to exhibit a protective role in gut epithelial cells (Liu et al., 2003). However, the mechanism by which such protective HSP70 is induced in the colon has not been determined.

We assumed roles of activation of the HPA axis and sympathetic nervous system in colon HSP70 expression as well as the contribution of commensal bacteria in stress-induced HSP70 expression, as basal HSP70 expression in gut epithelial cells has been reported to be dependent on commensal bacteria through interactions with TLR4 (Rakoff-Nahoum et al., 2004).

Interestingly, TLR4, which is responsive for bacterial lipopolysaccharides in the intestinal tissue, was shown to be located mainly at the lamina propria and absent at the epithelial surface (Rumio et al., 2006). TLR4 in the colonic tissue would also exhibit a similar distribution. For luminal bacterial components to interact with TLR4, we postulated that tight junction integrity of the gut epithelia may be altered under stress facilitating access of luminal bacterial components to the lamina propria TLR4 and finally inducing HSP70.

In the present study, therefore, we first examined colonic HSP70 under acute stress, and then examined the involvements of luminal bacteria and stress-induced corticosterone in HSP70 induction in colonic epithelia. In addition, we examined the expression of the tight junction component ZO-1 in the colonic tissue before and after stress.

2. Methods

2.1. Animals

Male C57BL/6 mice and green fluorescence protein (GFP) transgenic mice (C57BL/6 background) were purchased from the mouse supply centre of Tohoku University School of Medicine, and used for the experiments at 10–12 weeks of age. Five or six mice were housed together per cage (30 × 25 × 17.5 cm) and were given free access to food and water. Animals were maintained under specific pathogen-free conditions on a daily 12-h light/dark cycle. Drinking bottles, cages, sawdust, and rodent chow for mice were all autoclaved before use as described previously (Kanemi et al., 2005). Mice were housed in freshly sterilized cages with fresh sawdust every day. All experiments were performed in accordance with the Guidelines and Regulations for Laboratory Animal Care of Tohoku University Medical School.

2.2. Restraint stress procedure

The stressor used in this study was restraint (Fukui et al., 1997; Kanemi et al., 2005; Sudo et al., 1997). Each experimental male C57BL/6 or GFP mouse was placed for 2 h in 50-ml centrifuge tubes. The walls of the tubes were stripped in part to avoid an acute rise in the body temperature. The restraint experiment was carried out in the morning between 8:00 and 10:00. Non-restraint mice of the same age were kept in their home cage throughout the restraint procedure. Animals were given no access to food or water during restraint stress treatment. Immediately after restraint stress, restrained and non-restrained mice were sacrificed by anesthesia.

2.3. In vivo RU486 and propranolol treatment

The glucocorticoid receptor antagonist RU486 was administrated orally (30 mg/kg) in aqueous solution containing 0.25% carboxymethylcellulose and 0.2% polysorbate 80 (Sigma, St. Louis, MO, USA) in a volume of 5 ml/kg through a gastric feeding tube (Concordet and Ferry, 1993; Zhang et al., 2005a,b).

RU486 or an equivalent volume of vehicle (0.25% carboxymethylcellulose and 0.2% polysorbate 80) was given 30 min before the 2-h restraint session.

2.4. Depletion of colonic commensal bacteria

Depletion of colonic commensal bacteria was performed according to the method reported by Rakoff-Nahoum et al. (2004) with minor modifications by daily administration of 0.2 ml of the following antibiotic cocktail *via* a ball-tipped gastric feeding tube for 7 days and by supplementing the drinking water with a 2-fold dilution of antibiotic cocktail during the same 7-day period. The antibody cocktail contained ampicillin (1 mg/ml), vancomycin (0.5 mg/ml), neomycin sulfate (1 mg/ml), and metronidazole (1 mg/ml) (all from Sigma).

2.5. Lipopolysaccharide (LPS) administration to antibiotic-treated mice

Following antibiotic treatment, mice were given a daily supplement of 0.2 ml distilled water containing 50 μg/ml of purified *Escherichia coli* 026:B6 LPS (Sigma) through a gastric feeding tube for 5 days. Drinking water during this period was supplemented with LPS (25 μg/ml). A lower dose of LPS did not induce stable augmentation of colonic HSP70. Mice in the control group received the same volume of distilled water according to the same protocol.

2.6. Anesthesia

Sevoflurane (Abbott Japan, Tokyo, Japan) inhalation was used to anesthetize the animals. A volume of less than 2 ml of anesthetic was evaporated into a container of approximately 500 ml at room temperature. The mice were then placed into the container and anesthetized within 15 s (Kanemi et al., 2005).

2.7. Blood sampling, catecholamines, and corticosterone assay

Blood samples were collected from the axillary artery of anesthetized mice and transferred immediately into either heparinized or untreated stock tubes for plasma and serum sampling, respectively. After blood sampling, animals were euthanized by deep anesthesia with sevoflurane.

Blood samples were centrifuged at 3000 rpm for 10 min at 4 °C to obtain plasma or serum. Plasma and serum samples were stored at −80 °C until assay. Catecholamines were quantified by column-switching high-performance liquid chromatography (HPLC) with fluorometric detection (Dutton et al., 1999; Nohta et al., 1987). Serum corticosterone level was measured using a rat corticosterone-H^3 RIA kit (ICN Biomedicals, Costa Mesa, CA, USA), in accordance with the manufacturer's instructions.

2.8. Rectal temperature

The rectal temperature of the mice was measured with a digital thermometer (CTM-303 model; Terumo, Kanagawa, Japan) at a distance of 2.5 mm from the anus. Measurements were obtained at ambient room temperature (20–23 °C). The rectal temperatures of control and stressed mice were measured at 30-min intervals, for a total of 120 min ($n = 5$).

2.9. Bacterial culture

For determination of colonic microflora, fecal matter was removed from the colon using sterile technique. The contents were diluted and plated on universal and differential media for the growth of anaerobes and aerobes under anaerobic or aerobic culture conditions. AnaeroPack (Mitsubishi Gas Chemical Company, Niigata, Japan) was used to detect anaerobic flora. The numbers of colonies per mg of feces (wet weight) were counted after incubation at 37 °C for 48 h (aerobes) and 72 h (anaerobes).

2.10. Colonic tissue preparation

The mid-portion, approximately 1 cm, of total colon—transverse colon—was excised immediately after the restraint session and stored at −80 °C until examination. After determining the frozen weight of colon samples, colonic tissue was lysed with lysis buffer consisting of 40 mM Tris (pH 7.5), 300 mM KCl, 1% Triton X-100, 2 mM EDTA, with protease inhibitor cocktail (1:20) (Sigma). Colonic tissue was dissected using scissors and further homogenized using a Polytron Aggregate homogenizer (Kinematica, Luzern, Switzerland). The extracts were cleared by centrifugation at 21,000g for 10 min. Aliquots of prepared samples were kept frozen at −80 °C until ELISA or Western blotting analysis.

2.11. ELISA for HSP70

HSP70 concentration in the extract was quantified by enzyme-linked immunosorbent assay (ELISA) using a commercially available HSP70 kit (StressXpress HSP70 ELISA; Stressgen Biotechnologies, Victoria, BC, Canada) in accordance with the manufacturer's instructions.

2.12. Western blotting analysis

Equal amounts of protein from the supernatant of colonic tissue samples quantified by the Lowry method were electrophoresed on 12% SDS–polyacrylamide gels and transferred electrophoretically onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk in phosphate buffered saline (PBS) containing 0.1% Tween 20 for 1 h at room temperature, membranes were incubated with rabbit polyclonal anti-HSP70 antibody (1:1000) (Stressgen), rabbit polyclonal anti-TLR4 (H-80) antibody (1:500) (Santa Cruz Biotechnology, CA, USA), rat polyclonal anti-ZO-1 antibody (1:250) (Santa Cruz), and mouse monoclonal anti-β-actin antibody (1:2000) (Sigma) in TBS (pH 8.0) containing 0.05% Tween 20 (TBST) at 4 °C overnight and washed three times for 5 min with PBS. The membranes were then incubated with either HRP-conjugated secondary anti-rat IgG (1:1000) or anti-mouse IgG (1:1000) (Cell Signaling Technology, MA, USA) antibody in TBST for 1 h at room temperature, washed three times for 5 min with PBS, and visualized by chemiluminescence using an ECL immunoblotting kit (Cell Signaling) with a digital luminescent image analyzer (LAS-1000; Fujifilm, Tokyo, Japan).

2.13. Immunohistochemistry

The colon samples from male C57BL/6 mice were rapidly embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan), frozen in isopentane, pre-cooled in liquid nitrogen, and finally transferred into liquid nitrogen. The tissue blocks were stored at −80 °C prior to cutting. Colonic tissues on slides were fixed in PBS containing 4% paraformaldehyde at room temperature for 20 min, blocked, and penetrated in blocking solution (Dako, Kyoto, Japan) containing 0.3% Triton X-100 for 30 min at room temperature, and incubated overnight with rabbit polyclonal anti-HSP70 antibody (1:500, Stressgen), goat polyclonal anti-TLR4 (L-14) antibody (1:200) (Santa Cruz), and rat polyclonal anti-ZO-1 (R40.76) antibody (1:50) (Santa Cruz) in TBS (pH 8.0) containing 0.01% Triton X-100 at 4 °C overnight. Washes were performed with PBS. The sections were incubated with the secondary antibodies, Alexa Fluor 555-conjugated donkey anti-rabbit IgG (1:400) (Invitrogen, Carlsbad, CA), Alexa Fluor 488-conjugated donkey anti-goat IgG (1:400) (Invitrogen), and Alexa Fluor 488-conjugated goat anti-rat IgG (1:400) (Invitrogen) with nuclear stain marker Topro-3 in TBS (pH 8.0) containing 0.01% Triton X-100 for 1 h at room temperature. The reaction was examined by confocal microscopy (Nikon, Tokyo, Japan).

2.14. Ligated colon loop assays and processing of colonic tissues

Mouse ligated colon loop assays were performed as described previously by Mantis et al. with some modifications (Mantis et al., 2002). During the procedure, mice were maintained under sevoflurane anesthesia. Alexa-LPS 548 was injected into ligated colon loops (1 cm) at a concentration of 1/10 μl. After 10 min, mice were sacrificed by cervical dislocation and the colon was excised immediately. Portions of tested loops were taken and fixed in Tissue-Tek OCT compound (Sakura Finetechnical), frozen in isopentane, pre-cooled in liquid nitrogen, and finally transferred into liquid nitrogen. Colon sections of the specimens were prepared using standard procedures for immunohistochemical analyses.

