

Strong exercise stress exacerbates dermatitis in atopic model mice, NC/Nga mice, while proper exercise reduces it

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Abstract: Atopic dermatitis is well known to exacerbate by stress. How the influence of exercise stress on the skin symptoms in patients with atopic dermatitis has not been clarified. The purpose of our research is to investigate how different strength of exercise stress acts on atopic dermatitis. Specific pathogen-free (SPF) and conventional NC/Nga male mice were used for the experiments. Conventional mice but not SPF group spontaneously develop dermal symptom similar to that of patients with atopic dermatitis at their age of 7 weeks. They were given two types of stress, mild (20 m/min for 60 min) or strong exercise (25 m/min for 90 min), using a treadmill four times per day. The dermal symptom of the conventional group was strongly exacerbated by strong exercise but ameliorated by mild exercise. Under the standard experimental conditions, plasma concentrations of α -melanocyte-stimulating hormone (α -MSH), transforming growth

factor- β (TGF- β) and substance P in conventional mice increased markedly with concomitant exacerbation of the symptom. The plasma concentrations of these proteins elevated after strong exercise but decreased after mild exercise. Under the conventional conditions, plasma levels of β -endorphin increased with time by some mechanisms enhanced by the mild exercise. These observations suggested that exercise-induced stress significantly affect the symptom of atopic dermatitis in a pivotal manner depending on the plasma levels of TGF- β , α -MSH, substance P and β -endorphin.

Key words: atopic dermatitis – β -endorphin – α -melanocyte-stimulating hormone – stress – substance P – transforming growth factor- β

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Introduction

Atopic dermatitis is a chronic inflammation associated with dry skin, erythema, eczema and pruritus (1,2). Psychological stress has been known to affect the symptoms of allergic diseases including atopic dermatitis (3–7). However, role of stress response on the symptom of atopic dermatitis and the underlying mechanism remain to be elucidated. Activation of hypothalamic–pituitary–adrenal axis (HPA axis) and/or sympathetic nervous system has been postulated to play important roles in the stress-induced modulation of allergic diseases (3,8). Activation of these systems has been known to induce changes in cytokine balance in the circulation and at the site of inflammation (3,9–15). These observations suggest that an appropriate response to various stresses through HPA axis and/or autonomic nervous system is necessary for the regulation of immunological systems required for the survival of animals (16,17). Recent

study suggested that HPA axis also play an important role in the modulation of chronic inflammation (18,19). Although stress has been postulated to be a negative factor for organisms, its effect varies significantly depending on its type, strength and duration of exposure. In fact, mild stress has been shown to reduce the symptoms in animals with pollen antigen-induced pollinosis (20). To elucidate roles of mild and strong stresses elicited by exercise in the modulation of immunological systems and inflammatory reactions, we studied their effects on the cytokine profiles and symptoms of mice with atopic dermatitis (21,22).

NC/Nga mice, an inbred strain of Japanese origin, have the healthy skin under a specific pathogen-free (SPF) environment. When they are kept under conventional conditions for a period of time, they develop chronic dermatitis and IgE hyperproduction (23,24), which may be because of mite infection and allergies (25). Spontaneous scratching, an itch-associated response, gradually increases with the

worsening of dermatitis in NC/Nga mice kept under conventional conditions, but not under SPF conditions (24). These symptoms are similar to those observed in human AD and NC/Nga mice have therefore been used as AD model mice.

The present work describes the effects of mild and strong stresses elicited by treadmill on the immunological reactions and symptoms of atopic dermatitis in the NC/Nga mouse.

Materials and methods

Animals

Conventional and SPF NC/Nga male mice (7 weeks old) were purchased from SLC (Hamamatsu, Japan). They were housed in rooms with 12-h light/12-h dark cycle, and sterile water was provided *ad libitum*. These animals were subjected to experiments according to the animal care regulations of Osaka City University Medical School. Conventional but not SPF mice spontaneously started to exhibit the symptoms characteristic to atopic dermatitis at their age of 7 weeks old. In this study, we divided a mouse into six groups ($n = 10$) and used at same time. In addition, each experiment was repeated three times.

