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Restraint Plus Water-immersion Stress (RWIS)-induced Stomach Ulcers in Mice are Depressed by a Conditioning Heat Treatment

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Abstract : A conditioning mild heat treatment can depress subsequent severe heat-induced biological damage by inducing thermal tolerance. In the work described here, the effect of a conditioning mild heat exposure at 37°C was examined on the formation of gastric mucosa damage induced by a restraint plus water-immersion stress (RWIS) treatment in mice. RWIS treatments induced stomach ulcers in about 42% of the control animals, but a mild conditioning heat treatment at 37°C was found to depress the appearance of ulcers, and only about 9% of the pre-exposed animals developed ulcers. In addition, RWIS treatment induced an apoptosis rate in the stomach of about 13%, but a conditioning mild heat treatment depressed the apoptotic rate to about 8%. Since the heat shock protein (HSP) inducer geranylgeranylacetone (GGA) also efficiently depressed stomach ulcer incidence rates, it is suggested that gastric mucosa damage may be depressed by HSPs induced by a mild conditioning heat treatment through the depression of apoptosis.

Key Words : heat stress, restraint plus water-immersion stress (RWIS), stomach ulcer, apoptosis, adaptive response

Introduction

Molecules, cells, organs, and entire living organisms can recognize and respond to various environmental stresses. Furthermore, organisms can attempt to adapt to changed environments[1)]. After being released from, or escaping from these altered or stress producing environments, organisms can reverse or abolish their stress induced responses and seek to return to their normal state, and to resume their normal level of homeostasis. Many kinds of stress proteins are induced after exposure to stresses

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and can act as protection against subsequent stresses. It is well established that a conditioning treatment with mild heat induces heat-tolerance in organisms ranging from bacteria to human. This phenomenon is caused by heat shock proteins (HSPs) induced in response to stress[2,3]. In addition, inhibitors of HSP induction depress thermotolerance[4−6]. Environmental changes or challenges can include radiation and ultraviolet (UV) light such as genotoxic stresses. Previous exposure to heat as a priming treatment, or to low doses of UV and radiation as priming treatments can lead to resistance to these agents at subsequent higher challenging doses[1,7,8]. The mechanisms involved in adaptive responses to harmful agents are very important from the viewpoint of radiation research, especially at the molecular level[9,10]. On the other hand, non-genotoxic stresses such as physiological stresses like restraint plus water-immersion stress (RWIS) have been reported to induce HSPs which act to depress stress-induced biological damage[11−14]. However, when organisms must respond to excessive changes, adaptation may not be successful for cells, organs or entire organisms. In such cases, if an organism cannot adapt, stressful environmental changes can become lethal.

RWIS has frequently been used as a model for ulcer formation in the stomach wall in mice. The appearance of stomach ulcers was used as an indicator for the degree stress. RWIS produces a psychologically severe stress, and a mild heat treatment was used in these experiments as a conditioning pre-heat treatment to investigate the appearance of any adaptive response.

Materials and Methods

Mice

Male C57BL/6N mice 8 weeks old were used. Mice were maintained at 22.5°C (room temperature,