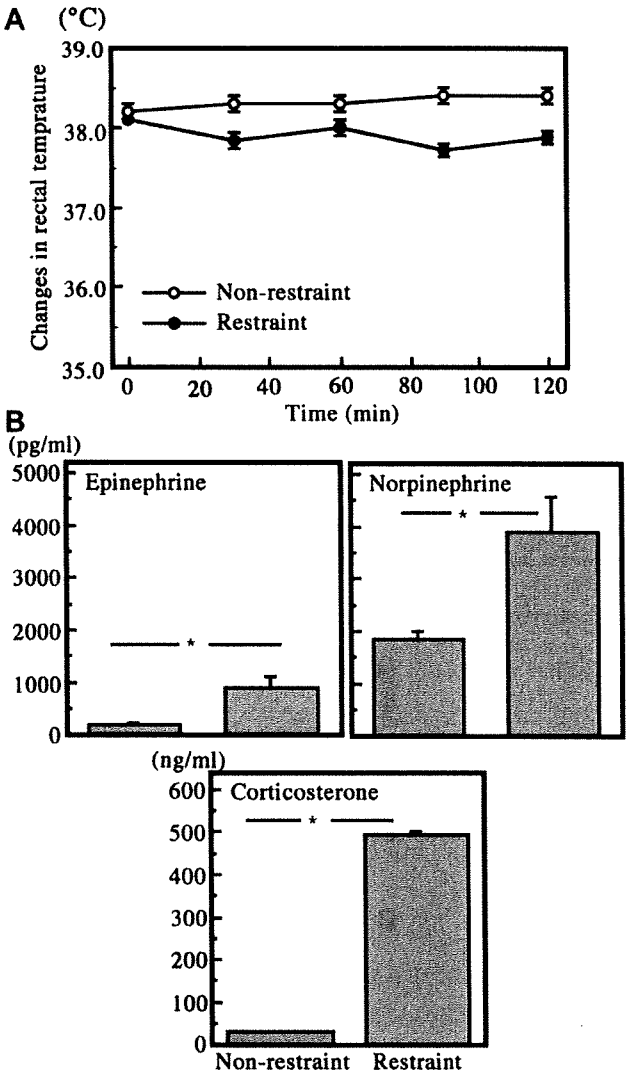


Fig. 1. Restraint did not raise the rectal temperature, but the levels of plasma catecholamine and serum corticosterone were markedly increased. (A) The mean rectal temperature of mice decreased during restraint stress. The results are shown as means ± SE of 5 mice ($^*P < 0.05$). (B) Plasma levels of epinephrine and norepinephrine was significantly elevated immediately after restraint stress. Results are shown as means ± SE of 6 mice ($^*P < 0.001$). Serum level of corticosterone was significantly elevated immediately after restraint stress. The results are shown as means ± SE of 5 mice ($^*P < 0.001$).

2.15. Statistical analysis

Results are presented as the means ± SE. We examined the statistical significance of differences using the two-way analysis of variance (ANOVA). *Post hoc* analysis was carried out using Scheffe's test. Statistical significance was defined as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Restraint stress did not raise the rectal temperature of mice

First, we examined whether there was a rise in rectal temperature in response to restraint stress, because a rise in the rectal temperature may involve HSP70 induction in the colonic tissue (Fig. 1A). The mean rectal temperature of mice decreased gradually during restraint stress, while there was virtually no change in the rectal temperature of non-restrained mice ($n = 5$, $P < 0.05$).

3.2. Restraint stress-induced elevation of plasma catecholamine and corticosterone levels

The plasma levels of epinephrine and norepinephrine were significantly elevated immediately after restraint stress ($P < 0.05$, Student's *t*-test) (Fig. 1B).

The serum level of corticosterone was significantly elevated immediately after restraint stress ($P < 0.001$, Student's *t*-test) (Fig. 1B).

The plasma levels of epinephrine and norepinephrine of antibiotics treated mice were significantly elevated immediately after restraint stress (non-restraint: epinephrine 260.0 ± 50.7 pg/ml, norepinephrine 1806.0 ± 593.9 pg/ml *vs.* restraint: epinephrine 1157.5 ± 193.2 pg/ml, norepinephrine 4438.8 ± 184.0 pg/ml, $n = 4$; $P < 0.05$, Student's *t*-test).

The serum level of corticosterone of antibiotics treated mice was significantly elevated immediately after restraint stress (non-restraint: 36.8 ± 12.7 ng/ml, restraint: 501.1 ± 28.5 ng/ml, $n = 4$; $P < 0.001$, Student's *t*-test).

Thus, antibiotics treatment did not affect the neurohumoral stress response to restraint.

3.3. Restraint stress enhances colonic HSP70

The effects of 2-h restraint on colonic HSP70 were examined both by ELISA (Fig. 2A) and by immunohistochemical analysis (Fig. 2B). HSP70 level in the colonic extract was significantly elevated immediately after restraint as compared to the non-restraint group ($P < 0.001$) (Fig. 2A). Immunohistochemical analyses indicated a detectable basal level of HSP70 expression, but that it was dominantly expressed at the apical or luminal cytoplasm of colonic epithelia. HSP70 expression was weaker in the cells in deeper crypts as compared to those closer to the tips (Fig. 2B). This expression pattern was unchanged after restraint stress, but the degree of HSP70 expression was enhanced after restraint.

3.4. Glucocorticoid receptor antagonist suppressed restraint-induced colonic HSP70 augmentation

Blockade of glucocorticoid by oral administration of RU486 before restraint completely abolished the augmentation of colonic HSP70 and even resulted in down-regulation to below the level of expression in non-restrained mice (basal level) (Fig. 2C). There were no significant differences in HSP70 level between non-restrained and restrained mice treated with RU486 (NS, Fig. 2C). Immunohistochemical analysis revealed no apparent morphological differences between RU486-treated and untreated colon tissue notwithstanding restraint (Fig. 2D). Colonic HSP70 augmentation after restraint was absent in RU486-treated mice.

3.5. Depletion of commensal bacteria abrogates restraint-induced colonic HSP70 augmentation

As commensal bacteria contribute to HSP expression in the gut tissue (Rakoff-Nahoum et al., 2004), the involvement of commensal bacteria in stress-induced HSP70 augmentation was examined.

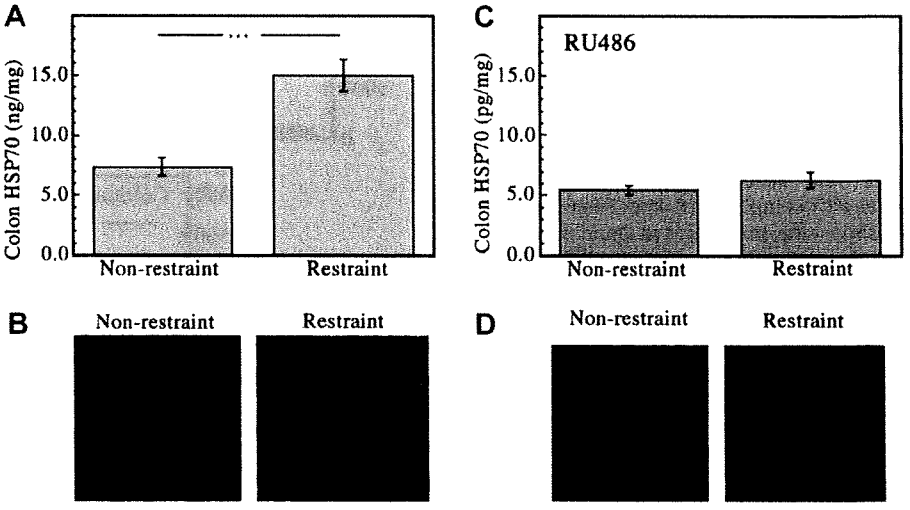
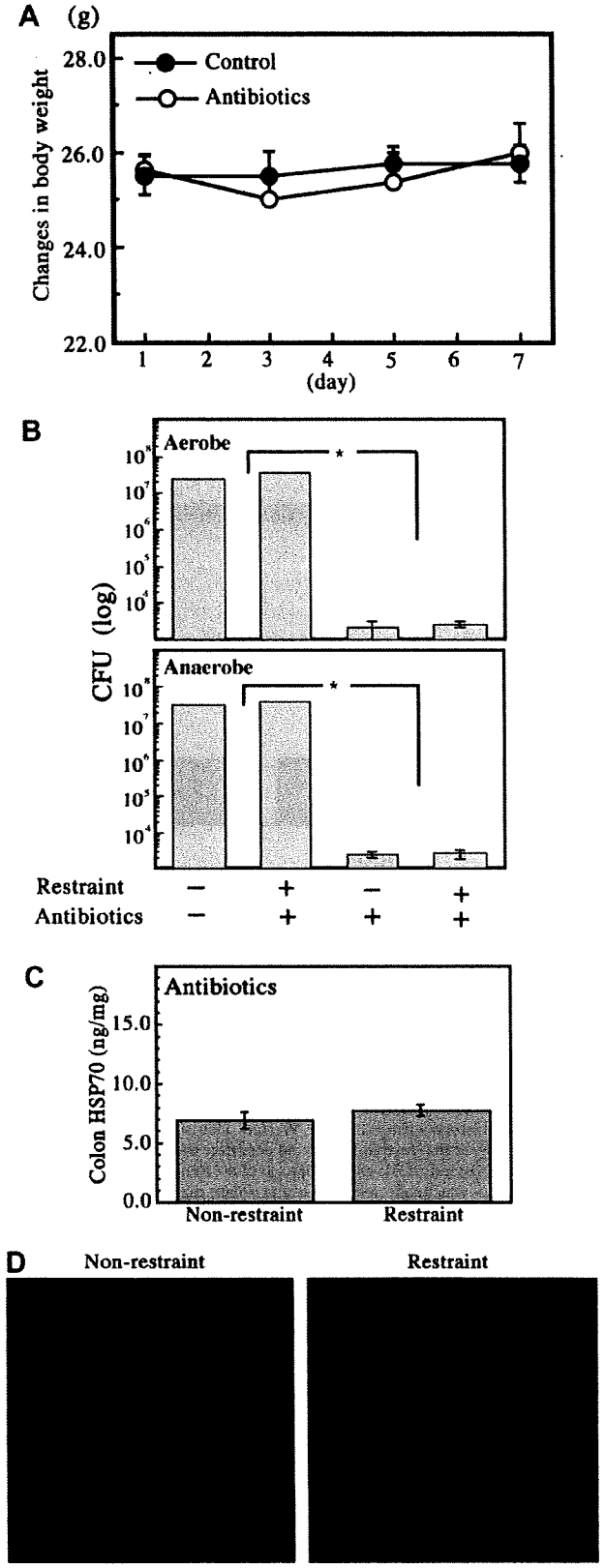


Fig. 2. Colonic HSP70 expression was markedly augmented after 2-h restraint. The augmentation of HSP70 expression was abrogated by RU486 administration. (A) The level of colonic HSP70 was significantly elevated immediately after restraint as compared with non-restrained controls. The results are shown as means ± SE of 8 mice ($^{***}P < 0.001$). (B) Immunohistochemical analysis indicated concentrated expression of colonic HSP70. HSP70: red; Topro-3: blue (200×). (C) No significant difference was found in the HSP70 level between non-restrained and restrained mice treated with RU486. The results are shown as means ± SE of 8 mice. (D) Immunohistochemical analysis revealed no apparent morphological differences in the levels of colonic HSP70 between non-restrained and restrained mice treated with RU486. No HSP70 augmentation after restraint was observed in RU486-treated mice. HSP70: red; Topro-3: blue (200×). There was a significant group × time interaction in repeated-measures ANOVA analysis ($p = 0.0005$, $F = 15.4$).