Voluntary and exercise protocols

To condition animals to a device for exercise, they were placed in a resting treadmill for 10 min followed by running at 5 m/min for 10 min and then at 10 m/min for 10 min. On day 2, animals were placed in an up-hill inclined device (at 10°) at a speed of 20 m/min for 60 min or 25 m/min for 90 min to give mild or severe stress, respectively. The latter conditions elicited strong fatigue (unable to move because of exhaustion) while the former conditions gave mild stress (no appreciable effect on their behaviour) as judged from their performance analysis for 60 min. These exercises were carried out every other day (totally four times of exercise).

To examine the running time, as a result of having examined time of running with 10 m/min, 20 m/min or 25 m/min for 60 min, or 25 m/min for 90 min, greatest fatigue (stress) effect of running time was shown at 25 min for 90 min. On the other hand, most few fatigue (stress) effect of running time was shown at 20 m/min for 60 min. We assumed a spontaneous momentum after the treadmill exercise an index of the fatigue, the run time with a few spontaneous momentums is severe and the most long run time when spontaneous momentum before a run does not change after a run is moderate.

Evaluation of inflammatory score

On day 9, the symptom of dermatitis in these animals was evaluated at their rostral skin, and their severity of oedema,

erythema and haemorrhage was scored (0, none; 1, slight; 2, moderate; 3, severe) as described previously (26).

Analysis of peptide hormones and cytokines in plasma

Under light ether anaesthesia, blood samples were obtained by cardiac puncture 24 h after giving final exercise. After centrifugation, plasma samples were obtained and analysed for proteins and peptide hormones. Plasma levels of IgE, TNF- α , TGF- β , Adrenocorticotrophic hormone (ACTH) and α -melanocyte-stimulating hormone (α -MSH) were determined by using ELISA kits according to manufacture's instructions. ELISA kits for IgE, TNF- α , TGF- β , ACTH and α -MSH were obtained from Yamasa Shoyu Co. (Chiba, Japan), Pierce Biotechnology, Inc. (Rockford, IL, USA), Promega (Madison, WI, USA), and Phoenix Pharmaceuticals (Burlingame, CA, USA), respectively.

Western blot analysis

The skin samples were homogenized in a lysis buffer containing 0.5% Nonidet P-40, 10% glycerol, 137 mM NaCl, 2 mM ethylenediamine tetraacetic acid and 50 mM Tris-HCl buffer (pH 8.0). After centrifugation at 3000 g for 10 min, the supernatant fractions were separated and stored at -80°C until use. The stored skin specimens and plasma samples were subjected to 12% polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS. The electrophoresed proteins in the gel were transferred to an Immobilon membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk at 4°C for overnight and subsequently incubated with primary antibodies against β -endorphin (Progen Biotechnic GmbH, Frankfurt, Germany) and substance P (Pharmingen, San Jose, CA, USA) at 25°C for 1 h and then with horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Glostrup CA, USA). Immune complexes thus formed were detected with Enhanced chemiluminescence (ECL) reagents (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

Statistical analysis

All data were expressed as the mean $\pm$ SD derived from six animals. The results obtained from the two animal groups were analysed by either Student's *t*-test or ANOVA using a computer software program. Differences were considered significant when $P < 0.05$.

Results

Effect of exercise on dermatitis

Conventional mice started to exhibit the symptom characteristic of atopic dermatitis including oedema, erythema and haemorrhage at their rostral skin (Fig. 1a–c). The dermal symptom was ameliorated in the animal group that

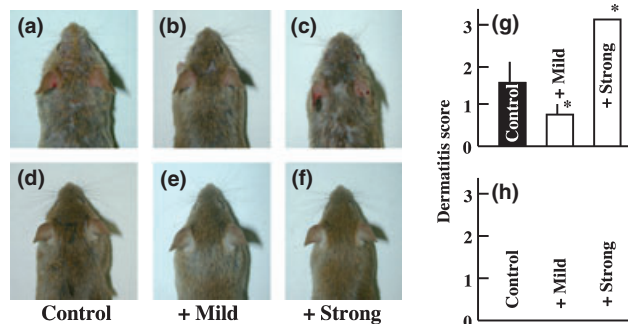


Figure 1. AD-like skin lesion of conventional NC/Nga mice (a, b, c). As for each conventional mouse, it was burdened strong exercise (c) and mild exercise (b). In the strong exercise load conventional mice (c), the skin symptom turned worse in comparison with the no burden exercise conventional mice, but was reduced with the mild exercise load conventional mice (b). (d, e, f) specific pathogen-free (SPF) NC/Nga mice did not develop any eczematous lesions. The dermatitis score (conventional NC/Nga mice: G; SPF NC/Nga mice: H) for the rostral back was assessed after final treadmill running (0, none; 1, slight; 2, moderate; 3, severe). This scoring was based on the severity of erythema/haemorrhage and pilosis.

received the mild exercise (Fig. 1g). In contrast, the symptom was deteriorated in animal group that received the strong exercise. The symptom of dermatitis was not apparent with the SPF group even if they were treated with strong exercise (Fig. 1d–f and h).