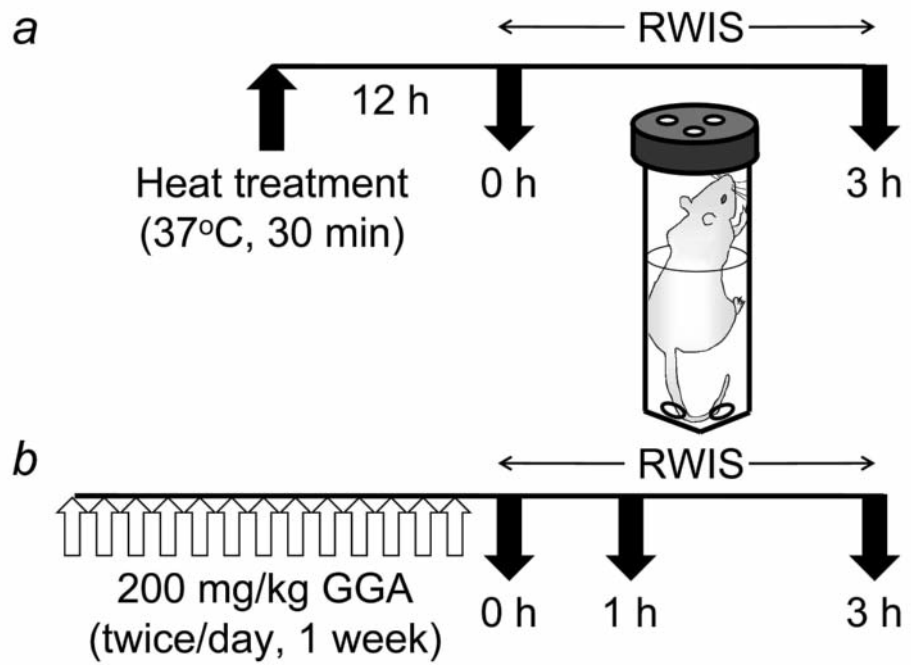

Fig. 1. Experimental schedules: ***a***) Conditioning pre-heat and RWIS treatments; ***b***) GGA and RWIS treatments.

RT). They were kept in a clean conventional environment, and given nutritional chow and water *ad libitum*. The mice were maintained on a 7 AM-7 PM light-dark cycle and were acclimated to laboratory conditions for 2 weeks before use. This study was conducted according to the Guidelines for Animal Welfare and Experimentation issued by the Nara Medical University.

Conditioning heat treatment and RWIS

Conditioning heat treatments were performed on 5 mice at 37°C. The mice were maintained at 22.5°C (AT-12R, Tomas Scientific, Swedesboro, NJ, U.S.A.). Control mice were always maintained at 22.5°C. The conditioning treatment was for 30 min at 12 h before RWIS treatment. For RWIS treatment, mice were kept in a 50 ml centrifuge tube (2345-050, Asahi Glass Co., Ltd., Funabashi, Japan) in a position in which their nose and mouth were kept above the water level. They were immersed into a water bath (Thermominder EX, TAITEC Co., Ltd., Saitama, Japan) at 37°C for a maximum of 3 h at 12 h after a conditioning treatment in an incubator (Fig. 1).

Geranylgeranylacetone (GGA) treatment

GGA was given to mice at a concentration of 200 mg/kg (as an emulsion with 5% gum arabic and 0.008% α-tocopherol)[13] twice per day directly through the mouth with a pipette beginning 1 week before stress treatments.

Stomach ulcer analysis

Immediately after a RWIS treatment, mice were sacrificed by dislocating neck vertebrae, and were then dissected. The inside surface of the stomach was photographed and three stages were used to define normal (white and pink) and damaged (red and carmine) tissues which can be scored by their different colors. Gastric mucosa damage was judgment of use of each small size square (0.75 mm^2).

Histological studies of apoptosis

For histological studies, samples were obtained from the stomach and fixed in a 10% neutralized formalin buffer solution. Apoptotic cells in the sections of mucosa were detected by staining with an ApopTag *in situ* Detection Kit® (Millipore Co., Billerica, MA, USA), which modifies genomic DNA with terminal deoxynucleotidyl transferase to detect positive cells using specific staining. In all samples, over 500 cells were counted in three random fields. Scoring for apoptosis was done in a blind manner, and the persons who performed the counting were not aware of the source of the samples. Five mice were used for each point in this study.

Statistical analysis

Levels of significance were calculated using the unpaired Student's *t*-test. $P < 0.05$ was considered significant.

Results and discussion

To establish conditioning heat treatment conditions, it was found that keeping the animals in a room at 45°C or 50°C for 30 min was lethal. On the other hand, the heat treatment of 40-30°C for 30 min did not kill any mice (data not shown). Therefore, temperatures adopted at 37°C as a conditioning heat treatment. Fig. 1***a*** shows the experimental schedules used for a conditioning heat treatment at 37°C for 30 min. Twelve hours after the mild conditioning treatment at 37°C, the mice were subjected to a RWIS treatment for 3 h. The stomach was examined from all of the animals after dissection. The stomachs