There were no differences between the changes in body weight of water- and antibiotic-treated mice (water *vs.* antibiotics NS) (Fig. 3A).



Both anaerobic and aerobic bacteria in the feces sufficiently dropped to less than 1/10,000 of the levels in untreated mice by antibiotic treatment ($n = 8$, $P < 0.001$, Fig. 3B). Restraint did not affect the number of colonies of either aerobic or anaerobic commensal bacteria.

Elimination of commensals abolished restraint-induced HSP70 augmentation (Fig. 3C, 3D). The level of colonic HSP70 of antibiotic-treated restrained mice was 7.75 ± 0.47 ng/ml ($n = 8$), while that in the non-restraint group was 6.93 ± 0.71 ng/ml ($n = 8$) (Fig. 3C). There was no significant difference in the level of HSP70 between the non-restrained and restrained mice. There were no apparent morphological changes, such as damage to the colonic epithelia after antibiotic treatment. Immunohistochemical analyses indicated no apparent enhancement of colonic HSP70 of restrained mice (Fig. 3D).

3.6. LPS administration partially restores restraint stress-induced colonic HSP70 augmentation of commensal bacteria-depleted mice

As it has been reported that TLR signaling is required for commensal bacteria-dependent HSP expression (Rakoff-Nahoum et al., 2004), LPS, the ligand for TLR4, was administered to antibiotic-treated mice.

LPS administration alone without restraint did not alter the level of HSP70 ($n = 8$), whereas LPS treatment significantly increased the level of colonic HSP70 in restrained mice ($n = 8$, Fig. 4).

Colonic HSP70 level of mice that received distilled water without LPS did not increase even after restraint stress. Immunohistochemical analyses revealed moderate enhancement of HSP70 in colonic epithelial cells, without apparent morphological changes.

3.7. TLR4 expression in the colonic tissue was unaffected by restraint stress

As TLR4 is known to be responsible for LPS-dependent expression of HSP70, the effect of restraint stress on the level of TLR4 expression was examined (Fig. 5).

Immunohistochemical analysis showed that the restraint session had no effect on the level or distribution of TLR4 (Fig. 5A). TLR4 was strongly positive in the muscularis externa as well as in the lamina propria. The level of TLR4 in the colonic tissue was not increased but was slightly down-regulated by restraint stress (Fig. 5B and C).

3.8. Passage of Alexa-LPS through the colon epithelia by restraint stress

Next, we examined whether LPS is translocated through the colonic epithelia into the lamina propria after restraint. Immediately after restraint, Alexa-LPS was injected into the looped colonic

Fig. 3. A cocktail of antibiotics was administered for 7 days to eliminate commensal bacteria. Up-regulation of colonic HSP70 expression by restraint was abrogated in antibiotic-treated mice. (A) Effects of antibiotic treatment on body weight changes in C57BL/6 mice. Antibiotic treatment did not affect body weight. The results are shown as means ± SE of 8 mice. (B) Effects of antibiotic treatment on colonic bacterial number (top: aerobe; bottom: anaerobe). Both the colony forming unit (CFU) of anaerobic and aerobic bacteria in the feces dropped to less than 1/10,000 of those from mice without antibiotic treatment. Restraint did not affect the number of colonies of either aerobic or anaerobic commensal bacteria. The results are shown as means ± SE of 8 mice (*$P < 0.001$). (C) There was no significant difference in HSP70 level between non-restrained and restrained mice treated with antibiotics. The results are shown as means ± SE of 8 mice. (D) Immunohistochemical analysis revealed no apparent morphological differences in the levels of colonic HSP70 between non-restrained and restrained mice treated with antibiotics. HSP70 augmentation after restraint was absent in antibiotic-treated mice. HSP70: red; Topro-3: blue (200×).

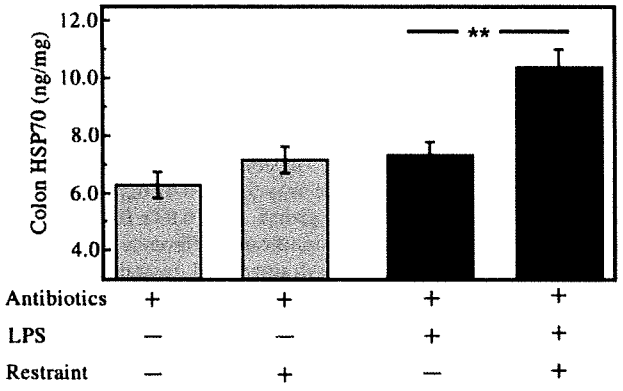


Fig. 4. LPS administration partially restores restraint-induced colonic HSP70 augmentation of commensal bacteria-depleted mice. LPS administration alone without restraint did not alter the level of HSP70, while LPS increased colonic HSP70 level of commensal-depleted restrained mice. Colonic HSP70 level of non-restrained mice, which received distilled water without LPS, did not change even after restraint stress. The results are shown as means ± SE of 8 mice ($^{*}P < 0.01$). There was a significant group × time interaction in repeated-measures ANOVA analysis ($p = 0.0404$, $F = 4.621$).

lumen. After 10 min of incubation, we examined the localization of Alexa-LPS (Fig. 6). Alexa-LPS staining was localized along the luminal surface on the apical membrane of epithelial cells in both control and restrained mice. Interestingly, however, Alexa-LPS staining was rarely detected in the lamina propria of non-restrained mice, but was detectable in the lamina propria of the restrained mice. This result clearly indicated that Alexa-LPS passed through the epithelial barrier of the colon and translocated into the lamina propria after restraint stress.

3.9. Tight junction component ZO-1 was down-regulated after restraint stress

As TLR4 was not expressed on the luminal surface of epithelial cells, we hypothesized that the integrity of tight junctions may be reduced under restraint for LPS or bacterial components to reach TLR4 in the lamina propria. As the level of ZO-1, a component of tight junctions, has been reported to correspond to permeability across a sheet of cultured epithelial cells (Boivin et al., 2007), we examined whether the level of colonic epithelium ZO-1 protein expression was affected by restraint stress (Fig. 7).

Immunohistochemical analysis indicated that ZO-1 staining was localized along the apical membrane of epithelial cells. Restraint resulted in a marked reduction of ZO-1 staining (Fig. 7A).

Western blotting analysis clearly demonstrated that ZO-1 protein level was down-regulated by restraint, in clear contrast to the up-regulation of HSP70 (Fig. 7B and C). The glucocorticoid receptor antagonist RU486 blocked restraint-induced down-regulation of ZO-1 as well as the augmentation of HSP70. ZO-1 level seemed to be enhanced under restraint when RU486 was administered.

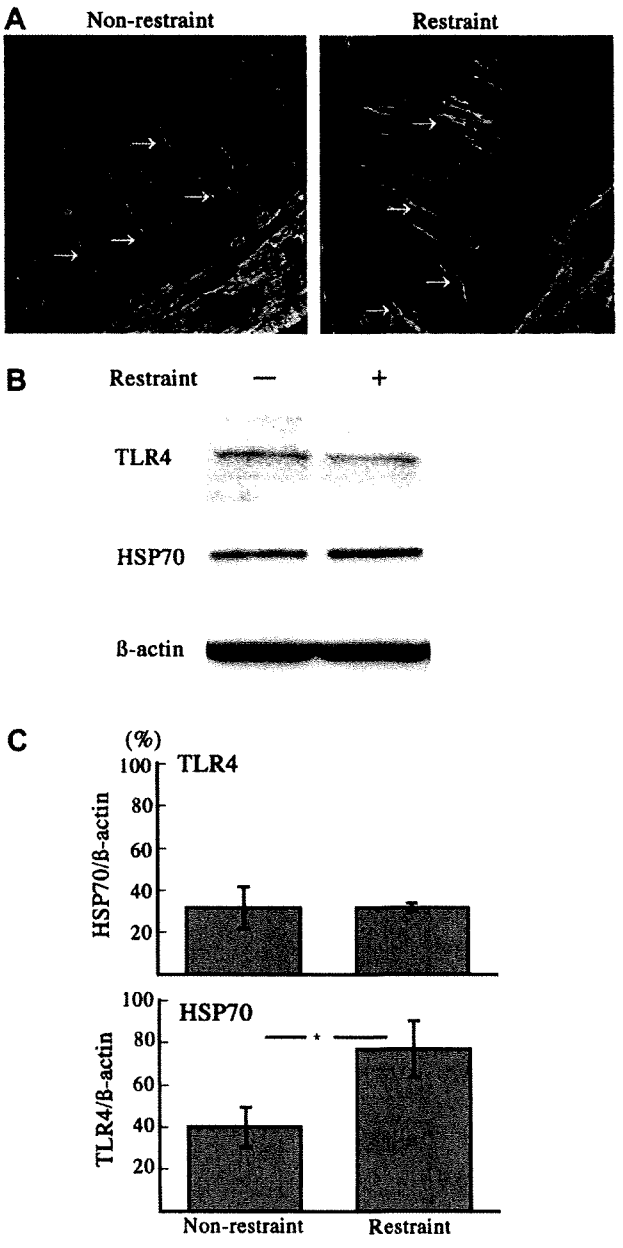


Fig. 5. Effects of 2-h restraint stress on TLR4 and HSP70 expression in colonic tissue (A, immunohistochemistry; B and C, Western blotting). (A) Immunohistochemical analysis showed that restraint stress did not affect the level or distribution of TLR4 (arrow). HSP70: red; TLR4: green; Topro-3: blue (400×). (B) The level of TLR4 in the colonic tissue was unaffected by restraint, while marked augmentation of HSP70 was observed after restraint ($n = 3$). (C) TLR4 expression of the colonic tissue was evaluated by Western blotting analysis in three separate experiments. Standard error of means (SE) is shown as bars.