Effect of exercise on plasma levels of cytokines and IgE

As shown in Fig. 2, plasma levels of TGF- β and TNF- α in the SPF animal group significantly increased by exercise stress. Under identical conditions, plasma levels of IgE remained low levels in SPF group. The plasma levels of TGF- β , TNF- α and IgE were significantly high when compared with those in SPF group particularly when the symptom of dermatitis became apparent. Plasma levels of TGF- β in the conventional group were significantly suppressed by receiving mild exercise but increased markedly by strong exercise. Plasma levels of TNF- α in the conventional group were decreased markedly by exercise stress irrespective of its strength. Although plasma levels of IgE were markedly elevated in the conventional group, no appreciable effect of mild and strong exercise was observed.

Effect of exercise on ACTH and related peptides

Because α -MSH is derived from the N-terminal domain of ACTH, exercise stress increased their plasma levels in the SPF group irrespective of the difference in its strength (Fig. 3). Plasma levels of ACTH were significantly higher in the conventional group than in the SPF group. However, their levels remained unaffected by exercise. In contrast, the elevated levels of α -MSH were suppressed by the mild exercise and elevated further by strong exercise. It should

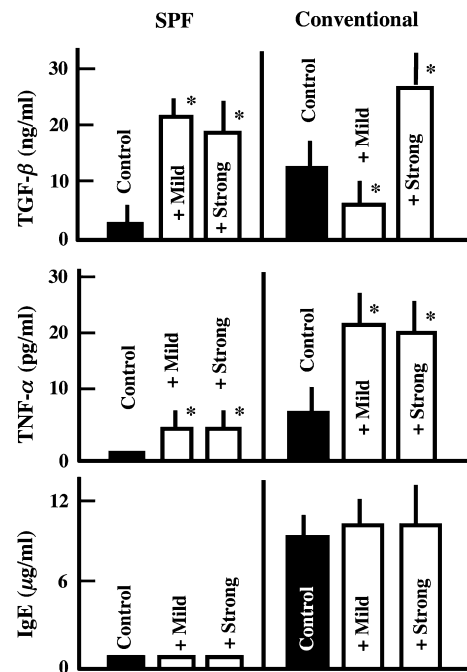


Figure 2. Plasma concentration of IgE, TNF- α and TGF- β in specific pathogen-free (SPF) and conventional NC/Nga mice. Plasma IgE level was not seen in the change in having exercise or not. In plasma TNF- α level, the exercise load mice significantly increased in comparison with the no exercise conventional mice, but did not change by the strength and weakness of the exercise. In plasma TGF- β , the strong exercise load mice increased in comparison with the no exercise mice, and the mild exercise load mice decreased in comparison with the no exercise mice. Values are mean $\pm$ SD derived from 10 animals. * $P \leq 0.05$ compared with no exercise SPF or conventional NC/Nga mice.

be noted that proopiomelanocortin (POMC) that involves ACTH/ α -MSH and β -endorphin within its molecule is also synthesized in dermal keratinocytes particularly when the skin is exposed to a variety of stress including ultraviolet B and inflammation (27,28). Thus, we also measured the dermal expression of β -endorphin at the site of inflammatory (Fig. 4). When the symptom of atopic dermatitis became apparent with the conventional group, the plasma levels of β -endorphin increased markedly. Although the plasma levels of β -endorphin were not affected by exercise in SPF group, its levels were further increased by the mild exercise but suppressed by the strong exercise in conventional group. Because substance P plays important roles in the modulation of the symptom of dermatitis, we also analysed its expression at the rostral skin. The dermal expression of substance P was significantly higher with conventional mice than with SPF group. The strong exercise enhanced the dermal expression of substance P both in SPF and in conventional groups. Although mild exercise did not appreciably affect the dermal expression of substance P in SPF group, it significantly suppressed the expression in the conventional group.

