 8:00 p.m.) and allowed to eat freely (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and drink freely tap water.

Stress Loading Method. SART Stress SART stress was loaded according to the method by Hata et al. Rearing cages were prepared in two rooms; one was the rearing room at room temperature (24 °C) and the other was the low temperature room (4 °C). Animals were moved between the two rooms and left in the 24 °C room for 30 min or longer in order to avoid direct effects of cold before being used for experiments.

Cold Stress Animals were reared for 2 days in the animal rearing chamber with inside temperature at 4 °C. Animals were used for experiments immediately after completing cold loading.

Preparation of Activated Charcoal Solution The 10% activated carbon solution was prepared by suspending activated charcoal in 3% aqueous solution of arabic gum.

Drugs Used Drugs used were cholinergic blocking agents; atropine sulfate monohydrate (Wako Pure Chemical Industries Ltd., Japan), methyl atropine (atropine methyl bromide, Sigma, St. Louis, MO, U.S.A.) and butyl scopo-
lamine (butyl scopolamine bromide, Sigma, St. Louis, MO, U.S.A.), a cholinesterase inhibitor; neostigmine (Wako Pure Chemical Industries, Ltd., Japan), and a pain remedy Neufigin® (NTP, Nippon Zoki Pharmaceutical Co., Ltd., Japan) which is non-protein extract from cutaneous tissue of rabbit inoculated with vaccinia virus, and has reported to be effective on many symptoms developed by SART stress. All drugs were dissolved in 0.9% physiological saline. Atropine and methylatropine were administered orally 50 min before the administration of charcoal activated (Wako Pure Chemical Industries Ltd., Japan). Butyl scopolamine and neostigmine were administered subcutaneously 30 min and 20 min, respectively, before the administration of charcoal. A control group received solvent of each drug. In experiments of daily administration of atropine and NTP, drugs were administered once a day up to the preceding day of the charcoal experiments.

Measurement of Charcoal Transportation Rate % Charcoal Transit (%CT) of the Small Intestine Activated carbon solution 0.1 ml/10 g body weight was administered orally to mice which fasted for 18 to 20 h at night. After a certain time, mice were sacrificed by cervical dislocation. Immediately thereafter, laparotomy and extirpation of the intestinal portion from the pyloric region of stomach to the cecum were performed. The total length of the small intestine and the distance between the pyloric region of stomach and the tip of charcoal migration were measured to calculate the %CT, which is percentage of charcoal migration against the full length of small intestine.

Statistical Processing Results of experiments were expressed in mean±standard error. Significant differences from results obtained from control mice were tested using Student’s t-test for comparisons between 2 groups and one way analysis of variance (one way ANOVA) and Tukey–Kramer’s test for comparisons among 3 or more groups. In both cases, p-values of less than 0.05 (p<0.05) were considered to indicate significant difference.

RESULTS

Time-Related Changes in %CT in the Mouse Small Intestine The %CTs were calculated successively during the period of 60 min after oral administration of charcoal (Fig. 1). The %CTs in both unstressed and stressed mice increased time-dependently and were approximately 80% in both groups 60 min after oral administration. The %CT was greater at any time in the SART-stressed mice than in the unstressed mice (p<0.01, two way ANOVA, F(1, 170)=49.240). Especially during 30 min after administration of charcoal, the %CTs in the SART-stressed mice were significantly greater (Tukey’s test). Based on this result, the efficacy of drugs was evaluated using the %CT 20 min after administration of charcoal as an index in the following experiments.

Figure 2 summarizes detailed results 20 min after charcoal administration. There was no significant difference in the total length of the small intestine between the unstressed group and the stressed group. The distance of charcoal migration was significantly greater in the stressed group and, correspondingly, the %CT was also significantly greater in the stressed group. The SART stress/unstress (S/U) ratio, or ratio of values for charcoal distance (mm) and charcoal transit (%) were 1.24 and 1.21, respectively. In other words, the small intestine of the SART-stressed group had approximately a 20% increase in charcoal transportation capacity.

Daily Changes in the Transportation Capacity of Small Intestine of SART-Stressed Mice after Cessation of Stress Loading Figure 3 shows daily changes in %CT after completion of SART stress loading.