4. Discussion

To understand how the colon tissue deals with physical stress, we investigated induction of HSP70 by physical stressors in colonic tissue *in vivo*. While baseline HSP70 expression in gut epithelial cells has been reported to be dependent on commensal bacteria (Arvans et al., 2005), we hypothesized that stress-inducible HSP70 expression was dependent on generalized stress reactions due to activation of the HPA axis and/or sympathetic activation.

Restraint-induced HSP70 augmentation was abrogated almost completely by prior administration of RU486, suggesting an essential role of the HPA axis in colonic HSP70 augmentation. Fukudo et al. (1997) reported that water-immersion induced HSP70 in the gut tissue as well as in the brain of rats. Similarly, Udelsman et al.(1994) showed increases in HSP70 in the aorta after restraint stress as well as after dexamethasone administration. They also showed that RU486 effectively reduced the induction of HSP70 in the aortic tissue (Udelsman et al., 1994). Therefore, the glucocorticoid dependence of HSP70 induction may be common in various tissues, including the colon.

Elimination of commensal bacteria by antibiotic treatment did not abrogate baseline HSP70 expression, but markedly abolished

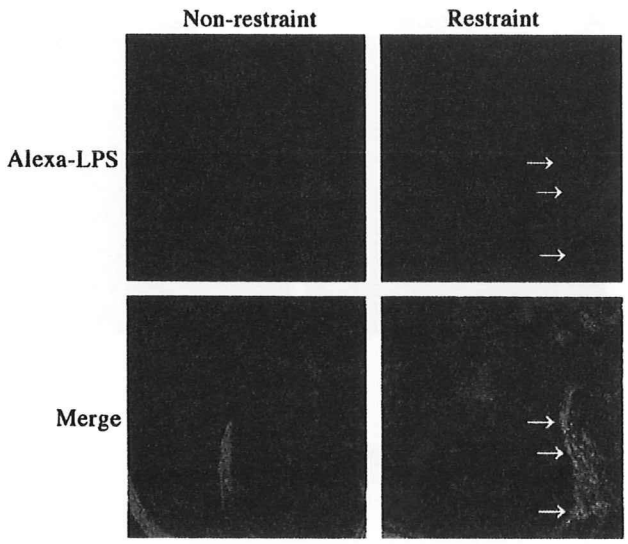


Fig. 6. LPS was translocated to the lamina propria after restraint stress. Immunohistochemical analysis showed that Alexa-LPS (arrow) passed through the mouse colon epithelium with restraint stress. GFP colon tissue: green; Alexa-LPS: red (200×).

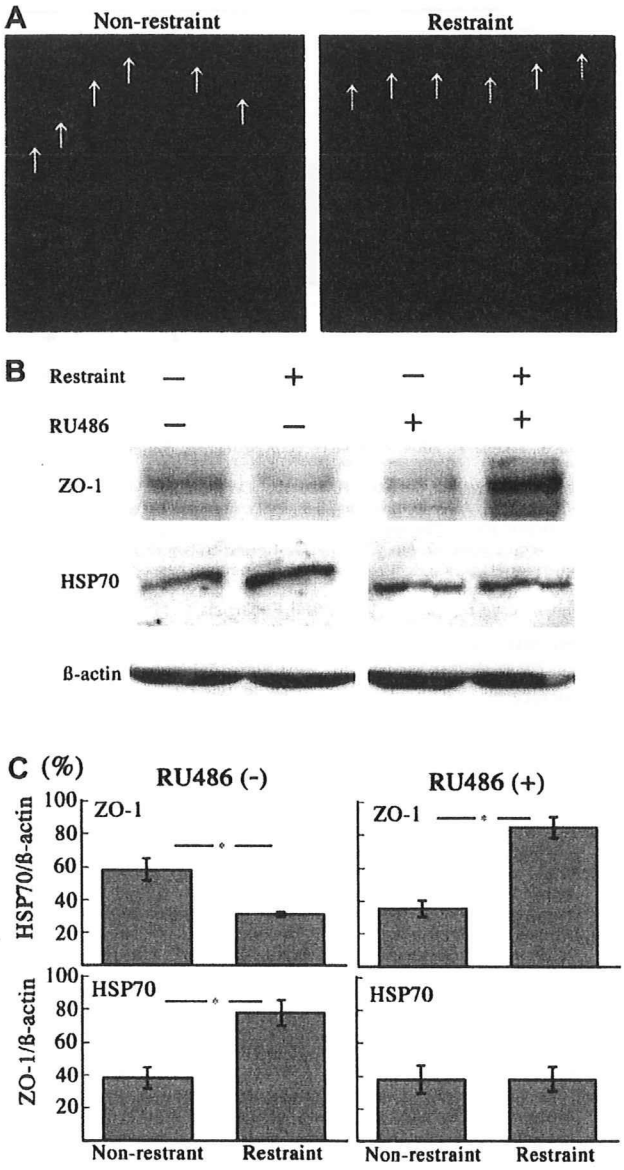


Fig. 7. Tight junction component ZO-1 was down-regulated after restraint. (A, immunohistochemistry; B and C, Western blotting). (A) Immunohistochemical analysis demonstrated that ZO-1 staining (arrow) was localized along the apical portion of the epithelia. Restraint resulted in marked reduction of ZO-1 staining ($n = 3$). ZO-1: green; Topro-3: blue (400×). (B) Western blotting analysis clearly demonstrated that ZO-1 protein level was down-regulated by restraint in clear contrast with the up-regulation of HSP70. The glucocorticoid receptor antagonist RU486 blocked restraint-induced down-regulation of ZO-1. ZO-1 level was rather enhanced under restraint when RU486 was administered ($n = 3$). (C) ZO-1 expression of the colonic tissue was evaluated by Western blotting analysis in three separate experiments. Standard error of means (SE) is shown as bars.

stress-induced HSP70 augmentation. As antibiotic treatment alone did not affect the baseline level of corticosterone or the elevation after restraint, our results suggest that stress-induced augmentation of colonic HSP70 requires both glucocorticoid augmentation and colonic microflora.

The requirement for commensal bacteria in restraint stress-induced HSP70 augmentation may be partially replaced by administration of LPS in commensal-depleted mice. As TLR4 is a specific receptor for LPS (Akira and Takeda, 2004), and Rakoff-Nahoum et al. (2004) showed that TLR4 signaling is required for colonic HSP70, the induction of HSP70 by restraint stress observed in this study was likely to be mediated by TLR4.

Interestingly, immunohistochemical examination revealed prominent expression of TLR4 in the lamina propria but not on the epithelial surface. Furrie et al. (2005) reported that TLR4 was only detected in the crypt epithelial cells and was lost as the cells matured and moved toward the gut lumen. Rumio et al. (2006) reported that functional TLR4 is not expressed in the epithelial layer, but its expression is much stronger in the smooth muscle cells and myenteric plexus of human and mouse intestines. They suggested that the low or absent expression of TLR4 on enterocytes may explain the intestinal epithelium hyporesponsiveness to the abundance of LPS in the intestinal lumen.

Then, we examined how luminal bacterial LPS could reach TLR4 in the lamina propria or the muscularis externa. For bacterial LPS to activate TLR4 signaling required for HSP70 induction (Rakoff-Nahoum et al., 2004), LPS must reach the lamina propria or beyond. Although the present study provided no direct evidence that LPS binds to TLR4 at the lamina propria, restraint stress markedly facilitated translocation of LPS to the lamina propria.

Tight junctions at the zonula occludens between epithelial cells form a strong barrier to macromolecules, and the expression and the localization of the tight junction proteins occludin and ZO-1 are known to be directly associated with the membrane permeability of cultured intestinal cells (Dokladny et al., 2006). Down-regulation of ZO-1 protein in the colonic epithelium by restraint stress in this study was glucocorticoid-dependent, as RU486 treatment effectively reversed the down-regulation of colonic epithelial ZO-1 expression. Therefore, we suggest that stress-induced glucocorticoid elevation down-regulated tight junction integrity of the colonic epithelium, resulting in increased translocation of LPS into the lamina propria. Although we lack direct evidence, LPS translocated into the lamina propria may interact with TLR4, resulting in HSP70 expression in the colonic epithelium.

In conclusion, we demonstrated that colonic HSP70 augmentation under physical stress is dependent on endogenous glucocorticoid elevation and luminal bacterial components. We demonstrated that endogenous glucocorticoid elevation reduced tight junction integrity in the colonic epithelium, which facilitated entry of luminal LPS into the lamina propria, where LPS could interact with TLR4 leading to epithelial HSP expression. Although

we still do not know the mechanism by which TLR4 activation in lamina propria led to epithelial HSP70 expression, the results of the present study demonstrated an elaborate and cooperative strategy of stress coping in the colon.

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Improving Gait Stability in Stroke Hemiplegic Patients with a Plastic Ankle-Foot Orthosis

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Stroke is the leading cause of long-term disability, and many stroke patients have hemiparesis. Hemiparesis induces ankle-control disturbances and equinovarus deformity, leading to difficulty in walking and an increased risk for falling. Plastic ankle-foot orthosis (PAFO) is frequently prescribed to correct ankle joint alignment and increase walking speed and stride length during ambulation. While several studies have shown that PAFO improves gait parameters, such as stride length and walking speed, in hemiplegic patients, the effect of PAFO on gait stability remains unclear. We quantitatively assessed the effect of PAFO on gait stability in 16 hemiplegic stroke patients (mean age 55.9 ± 11.8 years; 5 female and 11 male subjects; and 11 hemorrhagic and 5 ischemic stroke) using an ink footprint record. Wearing PAFO significantly improved the stride length, step length on the unaffected and affected sides, step width, walking speed, step frequency and functional ambulation ability. The coefficient of variation (CV), as an index of stability of movement from trial to trial, provides a measure that defines motor skills for a given task. Unaffected-side step-length CV and step-width CV were significantly decreased, when using PAFO. Furthermore, the correlation was found only between unaffected-side step length and its CV. The decrease in CV indicates that PAFO improved gait stability. We concluded that in addition to providing a faster gait, PAFO improves gait stability during walking. Gait stability and gait efficiency need to be considered separately in evaluating the effects of ankle-foot orthosis on gait performance in hemiplegic patients.