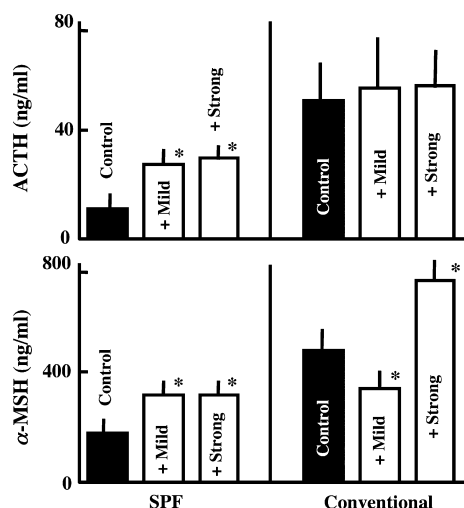


Figure 3. Plasma concentration of ACTH and α -MSH in specific pathogen-free (SPF) and conventional NC/Nga mice. Plasma ACTH level was not seen in the change in having exercise or not. In plasma α -MSH level, the strong exercise mice increased in comparison with the no exercise mice, and the mild exercise mice decreased in comparison with the no exercise mice. Values are mean $\pm$ SD derived from 10 animals. * $P \leq 0.05$ compared with no exercise SPF or conventional NC/Nga mice. α -MSH, α -melanocyte-stimulating hormone.

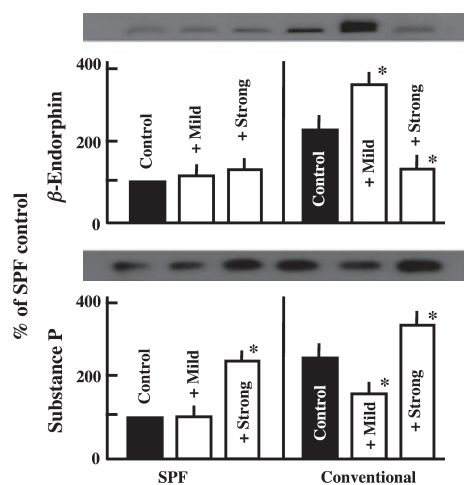


Figure 4. Expression of β -endorphin and substance P were determined by western blot analysis. In the plasma levels of β -endorphin, the mild load mice increased in comparison with the no exercise mice, and strong exercise mice decreased in comparison with the no exercise mice. Conversely, the substance P expression in the inflammation skin increased with the strong exercise mice in comparison with the no exercise mice and decreased with the mild exercise mice. Values are mean $\pm$ SD derived from 10 animals. * $P \leq 0.05$ compared with no exercise specific pathogen-free or conventional NC/Nga mice.

Discussion

The present work demonstrates that atopic dermatitis of the conventional mice was exacerbated by strong exercise but ameliorated by mild exercise. Exercise has long been

considered associated with oxidative stress based on experimental data showing an increase in oxidative stress markers after high-intensity exercise and on the unsupported assumption that the production of oxygen free radicals is linearly related to the rate of mitochondrial oxygen uptake (29,30). However, the effects of moderate exercise a series of beneficial effects in ageing mice, observed at the upregulation of antioxidant enzyme activity, decrease in oxidative stress markers (31).