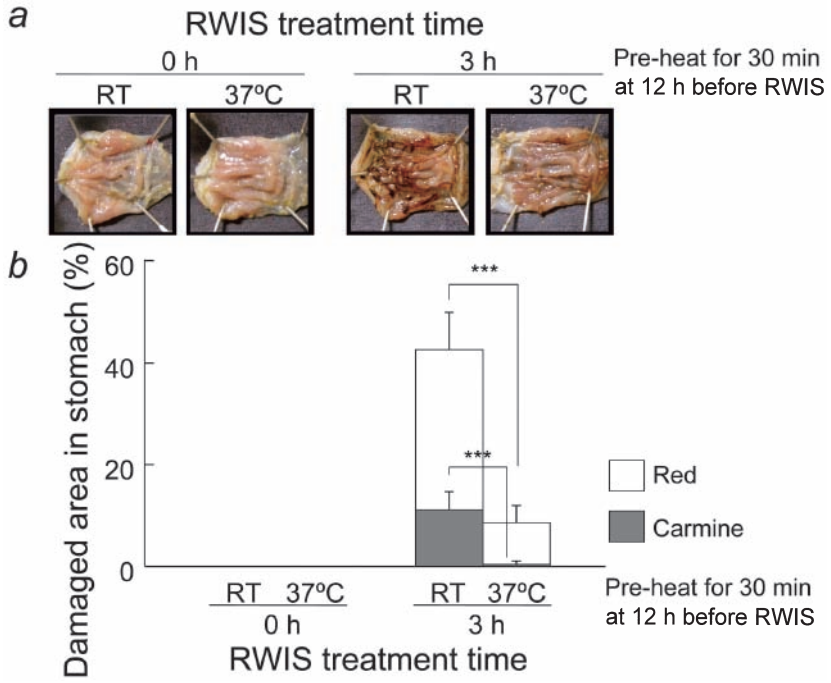

Fig. 2. Effect of pre-heat treatments on stomach ulcers induced by RWIS : ***a***) typical photographs of stomach tissue ; ***b***) statistical analysis. RT indicates mice were kept at room temperature until RWIS treatment. 37°C indicates that the mice were exposed to a conditioning heat treatment prior to the RWIS treatment. 0 h and 3 h indicate the length of the RWIS treatment times. The error bars indicate standard deviations. Asterisks (***) indicate a highly significant difference ($P<0.001$) with the Student's *t*-test.

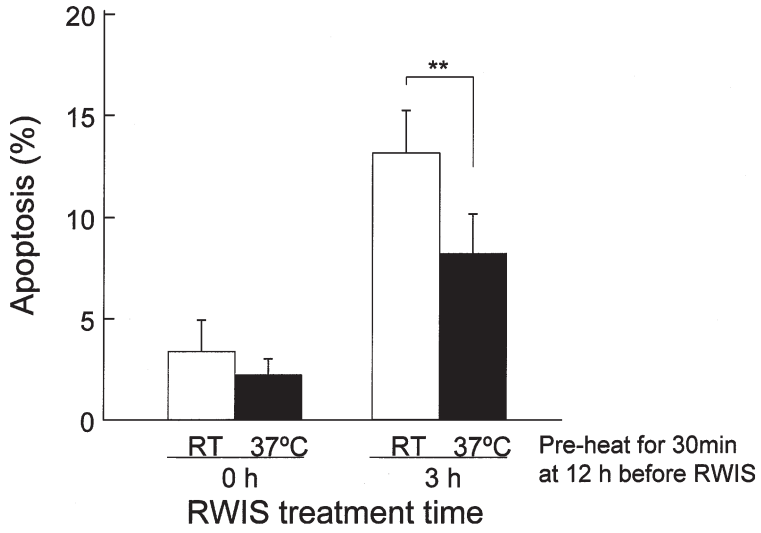

Fig. 3. Effect of a pre-heat treatment on the percent of cells which stain positive in the stomach for RWIS induced apoptosis. RT indicates mice were kept at room temperature until RWIS treatment. 37°C indicates that the mice were exposed to a conditioning heat treatment prior to the RWIS treatment. 0 h and 3 h indicate the length of the RWIS treatments. Asterisks (**) indicate a highly significant difference ($P<0.01$) with the Student's *t*-test.