——— gait analysis; ankle foot orthosis; gait stability; hemiplegia; coefficient of variation.
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Walking ability after stroke is often disrupted because of muscle weakness, spasticity, and impaired sensorimotor control. Yet regaining the ability to walk is a major goal during rehabilitation of stroke patients. Ankle-foot orthosis (AFO) is frequently prescribed for hemiplegic patients to correct ankle joint alignment, increase walking speed, and reduce energy expenditure during ambulation (Corcoran et al. 1970; Lehmann 1979; Lehmann et al. 1983; Mojica et al. 1988; Chen et al. 1999; Hesse et al. 1999; Tyson and Thornton 2001; Gok et al. 2003). Various AFO designs make use of metal as well as plastic materials. Because metal orthoses are heavy and have a poor cosmetic appearance, they have been gradually replaced by plastic AFO (PAFO) (Corcoran et al. 1970). Conventional PAFO, designed as a posterior leaf type, is fabricated by lamination or a vacuum-forming technique over a positive plaster model of the limb. PAFO is the most commonly prescribed AFO (about 70%) in Japan (Sumiya et al. 1993). Typically, AFO is used for patients with gait abnormalities such as foot drop during swing, mediolateral ankle instability, and insufficient push off during the stance phase of the gait cycle.

Results of previous studies have shown that AFO improves gait parameters such as stride length, cadence, and walking speed (Corcoran et al. 1970; Lehmann 1979; Lehmann et al. 1983; Mojica et al. 1988; Tyson and Thornton 2001; Gok et al. 2003). When Mojica and associates (1988) investigated the effects of PAFO on body sway in 8 hemiplegic post-stroke patients, they noted that when patients were not wearing a PAFO, the center of foot pressure moved toward the unaffected limb and body sway was excessive. With a PAFO, the center of foot pressure shifted to the mid-position and body sway decreased. Accordingly, PAFO clearly improves the static balance of standing hemiplegic patients (Mojica et al. 1988; Chen et al. 1999). However, the effect of PAFO on gait stability in hemiplegic patients has not been clarified.

Human ambulation is a repetitive rhythmic movement

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with considerable diversity. Ambulation pattern and movement constancy can be suboptimal in patients with motor impairment. Disturbances in gait pattern and its constancy can be assessed by the coefficient of variation (CV) of gait characteristics such as stride length, step length, and step width (Isaacs 1982; Gabell and Nayak 1984). Moreover, previous studies (Isaacs 1982; Gabell and Nayak 1984; Maruyama and Nagasaki 1992; Vieregge et al. 1997; Stolze et al. 2000; Brach et al. 2001) have proposed that variation in gait represents a measure of gait performance stability.

This study used a footprint method to quantitatively assess the effect of PAFO on gait stability in hemiplegic stroke patients.

Materials and Methods

Subjects

Sixteen hemiplegic patients who had been prescribed PAFO were recruited as subjects. Criteria for selection were (1) unilateral hemiparesis caused by cerebrovascular disease, (2) ability to walk at least 8 m, 4 times bare feet without external support except from a cane, (3) ability to follow simple verbal commands or instructions [functional independence measure (FIM; Fiedler and Granger 1996), comprehension score of 3 points or greater], (4) freedom from neglect phenomena, defined as a visuospatial perception score of 3 points according to the Stroke Impairment Assessment Set (SIAS) (Chino et al. 1994), and (5) no history of orthopedic problems related to the lower extremity. Table 1 shows the demographic and etiologic characteristics of the subjects.

Ethical approval was obtained from the ethics committee of Tohoku University Graduate School of Medicine. Informed consent was obtained from all participants.

The subjects were assessed for hemiparesis according to Brunnstrom's lower limb motor stage (Brunnstrom 1966), for kinesthesia according to the SIAS sensory scale (Chino et al. 1994), and for ankle dorsiflexion based on the passive range of motion. The subjects were prescribed one of the following PAFOs: Shoehorn-type PAFO (9 subjects), Gillette double-flexure joint AFO (Becker Orthopedic, Troy, MI, USA) (6 subjects), or the Tamarack flexure joint AFO (Becker Orthopedic) (1 subject; Table 2). The PAFO was made to order for several patients.

The shoehorn-type PAFO is a posterior leaf-type orthosis that resists all movement allowing no planter flexion or dorsiflexion. The Gillette and Tamarack hinged PAFOs have lateral hinges at the ankle joint. Hinged AFO was selected for patients with less impaired lower limb function.

Instruments

Paper walkways were used to assess spatial gait characteristics. A paper of 8 m length was divided into a 5-m walkway, with 1.5-m area left at each end to establish start and finish lines. Small stickers (2 cm × 2 cm, with felt adhesive backing soaked in ink) were attached to the subject's sole at the head of the metatarsus of the fifth toe (Fig. 1).

Procedure

Subjects ambulated 8 m on the paper walkway with ink patches on their foot soles, which left behind a footprint record. The first and last 1.5 m of the walk was not used because of changes in walking speed that occur when a person starts and stops walking.

Subjects were asked to walk with or without PAFO at their most comfortable (i.e., self-selected) speed along the 8-m walkway. When walking, the subjects used ambulation aids as shown in Table 2. The order of testing (with and without a PAFO) was randomized. Testing was repeated twice for two conditions, i.e., barefoot and wearing a PAFO, and mean values for the stride length, step length, symmetry, and step width were calculated for each trial. Measurements of spatial parameters were made by one of two authors (NS, KS) kept unaware of the subject's gait status and who had not participated in ambulation

Table 1. Demographic and etiologic characteristics of the hemiparetic subjects.

Subject	Gender	Age (years)	Diagnosis	Affected limb	Height (cm)	Weight (kg)	Interval from onset (months)
1	M	62	INF	R	163	46	5.3
2	M	29	ICH	R	183	99	2.6
3	M	55	ICH	L	168	66	4
4	M	73	INF	L	160	54	4.4
5	M	45	ICH	R	172	57	10.4
6	M	55	ICH	R	170	61	50.4
7	F	48	INF	R	150	55	5.3
8	F	57	ICH	L	149	54	15.7
9	F	70	INF	L	147	45	32.6
10	M	47	INF	R	165	65	2
11	M	52	ICH	R	167	72	5.8
12	M	79	ICH	R	160	60	55.4
13	M	55	ICH	L	165	67	28.7
14	F	55	ICH	L	155	71	111.8
15	M	56	ICH	R	168	80	113.8
16	F	58	ICH	R	152	52	49.5

F, female; ICH, intracerebral hemorrhage; INF, infarction; L, left; M, male; R, right.

Table 2. Clinical characteristics of the hemiparetic subjects.

Subject	Ambulation aids	PAFO	BRS (L/E)	FAC (barefoot)	Position sense	ROM (degrees)
1	SPC, AFO	Hinge[a]	IV	3	3	20
2	SPC, AFO	Hinge[a]	IV	3	3	20
3	SPC, AFO	SHB	IV	3	3	5
4	SPC, AFO	SHB	IV	3	3	10
5	SPC, AFO	SHB	IV	3	0	0
6	SPC, AFO	Hinge[a]	IV	3	3	0
7	SPC, AFO	SHB	III	3	3	15
8	SPC, AFO	Hinge[a]	IV	4	3	15
9	SPC, AFO	SHB	IV	4	3	10
10	SPC, AFO	Hinge[a]	IV	3	3	20
11	SPC, AFO	Hinge[a]	IV	3	2	20
12	SPC, AFO	SHB	III	4	3	20
13	SPC, AFO	Hinge[b]	III	4	0	15
14	AFO	SHB	IV	4	3	20
15	AFO	SHB	III	4	0	0
16	AFO	SHB	III	4	3	15

BRS, Brunnstrom recovery stage; FAC, functional ambulation category; L/E, lower extremity; PAFO, plastic ankle-foot orthosis; ROM , range of motion for ankle dorsiflexion with the knee flexed 90°. (The angle between the fibular shaft and the fifth metatarsal bone was measured. The right angle between the fibular shaft and the fifth metatarsal bone was defined as 0° of dorsiflexion); SHB, shoehorn brace; SPC, single-point cane; [a], Gillette double flexure joint; [b], Tamarack flexure joint.

Position sense was assessed in the great toe according to the Stroke Impairment Assessment Set. When no position change was detected by the patient after the maximum possible motion, a score of 0 was given. A score of 1 indicated that the patient recognized movement of the digit but not the direction, even at maximal excursion. When the patient correctly perceived the direction of a moderate excursion, the score was 2. A score of 3 indicated that the patient could correctly identify the direction of a slight movement.

testing.

Measurements

Gait parameters

The 5-m walk was timed using a stopwatch, and the steps were counted. These were converted into units of walking speed (m/min) and cadence (the numbers of steps per unit time, steps/min). Stride length for the right leg was measured as the linear distance between successive ipsilateral ink marks. Step length was measured as the linear distance between contralateral ink marks. Step width was measured as the transverse linear distance between the borders of two consecutive footprints (Fig. 2).

The functional ambulation category (FAC) (Holden et al. 1984; Holden et al. 1986) is an ordinal scale used to assess functional ambulation ability. The physical therapist who treated or tested subjects in this study determined the FAC values of the subjects.

Statistical Analysis

Step-length symmetry was evaluated by dividing the values for unaffected-side step length by those for affected-side step length. Furthermore, symmetry ratios for the two conditions were compared in terms of the absolute value of the difference from 1. CV, used to represent the variation in each spatial parameter (stride length, step length, and step width), was calculated as a percentage by dividing the standard deviation (SD) by the mean value and multiplying the result by 100.

Each parameter was compared between gait measurements with and without a PAFO, using a paired *t*-test or the Wilcoxon's signed-rank test. FAC ratings with and without a PAFO were compared using the Wilcoxon's signed-rank test. CV values of parameters exhibiting a significant difference between the two conditions were examined for correlation with individual gait parameters using Pearson's correlation coefficient or Spearman's rank correlation. SPSS for Windows version 11.0J (SPSS, Chicago) was used for the analysis and a P value of < 0.05 was considered statistically significant.

Results

For one patient (no. 1), only the second trial measurement was used for analysis because he almost fell when ambulating without a PAFO during the first trial. Comparisons of mean values for each gait parameter between conditions with and without a PAFO are presented in Table 3.

Gait parameters

Significant increases were found in walking speed (126.5%, $P < 0.01$), cadence (109.7%, $P < 0.05$), stride length (115.5%, $P < 0.01$), and step length of the unaffected (119.8%, $P < 0.01$) and affected sides (111.8%, $P < 0.05$) when walking with a PAFO compared to walking without a PAFO. Step width was increased significantly when wearing a PAFO ($P < 0.05$), but by only 3%. The variance in

Fig. 1. Timed ambulation and ink footprint record.