IgE has been well documented to play key roles in the onset of type I allergic diseases including atopic dermatitis (32). In fact, when the symptom of atopic dermatitis appeared in the conventional group, plasma levels of IgE increased markedly. Because neither strong nor mild exercise affected the plasma levels of IgE both in SPF and in conventional groups, exercise stress *per se* does not seem to affect the synthesis of this immunoglobulin. The immunological reactions and their symptoms have been known to be affected by a variety of stress and cytokines (33). It is known by the atopic dermatitis that TGF- β level increases with exacerbation of the inflammation (34). In this study, plasma levels of TGF- β were significantly higher in the conventional group than in SPF group; their levels in the former were increased further by strong exercise but decreased by mild stress, suggesting that this cytokine might play important roles in the exacerbation of atopic dermatitis. In fact, TGF- β has been known to interact with TGF- β RI and RII receptors thereby reinforcing the Th-2 type immunity to enhance Th2-dependent inflammatory reactions (35). Because both mild and strong exercise also increased the plasma levels of TGF- β in SPF group, immunological abnormality elicited in the conventional group seems to be prerequisite to the enhancement of the symptom by this cytokine. TGF- β -RI and RII receptors are expressed in a variety of cells that produce POMC, such as neurons in the hypothalamus (36). Recent studies revealed that TGF- β -RI mediates TGF- β -induced signalling from cytoplasm to nucleus through Smad family proteins (37). When phosphorylated by TGF- β -RI, Smad 2 and Smad 3 form heterodimeric complexes with their partner proteins, and the resulting complexes translocate into nucleus to modulate gene expression. In fact, the occurrence of TGF- β 1 signalling in POMC neurons has been suggested from the observation that TGF- β 1 stimulated the gene expression of POMC. POMC and related peptides have been well documented to play key roles in stress response and immunological modulation (38,39). It has been well documented that HPA axis and POMC-related peptide hormones, such as ACTH, MSH and β -endorphin, play important roles in the stress response and immunological regulation (40). Although the plasma levels of ACTH were elevated markedly in the conventional group, they were unaffected either by strong or by mild exercise despite the marked effect of

these stresses on the symptom of dermatitis. Thus, the stress elicited by the symptom of dermatitis, such as pruritus, might be strong enough to increase ACTH levels maximally in plasma. In addition, in this study, the plasma ACTH levels of stress addition mice in the conventional group were not significant different in comparison with the control group. As this reason, it was thought that this phenomenon is induced by lowering the ACTH level, but the details are unclear. Plasma levels of α -MSH were also higher in the conventional group than in SPF group. However, its plasma levels were increased further by strong exercise but decreased by mild exercise. This observation suggests that α -MSH-induced signalling might participate in the exacerbation of the symptom of atopic dermatitis. In fact, α -MSH has been shown to interact with melanocortin receptor (MCR) receptors on a variety of leucocytes thereby modulating inflammatory reactions induced by endotoxin and related cytokines including IL-1, TNF- α and IFN- β (41–44). α -MSH has been shown also to enhance the production of TGF- β 1 (45). Thus, the TGF- β /TGF- β -R signalling pathway might seem to activate POMC system to increase plasma levels of α -MSH thereby exacerbating the symptom of atopic dermatitis. Although both ACTH and α -MSH were derived from POMC by some stress-induced mechanism, they showed different responses to the stress elicited by mild and strong exercise. The processing of POMC to form ACTH and/or α -MSH might seem to be regulated differently depending on the properties and/or strength of stress. It should be noted that POMC also involves β -endorphin in its C-terminal domain. Recent study demonstrated that the TGF- β signalling pathway also operated in β -endorphin-expressing POMC neurons (46). β -Endorphin secreted in the circulation has been shown to play important roles in the modulation of immunological reactions to augment natural immunity (47). The present work also demonstrates that, although plasma levels of β -endorphin were significantly higher in the conventional group than in SPF group, mild exercise increased its plasma level with concomitant amelioration of dermatitis while strong exercise decreased the plasma level and exacerbated the symptom. Thus, the different strength of the exercise modulated plasma levels of α -MSH and β -endorphin in a pivotal manner and affected the symptom of dermatitis. The stress-dependent mechanism for the processing of POMC-related peptides and its relationship with the symptom of atopic dermatitis should be studied further. The present work also shows that the expression of substance P in the rostral skin was increased by strong exercise but decreased by mild exercise. This neuronal peptide has been postulated to stimulate mast cells thereby enhancing pruritus in inflammatory diseases including allergic dermatitis (5,48–52). The present observation that strong exercise but decreased by mild exercise is consistent with the

hypothesis that substance P is a key player in stress-enhanced inflammation and pruritus in atopic dermatitis (50,51). Substance P also stimulates infiltration of eosinophils (53) and cytokine production by T-lymphocytes (54) thereby exacerbating the symptom of atopic dermatitis. The aetiology of atopic dermatitis has been postulated to involve imbalance between Th1 and Th-2 immune systems in which the former predominates in its chronic phase while the latter predominates in its acute phase. Furthermore, the potential involvement of Th17-type immune system has been suggested in patients with atopic dermatitis (55). Thus, the balance among Th1, Th2 and Th17 immune systems seems to play important roles in the pathogenesis and modulation of atopic dermatitis (56). The mechanisms by which different strength of exercise affect on the balance of these immune systems require further study.

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