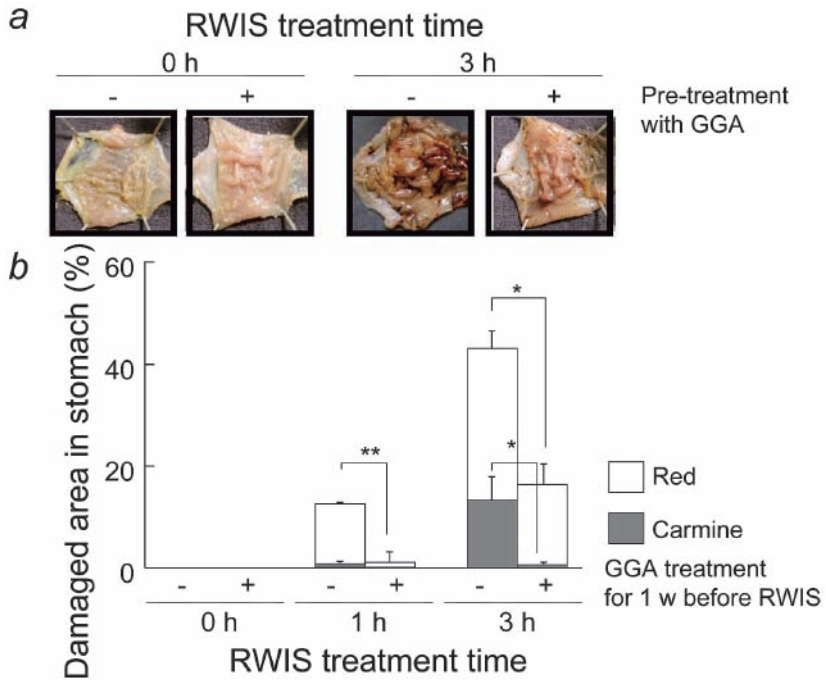

Fig. 4. Effect of GGA treatment before heat exposure on stomach ulcers induced by RWIS. The times 0, 1, and 3 h indicate the length of the RWIS treatment. ***a***) typical photographs ; ***b***) statistical analysis. The symbols＋and－indicate the presence or absence of GGA treatment. Asterisks (* and **) indicate a highly significant difference ($P<0.05$ and 0.01, respectively) with the Student's *t*-test.

were classified into one of three grades : no effects ; moderate effects (red) : and severe effects (carmine).

The color of the stomachs of mice exposed to 37°C at 0 h was almost the same as in control mice which had been subjected to no treatment. The control mice were maintained at RT (Fig. 2***a***). In contrast, after mice had been subjected to RWIS treatment, the colors seen in the stomachs of mice which had been maintained at RT (with no pre-treatment) changed to colors indicating a stomach ulcer (Fig. 2***a***). However, when mice were exposed to a conditioning heat treatment at 37°C for 30 min, almost no ulcers were found (Fig. 2***a***). It is clear that a mild heat pre-treatment depressed stomach ulcer formation induced by RWIS treatment. The stomach ulcers were classified into three grades : red and carmine colors were seen in 42.47±7.34% without a conditioning treatment, and in 8.59±3.40% of the mice with a conditioning pre-heat treatment ($P<0.001$) (Fig. 2***b***).

RWIS-induced apoptosis rates were analyzed with immunohistochemical staining (Fig. 3). Though RWIS treatment induced apoptosis rates of 13.20±2.05% in the stomach, a conditioning mild pre-heat treatment depressed this to 8.20±1.92%. Though it does not understand whether there is clinically significant difference, it thus appears that a mild pre-heat treatment was able to depress the rate of RWIS induced apoptosis in the stomach.

Mice were pre-treated with GGA 1 week before a RWIS treatment, and the effect on stomach ulcers induced by RWIS treatment was investigated (Fig. 1***b***). When mice were exposed to a 1 h RWIS treatment, the damaged stomach area in control mice with no pre-treatment was 12.55±0.25%. In

contrast, the affected stomach area was 1.18±2.04% in mice pre-treated with GGA. When mice were exposed to a 3 h RWIS treatment, the ulcer stomach areas were 43.37±3.08% in mice which were not exposed to GGA, and 16.51±3.93% in mice treated with GGA (Fig. 4). Thus, pre-treatment with GGA apparently depressed the extent of the damaged area induced by RWIS treatment.

Experiments using RWIS to provide a non-genotoxic stress have frequently been utilized to provide a model which leads to gastric mucosa damage induced by acidic gastric secretions in mice exposed to stress. Even when animals were exposed to non-genotoxic stresses, Hirakawa *et al* (1996)[11] reported that these stresses activated HSF1 and induced Hsp70 within 60-90 min in the stressed rats. In addition, Roktan *et al* (2000)[12] reported that the induction of Hsp70 induced by GGA efficiently depressed gastric mucosa damage in rats. It was assumed that the induction of HSPs by a conditioning heat treatment protected cells from cytotoxicity through chaperone-dependent activities as well as through chaperone-independent activities[15]. The work presented here shows that a conditioning heat treatment can depress RWIS treatment induced gastric mucosa damage and apoptosis (Figs. 2 and 3). Although further studies are still required to define the induction of HSPs in stomach, it appears possible that much of this tissue damage is suppressed by HSPs which are induced by a conditioning pre-heat treatment.

Acknowledgements

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