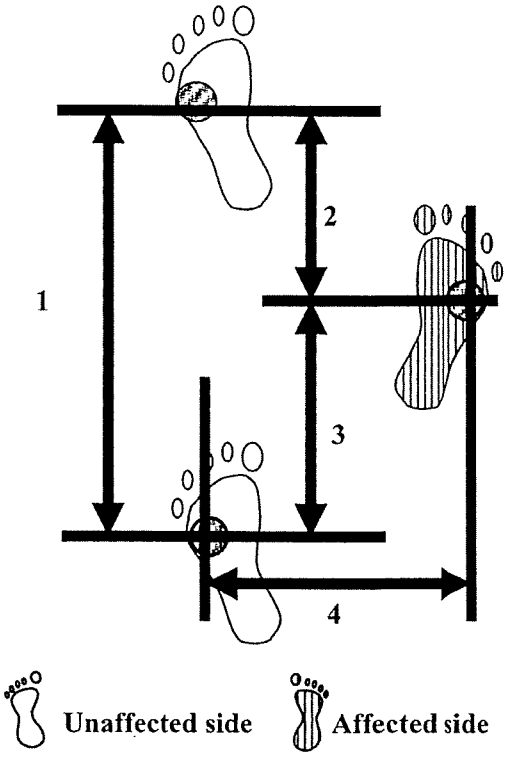

Fig. 2. Measurement of spatial parameters from ink footprints on a paper walkway in patients with right hemiparesis. 1, stride length; 2, unaffected-side step length; 3, affected-side step length; 4, step width.

Table 3. Comparison of gait parameters assessed with and without a PAFO.

	Without PAFO	With PAFO	*P* value
Stride length (cm)*	56.9 ± 13.6	65.7 ± 13.6	0.0041
CV%	7.4 ± 2.5	6.4 ± 2.7	0.26
Unaffected side step length (cm)*	26.3 ± 8.2	31.5 ± 5.9	0.0011
CV%*	15.5 ± 8.0	9.5 ± 3.9	0.0019
Affected side step length (cm)*	30.4 ± 9.4	34.0 ± 10.0	0.044
CV%	10.5 ± 7.5	9.2 ± 4.4	0.45
Step width (cm)**	28.2 ± 5.0	29.8 ± 4.4	0.034
CV%**	7.7 ± 4.6	5.5 ± 1.8	0.0038
Velocity (m/min)*	18.1 ± 8.1	22.9 ± 6.8	0.0032
Cadence (step/min)*	66.8 ± 21.0	73.3 ± 15.8	0.015
Step-length symmetry ratio	0.36 ± 0.45	0.25 ± 0.23	0.11
Step-length symmetry ratio range	0.003 – 1.84	0.01 – 0.99	

CV, coefficient of variation; PAFO, plastic ankle foot orthosis. Values are mean ± S.D.
*Significant at $P < 0.05$ by paired *t*-test. **Significant at $P < 0.05$ by Wilcoxon's signed-rank test.

Table 4. Correlation of unaffected-side step-length CV and step-width CV with gait parameters.

	Unaffected-side step-length CV[a]		Step-width CV[b]	
	Without PAFO	With PAFO	Without PAFO	With PAFO
Velocity	–0.20	–0.25	0.27	0.09
Cadence	–0.07	0.1	0.15	0.27
Stride length	0.22	–0.18	0.37	0.24
Unaffected-side step length	–0.76**	–0.78**	0.21	0.24
Affected-side step length	–0.30	–0.47	0.33	0.33
Step width	–0.40	–0.04	–0.53*	–0.35

CV, coefficient of variation; PAFO, plastic ankle foot orthosis; [a], Pearson's correlation coefficient; [b], Spearman's rank-order correlation coefficient. Step-width CV did not follow a normal distribution.
*$P < 0.05$; **$P < 0.01$.

Table 5. Comparison of FAC in hemiplegic patients with and without PAFOs.

FAC	Subject number (%)	
	Without PAFO	With PAFO
0	0 (0)	0 (0)
1	0 (0)	0 (0)
2	0 (0)	0 (0)
3	9 (56.3)	1 (63)
4	7 (43.8)	5 (31.3)
5	0 (0)	10 (62.5)

A significant improvement in FAC ($P < 0.001$) was found by Wilcoxon's signed-rank test. FAC, functional ambulation category; PAFO, plastic ankle foot orthosis.

step-length symmetry decreased when wearing a PAFO. Step-length symmetry ratios of 10 subjects (no. 1, 3, 5, 6, 7, 8, 9, 13, 15, and 16) increased to 1, while those of others (no. 2, 4, 10, 11, 12, and 14) decreased from 1. Overall, the step-length symmetry ratio was decreased (69.4%), but the difference was not significant ($P = 0.11$).

CV of spatial parameters

Unaffected-side step-length CV and step-width CV decreased significantly while wearing a PAFO ($P < 0.01$ each). However, affected-side step-length CV and stride-length CV did not differ between the two conditions ($P = 0.4$, Table 3).

Relationship between spatial-parameter CV and gait parameters

There was a significant difference between the use and nonuse of PAFO when we examined unaffected-side step-length CV and step-width CV, and this correlated with walking speed, cadence, and spatial parameters. Results showed a significant correlation between step-length CV of the unaffected side and step length of the unaffected side, both with ($r = -0.76$) and without a PAFO ($r = -0.78$). A significant relationship was observed between step-width CV and step width while walking without a PAFO ($r = -0.53$, Table 4).

FAC

Wearing a PAFO improved FAC ($P < 0.001$, Table 5); the number of participants who required considerable supervision (FAC 3) decreased from 9 (56.3%) to 1 (6.3%), while the number of participants who were able to ambulate independently on a level surface but required supervision or physical assistance for stairs, inclines, or nonlevel surfaces (FAC 4) decreased from 7 (43.8%) to 5 (31.3%). The number of participants who were able to ambulate independently on both nonlevel and level surfaces, stairs, as well as inclines (FAC 5) increased from none to 10 (63%).

Discussion

The simple walkway method used in the present study is suitable for clinical situations and previous studies have demonstrated its reliability, accuracy, and validity (Boenig 1977; Holden et al. 1984; Rigas 1984; Holden et al. 1986; Tyson and Thornton 2001). The longer do subjects walk, the higher became the reliability of measuring spatial parameters. However, walking a longer distances is difficult for patients with disabilities. Consequently, a 5-m walkway is usually used for patients with severe hemiplegia (Tyson and Thornton 2001). Moreover, preparation for a long-distance walkway is difficult in a clinical setting. Holden et al. (1984; 1986) reported that the reliability of gait parameters measured using a 20-foot (< 7 m) paper walkway was sufficient. Brach et al. (2001) used a 4-m walkway for hemiparetic patients to compare spatial parameter CVs among different conditions at various speeds. Using a 5-m paper walkway, Tyson and Thornton (2001) reported that spatial walking parameters for hemiplegic patients were better with an AFO than without it. Our study used a 5-m paper walkway, considering the demonstrated reliability of measurement and practicality in a clinical situation. We believe that this is a valid method for examining the effects of PAFO in hemiplegic patients.

The CVs of gait parameters are interpreted as indicat-

ing constancy or stability of movement from trial to trial; they therefore provide a measure of motor skill for a given task (Maruyama and Nagasaki 1992). Temporal (e.g., stride time) and spatial (e.g., stride length) parameters are used to examine variation in human gait. Gait stability has been assessed efficiently using the variability measure not only in elderly subjects (Gabell and Nayak 1984; Stolze et al. 2000), but also in physically disabled post-stroke patients (Zverev et al. 2002) and patients with Parkinson's disease (Vieregge et al. 1997). In fact, Gabell and Nayak (1984) reported that in both older and younger groups, the gait-patterning mechanism (step length and stride time) is more consistent than the balance-control mechanism (step width and double-support time) and that increased variability in gait is not necessarily a normal concomitant of old age. Furthermore, step length variability is greater in hospitalized patients with a history of falls than in those with no such history (Isaacs 1982). Therefore, the variability measure can be used as an index of gait performance stability (Maruyama and Nagasaki 1992).

In the present analysis, step-length CV of the unaffected side and step-width CV were decreased when wearing a PAFO. However, affected-side step-length CV was not decreased, suggesting that PAFO improved stability during the affected-side stance phase.

Step width was increased by wearing a PAFO. A larger step width could reflect increased postural gait impairment. In this study, the step width increase was as small as 1 cm (without PAFO, 28.2 ± 5.0 cm; with PAFO, 29.1 ± 4.4 cm). We interpret that the step-width increased because of the widening of toe out angles due to wearing a PAFO and not because of gait instability.

Previous studies (Lehmann 1979; Lehmann et al. 1983; Mojica et al. 1988; Chen et al. 1999; Tyson and Thornton 2001; Gok et al. 2003) have shown that wearing of PAFO increases walking speed, cadence, and stride length and improves FAC of subjects. These findings were confirmed in the present study. In addition, this report describes increases in the unaffected-side step length, affected-side step length, and step width when wearing a PAFO. There are a few studies that have focused on the effects of PAFO on step length in hemiplegic patients. Burdett et al. (1988) indicated that step length increases on the affected side while wearing PAFO when compared with that without a brace; however, the unaffected-side step length does not increase. This discrepancy between studies by Burdett et al. (1988) and ours may be because of differences in subject's impairments. However, this cannot be confirmed since they have not provided any details about patient's impairments in their study. Moreover, we found that the change in the step length symmetry ratio was inconsistent; ratios of some subjects rose to 1, while those of others fell from 1. We therefore concluded that a PAFO does not necessarily improve gait symmetry. Thus, these findings may suggest that assessment of step length rather than stride length reflects the effect of wearing PAFO in hemiplegic patients.

Gait stability improvement using a PAFO was assumed based on increased walking speed, cadence, and stride length. However, some gait parameters might not directly represent gait stability during walking. In this study, a good correlation was found only between unaffected-side step length and its CV.

Hesse et al. (1999) reported that the use of orthoses reduces double-stance duration and increases single-support duration upon the hemiparetic leg. Furthermore, the brace provides a feeling of security, reducing the patient's inclination to shift weight quickly to the nonparetic limb. Therefore, we concluded that in addition to improving gait stability during walking, PAFO also provides a more efficient gait. Gait stability and gait efficiency need to be considered separately when evaluating the effects of PAFO on gait performance in hemiparetic patients.