The %CTs increased by approximately 20% following SART stress loading. Increases in %CTs due to stress were hardly changed 1, 3 and 7 days after cessation of stress loading and persisted for at least 1 week.

Effects of Cold Stress on the Transportation Capacity of the Small Intestine Figure 4 shows effects of cold stress, which was used as control to SART stress on the side of low temperature, on %CT. The %CT in cold-stressed mice
20 min after charcoal administration was 42.2 ± 1.05%, which was similar to the %CT of unstressed mice (41.5 ± 0.8%). The %CT of SART-stressed mice at the time was 52.2 ± 1.2%, which was significantly greater (*p* < 0.01) than that in either unstressed mice or cold-stressed mice.

**Effects of Drugs on the Charcoal Transportation Capacity of the Mouse Small Intestine**

Figure 5 shows effects of atropine on %CT. The %CT in the unstressed group was significantly suppressed by atropine 5 mg/kg or more. The stressed group was sensitive to a small dose of 0.5 mg/kg and above and the %CTs were suppressed dose-dependently. The S/U ratios were between 1.13 and 1.02, and less than that in the control group which was 1.22.

**Effects of Other Cholinergic Blocking Agents and a Cholinesterase Inhibitor**

Table 1 lists results of single oral administration of methylatropine, butyl scopolamine and neostigmine and daily administration of atropine.

In order to suppress %CT, high doses of methylatropine, 10 mg/kg or higher, were needed in the unstressed group, whereas 2 mg/kg or higher were needed in the stressed group. The S/U ratios were between 1.15 and 1.03, decreasing dose-dependently.

Similarly, the S/U ratios decreased dose-dependently with butyl scopolamine.

In neostigmine, the %CTs in both unstressed and stressed groups increased dose-dependently. Increases in the SART-stressed group were markedly smaller than those in the unstressed group.

Unlike single administration, daily administration of atropine did not affect %CT in the unstressed group. In the SART-stressed mice, the %CTs decreased dose-dependently, reaching the level of %CT of the unstressed control group.

**Anti-SART Stress Effect of Daily Administered NTP**

Figure 6 shows effects of NTP on %CT. NTP has been reported to be effective on many symptoms developed by SART stress. Following consecutive daily administrations of NTP 200 and 400 noirotopin unit (NU)/kg, the %CTs in the stressed group were suppressed dose-dependently, nearing the level in the unstressed control group, whereas the %CTs of the SART-stressed mice were not affected by consecutive daily administrations of 200 NU/kg.
In the present study, the transportation capacity remained elevated 7 days after stress loading discontinued. Unlike in general stress, adaptation to stress was not observed in SART stress.

Effects of drugs are discussed below. Single administration of cholinergic blocking agents including atropine, methylatropine and butyl scopolamine suppressed the charcoal transportation capacity of the small intestine in both unstressed and stressed mice, the latter of which was suppressed more greatly than the former. Neostigmine, a peripheral cholinesterase inhibitor, increased the charcoal transportation capacity of the small intestine. The increases were less in the SART-stressed mice. Parasympathetic nerves play important roles in intestinal contraction. Changes in IBS symptoms such as diarrhea and constipation should also be affected by contraction abnormalities of the intestine. SART-stressed animals are of systemic parasympathetic hypertonicity.7 Said parasympathetic hypertonicity may play a large role in the mechanism of IBS-like changes such as elevation of the transportation capacity of the small intestine demonstrated in the present study. NTP improved the elevated transportation capacity of the small intestine in the present study. NTP is an extract from the inflamed skin of rabbits inoculated with vaccinia virus and used widely as an analgesic to treat chronic pain caused by intractable neuropathic pain. The drug has recently been reported to be effective to fibromyalgia.22) NTP has anti SART-stress effects on many SART stress-induced symptoms including hyperalgesia.23) The effect of NTP in the present study is thought to be also due to said anti-SART stress effects, that is, regulating effects on autonomic imbalance seen in SART-stressed animals which deflect toward different ways by regions of body, suggesting that the drug has anti-IBS effects.