Our study has several limitations. First, we did not include patients who were unable to walk without a PAFO. Hemiplegic patients often have difficulty in ambulation even after being prescribed an orthosis. Second, all subjects improved over the 2 weeks following prescription of the PAFO, so results might have been influenced by the individual adaptations of the subjects to their PAFO. Consequently, the immediate effects of a PAFO on gait stability are not clear. Additional studies should investigate the time course of gait characteristics after initiating PAFO use. Third, we used CV parameters to indicate gait stability. For example, if movement of body parts becomes steady by the simple mechanism of an orthosis, the CV will become smaller. Thus, CV parameters might have reflected not only gait stability, but also other gait characteristics. Fourth, no "gold standard" exists for measuring gait stability. We need to determine the parameters that best reflect gait stability, considering temporal CV, acceleration, and the like.

In conclusion, the use of PAFO leads to a more dynamic and stable gait in hemiparetic patients. Gait stability can be assessed quantitatively using the distance parameter CV. We believe that the methods used in this study can contribute to prescribing appropriate PAFO for stroke patients in a clinical environment.

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Effects of Low-frequency Repetitive Transcranial Magnetic Stimulation in Parkinson's Disease

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Objective: The therapeutic effects of low-frequency repetitive transcranial magnetic stimulation (rTMS) were investigated in Parkinson's disease with cognitive dysfunction known as impaired set switching.
Methods: Six patients with Parkinson's disease exhibiting impaired performances on the Wisconsin card sorting test (WCST) were enrolled. Under electroencephalogram (EEG) monitoring, rTMS was performed using a concave circular coil once a week for three months. A 0.2-Hz rTMS was applied over the frontal region (Fz) at an intensity of 1.2 x the motor threshold of the abductor pollicis brevis (APB) for a total of 100 stimuli per session. The Trail Making Test part B (TMT-B), WCST, Wechsler Adult Intelligence Scale Revised (WAIS-R), Self-rating Depression Scale (SDS), Functional Independence Measure (FIM), and 20 m Walk time were evaluated before and after rTMS. Subjective symptoms and objective findings were also evaluated.
Results: Significant improvements in the TMT-B and WCST scores after rTMS were observed for all six patients. In addition, the subjective symptoms and objective findings also improved. The 20 m walk time decreased significantly in all four subjects after rTMS. The SDS scores improved in four of the five subjects, although the differences between the baseline and follow-up scores were not significant. No significant improvements in the WAIS-R, FIM scores were observed.
Conclusions: Low-frequency suprathreshold rTMS applied over bilateral prefrontal areas alleviated impaired set switching in Parkinson's disease. These results suggest that rTMS can affect the functional recovery of the frontostriatal circuit.

Key words: Parkinson's disease, repetitive transcranial magnetic stimulation, impaired set switching, prefrontal area, Wisconsin card sorting test

INTRODUCTION

Parkinson's disease is a chronic progressive neurodegenerative disease caused by abnormal degeneration and the detachment of dopaminergic neurons in the ventral tegmental area and substantia nigra pars compacta in the midbrain, with subsequent basal ganglion damage. It is characterized by four major motor system disorders (tremor, rigidity, akinesia and impaired postural reflex) and non-motor disorders (cognitive disorder and higher brain dysfunction). The main treatment are drug therapy and rehabilitation, but surgical therapies, such as stereotactic surgery including basal ganglion destruction and deep electric brain stimulation, have also been performed. While the effects of these surgical treatments require clarification, some studies have reported complications, such as brain edema and bleeding, as well as neurologic symptoms, such as hallucination, delusion, mood disorder and cognitive dysfunction [1, 2]. In Parkinson's disease accompanied by intractable depression, the therapeutic effects of electroconvulsive therapy have also been documented [3]. However, this is an invasive treatment conducted under general anesthesia. Therefore, noninvasive therapies that can be combined with drug therapy and rehabilitation are required.

In recent years, transcranial magnetic stimulation (TMS) has been closely examined as a possible noninvasive treatment. Ever since Barker *et al.* [4] performed single-session magnetic stimulation and recorded evoked potentials from hand muscles in 1985, this procedure has been widely used to assess motor function in the central nervous system.

After the development of repetitive transcranial magnetic stimulation (rTMS), the cortex can be repeatedly stimulated at a specific frequency and an adjustable intensity. Since Pascual-Leone *et al.* [5] first used rTMS in humans in 1991, it has been used for the treatment of various diseases. Pascual-Leone *et al.* [6] were the first to perform rTMS in Parkinson's disease, and Mally *et al.*, Shimamoto *et al.* and Siebner *et al.* reported that rTMS was useful for reducing motor impairment [7, 8, 9]. On the other hand, Ghabra *et al.* and Boylan *et al.* reported that it was ineffective [10, 11]. As these studies used different parameters of magnetic stimulation and assessments, no definite conclusion regarding the effects of rTMS on the motor functions and activities of daily living (ADL) in Parkinson's disease has been made. Furthermore, some safety issues also remain.

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Table 1 Profiles of patients and summary of clinical findings during baseline period

Subject	1	2	3	4	5	6
Age (years)	64	66	71	69	62	69
Sex	F	M	M	F	F	M
H&Y	3	2	3	2	2	2
Disease duration (years)	7	8	11	6	2	9
MMSE	29	30	27	26	26	29
WAIS-R (TIQ)	103	101	101	100	72	100
(VIQ)	121	103	115	109	69	113
(PIQ)	79	98	86	90	79	85
WCST (CA)	2	4	0	4	2	0
(PEN)	7	0	10	2	4	19
(TE)	19	15	35	12	23	48
TMT-B	323.2	204.8	337.4	191.2	329.4	806.6
SDS	49	68	64	77.5	57.5	-
FIM (total)	68	122	114	90	126	120
(motor)	37	87	79	55	91	85
(cognitive)	31	35	35	35	35	35

The SDS was evaluated in 5 of the 6 subjects. H & Y: Hoehn and Yahr classification; WCST, Wisconsin card-sorting test (Keio version); CA, categories achieved; PEN, perseverative errors of Nelson; TE, total errors; TMT-B, trail-making test B; WAIS-R, Wechsler adult intelligence scale – revised; TIQ, total intelligence quotient; VIQ, verbal IQ; PIQ, performance IQ; SDS, self-rating depression scale of Zung; FIM, functional independence measure.

In most previous studies, rTMS was applied to a motor area. The functional failure of the frontostriatal circuit has been suggested to be the neural basis of cognitive dysfunction in Parkinson's disease; therefore, the stimulation of the frontal cortex might reasonably be expected to be effective in Parkinson's disease based on this neural basis and the observation that the application of rTMS to the prefrontal cortex can effectively alleviate depression. In fact, studies have reported that low-frequency repetitive magnetic stimulation over the dorsolateral prefrontal area is effective for improving depression in Parkinson's disease and cognitive task performance in patients with major depression [12, 13].

In the present study, the therapeutic effects of rTMS on cognitive dysfunction, particularly on impaired set switching, in Parkinson's disease were evaluated by simultaneously stimulating bilateral dorsolateral prefrontal areas using a concave circular coil over the frontal region (Fz).

MATERIALS AND METHODS

1. Subjects

Six patients (3 men and 3 women; between 64 and 71 years of age; mean age, 66.8 years) with Parkinson's disease referred to the Department of Rehabilitation Medicine, Tokai University Hospital, between June 2002 and March 2003 were enrolled. All the patients had displayed an impaired performance (≤ 4 categories achieved) on the Wisconsin card sorting test (WCST; Keio version) [14] and had a Mini Mental State [15, 16] score of ≥ 26 points (Table 1). None of the patients had a past history of cerebrovascular disorder, and all the patients had been diagnosed as Parkinson's disease by a neurologist. All the patients were receiving drug therapy, and their disease duration ranged from 2 to 11 years (Table 1). All the subjects gave their informed consent to participate in this research project, which was approved by the Ethics Committee of Tokai University School of Medicine.

2. Methods

(1) Repetitive transcranial magnetic stimulation (rTMS)

rTMS was delivered using a MagLite™ (Dantec, Skovlunde, Denmark) with a concave circulation coil (MMC-140; Dantec). The coil had a diameter of 140 mm, and the degree of change in the magnetic flux was highest at the central area adjacent to the coil surface, while the induced current was highest within a radius of 2 cm.

(2) Experiment procedure

Each subject was asked to sit in a reclining chair with their hip and knee joints at 90°. Using the International 10-20 method for electroencephalography (EEG), the Cz and Fz of the head were determined.

Disc surface electrodes were placed for motor evoked potential (MEP) recording on the right abductor pollicis brevis (APB) muscle according to the belly-tendon method to determine the optimal stimulation site and threshold for the motor cortex. The optimal stimulation site was determined by moving the coil around the left side of the scalp Cz until the maximum MEP response was achieved. The threshold was defined as the minimum stimulation intensity, required to elicit reproducible MEPs of at least 50 μV during at least four out of eight stimulations at the optimal stimulation site at rest.

Next, after attaching an EEG cap, the backrest was lowered to adjust the trunk angle to 45° in a resting sitting position. rTMS was delivered over the Fz at 1.2 times the motor threshold of the APB at 0.2 Hz for a total of 100 times once a week. The MEPs were also recorded during rTMS.

Over a period of about 3 months, rTMS was performed for a total of 1200 stimulations. The period was divided into three in relation to the therapeutic

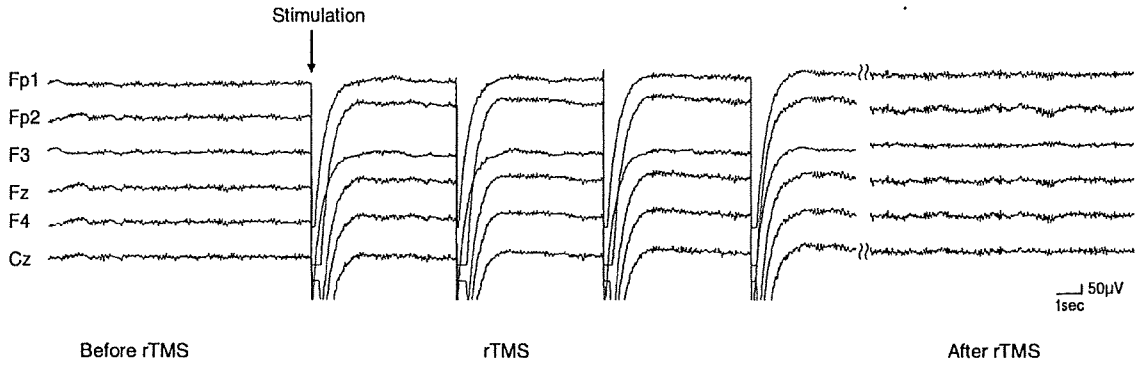

Fig. 1 EEG before, during and after rTMS.
The brain waves were primarily within a range of 8 – 10 Hz. Even after stimulation of the Fz region, no abnormal waves were observed at any of the monitoring sites (Fp1, Fp2, F3, Fz, F4, and Cz).

schedule: the baseline period (about 1–2 months before the rTMS period), the rTMS period (about 3 months), and the follow-up period (about 1–2 months after the rTMS period). Over the period, no changes were made in drug therapy and rehabilitation. During the baseline and follow-up periods, we performed the neuropsychological tests described below and evaluated the patients' activities of daily living. During the follow-up period, these tests and evaluations were performed, starting one week after the end of the rTMS period.