IBS is a chronic functional disease without organic lesions. Psychological and physical stresses affect greatly the onset of IBS.2—4 Recent studies have found that mental factors such as anxiety and depression play important roles in the manifestation of IBS. Major symptoms of IBS are motility abnormalities of the intestine and hyperesthesia accompanied by abdominal pain and dejection abnormalities. In small intestine of SART-stressed mice, contractile response to ACh9 and number of muscarinic receptors were decreased.10,11) Other manifestations include hyperalgesia, depression,25) anxiety and behavioral changes.27,28) We find that these symptoms are improved by some antianxiety drugs (data not shown). Many of these abnormalities resemble human IBS symptoms, suggesting that SART-stressed animals can be an IBS model and a useful tool for elucidation of the mechanism of IBS.

**DISCUSSION**

Diagnostic criteria of human IBS are based on the Rome III Criteria compiled in 2006,13 which include the persistence of abnormal defecation (constipation, diarrhea or alternating normal defecation etc.), abnormal test results such as hyperkinesia of the intestine and absence of organic changes of tissues.5) SART-stressed animals used in the present experiments are the model of autonomic imbalance in the type of parasymptathetic hypertonicity with sympathetic hypotonicity.7) Additionally, the animals have a slight diarrhea with loose stools.

IBS patients are found to have no abnormalities in the length of the small intestine or organic changes in the tissues. The total length of the small intestine is shorter than normal in patients with inflammatory intestinal diseases such as ulcerative colitis and Crohn’s disease.24) It is known that the total length of the colon is found to be shorter in model animals of inflammatory intestinal diseases, which are created by administering sodium dextran sulfate or 2,4,6-trinitrobenzenesulfonic acid.13—15) The present experiments demonstrated that there was no change in the length of the small intestine of SART-stressed mice.

In the present study, any change was not found in the shape and the full length in the small intestine isolated from unstressed and stressed groups. Thus, organic changes such as inflammation may not occur in SART-stressed mouse small intestine. The charcoal transportation capacity of the small intestine was greater in the SART-stressed mice than in unstressed mice, indicating hyperkinesis of the small intestine and absence of organic changes of tissues.5) SART-stressed animals used in the present experiment are the model of autonomic imbalance in the type of parasymptathetic hypertonicity with sympathetic hypotonicity.7) Additionally, the animals have a slight diarrhea with loose stools.

It was reported that the transportation capacity of the small intestine was suppressed by restraint stress in rats and by fasting in mice.15) The capacity was not affected by sound stress.10) In the present study, the transportation capacity of mouse small intestine increased by SART stress and was hardly affected by cold stress. Varied effects of stress on the transportation capacity of the small intestine may be due to types of stressors, duration of stress loading and stress being acute or chronic. More importantly, increased transportation capacity of the small intestine due to SART stress appears to be affected not simply by cold factor but by the psychological factor that it is impossible to pull oneself out of the environment of stress loading.

Cold stress is used as an acute physical stress. SART stress is well known to be not only physical stress but also chronic stress with a psychological aspect, and symptoms and changes persist even after stress loading is discontinued.20,21) In the present study, the transportation capacity remained elevated 7 days after stress loading discontinued. Unlike in general stress, adaptation to stress was not observed in SART stress.

The charcoal transportation capacity in the mouse small intestine was measured 20 min after oral charcoal. Data show the mean±S.E.M. from 8—6 mice. **p<0.01 vs. respective unstressed group, and ***p<0.01 vs. respective control group (Tukey–Krammer’s test).

**Fig. 6. Effects of Neurotropin on Stress-Induced Changes in Charcoal Transit in the Mouse Small Intestine**

□, U; unstressed mice. ■, S; SART-stressed mice. NTP: Neurotropin. NU: Neurotropin Unit. NTP was daily administered orally. Charcoal distance in small intestine was measured 20 min after oral charcoal. Data show the mean±S.E.M. from 8—6 mice. **p<0.01 vs. respective unstressed group, and ***p<0.01 vs. respective control group (Tukey–Krammer’s test).
As a conclusion, in SART-stressed mice, the transportation capacity in the small intestine increased, and on this phenomenon the parasympathetic hypertonicity played a role. These animals can be a useful tool for elucidation of the mechanism of IBS. In further studies on stress and IBS, the relationship will be investigated from the aspect of not only cholinergic nervous system as in the present experiments but also serotonergic nervous system.

REFERENCES