(3) EEG

EEG was monitored and recorded with the patient's eyes open during and for 30s before and after rTMS. According to the International 10–20 method, electrodes were placed at Fpz, Fp1, Fp2, F3, Fz, F4 and Cz. A signal processor (DP1100; NEC Medical Systems, Tokyo, Japan) was used for waveform reading, and Hyper Wave (Kissei Comtec, Tokyo, Japan) with a bandpass of 0.5–30 Hz was used to record the wave.

(4) Neuropsychological tests, assessment of ADL, Unified Parkinson's Disease Rating Scale (UPDRS), and 20 m walk

With regard to the neuropsychological tests, each of the six subjects were asked to perform the trail-making test part B (TMT-B), the Wisconsin card-sorting test (WCST; Keio version), and the WAIS-R. The self-rating depression scale (SDS) [17] was assessed in five of the six subjects. With the TMT-B, the task execution time(s) was measured using a stopwatch.

With regard to the assessment of ADL, the functional independence measure (FIM) was evaluated in all six subjects. The Japanese version of the Unified Parkinson's Disease Rating Scale (UPDRS) [18] was also used to evaluate the severity of the disease. In these subjects, no diurnal variations were observed and the assessments were made during the "on" time. In four of the six subjects, a 20 m walk test was administered: the length of time required for each subject to walk 20 m down a flat linear corridor at their own pace was measured.

The TMT-B and 20 m walk test are measured five times during the baseline and the follow-up period and then averaged for analysis. The other tests are evaluated once during the second week of the baseline and the follow-up period.

Changes in subjective symptoms and objective findings were also assessed. During the follow-up period, each subject and his family were asked once a week about changes in subjective symptoms and objective findings in daily life. Physical therapists were also interviewed regarding the conditions of the patients.

(5) Statistical analysis

The neuropsychological tests, assessments of activity of daily living, UPDRS, and 20 m walk test between the baseline period and the follow-up period were compared. SPSS[C] was used for the statistical analysis, and the Wilcoxon signed rank test was used, with a level of significance set at $p < 0.05$.

RESULTS

(1) EEG and MEP

Fig. 1 shows a representative EEG pattern during magnetic stimulation. No abnormalities were observed during the 30-s periods of EEG monitoring before and after stimulation. During the stimulation period, the EEG mostly showed approximately 8-Hz α-waves at rest; no abnormalities were seen in any of the subjects. None of the subjects showed abnormal waveforms during the 30s periods of EEG monitoring before and after stimulation.

The MEPs from the right abductor pollicis brevis monitored and recorded during magnetic stimulation were less than 50 μV in all the subjects.

(2) Changes in neuropsychological tests, assessments of ADL, UPDRS, and 20 m walk

During the baseline period, the average TMT-B execution time was 365.4 seconds, which was longer than the average time for healthy people in their 60s [19] (Table 2). The WAIS-R TIQ was ≥ 100 points in five of the six subjects, and although the results were comparable to the average for healthy adults, differences of more than 15 points in the WAIS-R between verbal and performance IQ were seen in four of the six patients (Table 2).

When comparing the baseline and follow-up periods, no significant changes were found in the WAIS-R subscales or in any of the FIM motor or cognitive scores (Table 2). The SDS scores improved in four of the five subjects, although the differences between the baseline and follow-up scores were not significant.

Table 2 WCST, TMT-B, WAIS-R, SDS and FIM scores before and after rTMS

Test	Before	After rTMS
WCST (CA)	2 ± 1.8	5.8 ± 0.4 *
(PEN)	7.0 ± 6.9	0.7 ± 1.0 *
(TE)	25.3 ± 13.7	10 ± 2.1 *
TMT-B	365.4 ± 225.7	207.7 ± 84.6 *
WAIS-R (TIQ)	96.2 ± 11.9	99.3 ± 14.4
(VIQ)	104.8 ± 19.0	108.3 ± 18.0
(PIQ)	86.2 ± 7.2	88.7 ± 11.2
SDS	63.2 ± 10.8	56.1 ± 16.1
FIM (total)	106.7 ± 22.9	106.2 ± 21.3
(motor)	72.3 ± 21.6	71.3 ± 21.0
(cognitive)	34.3 ± 1.6	34.8 ± 0.4

*P < 0.05; value indicates results of Wilcoxon signed-rank test (n = 6). Values represent means ± SD. Trail-making test B scores represent time in seconds required to complete test. WCST, Wisconsin card-sorting test (Keio version); CA, categories achieved; PEN, perseverative errors of Nelson; TE, total errors; TMT-B, trail-making test B; WAIS-R, Wechsler adult intelligence scale – revised; TIQ, total intelligence quotient; VIQ, verbal IQ; PIQ, performance IQ; SDS, self-rating depression scale of Zung; FIM, functional independence measure

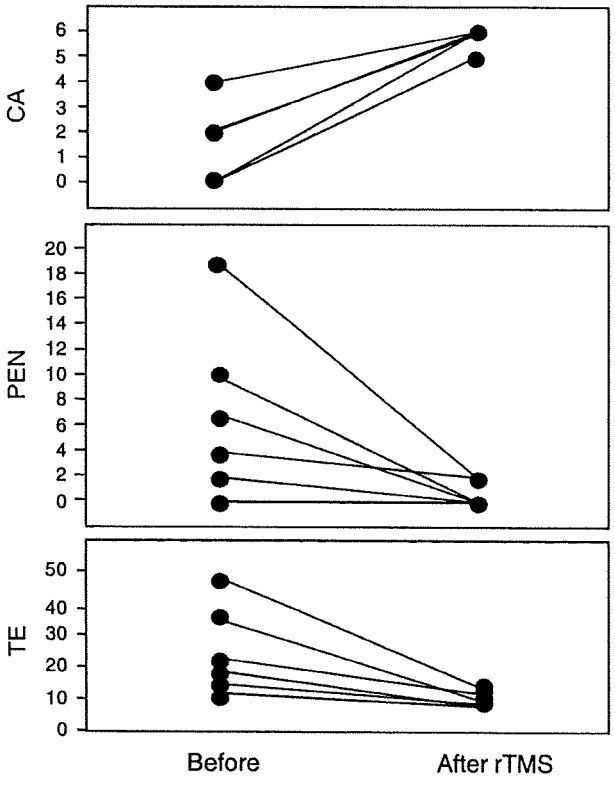

Fig. 2 Changes in the categories achieved (CA), perseverative errors of Nelson (PEN) and total errors (TE), as assessed using the WCST before and after three months of rTMS.
After three months of rTMS, the CA increased and the PEN and TE decreased in all the subjects ($p < 0.05$). CA: Number of categories in which six consecutive correct responses were achieved. PEN: Number of categories where wrong responses were given before and after rTMS. TE:Total number of errors.

The number of achieved WCST categories increased significantly ($p < 0.05$). In addition, the numbers of perseverative errors of Nelson and the total errors decreased significantly ($p < 0.05$) (Table 2 and Fig. 2).

The TMT-B execution time did not change significantly during the base line period, but significantly decreased after rTMS ($p < 0.05$) (Table 2 and Fig. 3) and maintained during the follow-up period. The 20m walk time did not change significantly during the base line period, but decreased significantly in all four subjects after rTMS and maintained during follow-up period. (Fig. 4). The decrease in the 20-m walk time was particularly marked in Subject 3.

With regard to the UPDRS, no changes were found in part I (mentation, behavior and mood), but improvements were seen in part II (activities of daily living) and part III (motor exam). In motor exam, there were some improvements in tremor at rest in legs, rigidity in legs, posture and body bradykinesia. No improvements were seen in tremor at rest in arm, rigidity in arm and rapid alternating movements in arm (Table 3).

(3) Changes in subjective symptoms and objective findings

During the follow-up period, changes in subjective symptoms and objective findings in daily life were reported by the subjects, their families, and the therapists. These changes included "faster reactions",

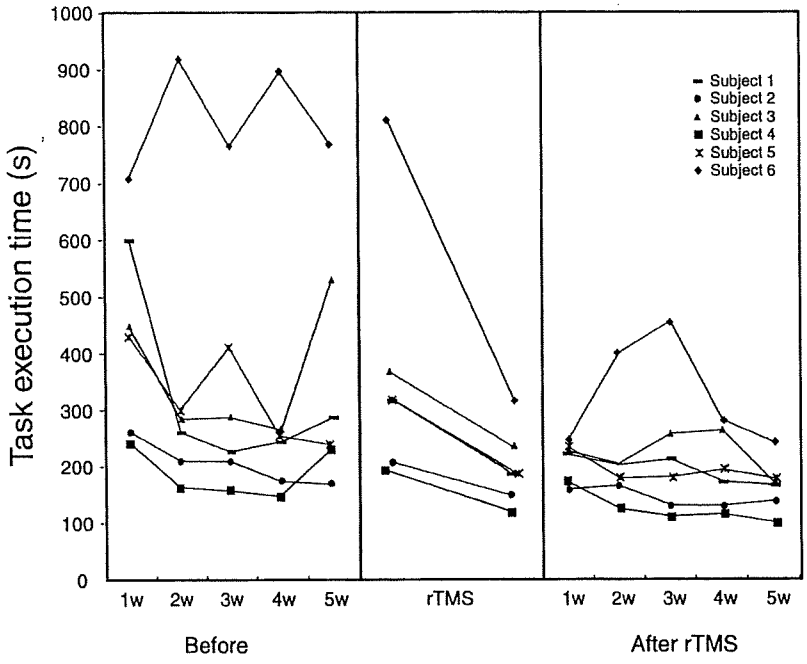

Fig. 3 Changes in TMT-B execution time during the baseline,before rTMS and during follow-up period, after rTMS. The straight lines in the central column represent the difference in the task execution times measured pre-rTMS (mean of five measurements) and post-rTMS (mean of five measurements) in individual subjects.
For all the subjects, the task execution time was significantly shorter after three months of rTMS ($p < 0.05$).

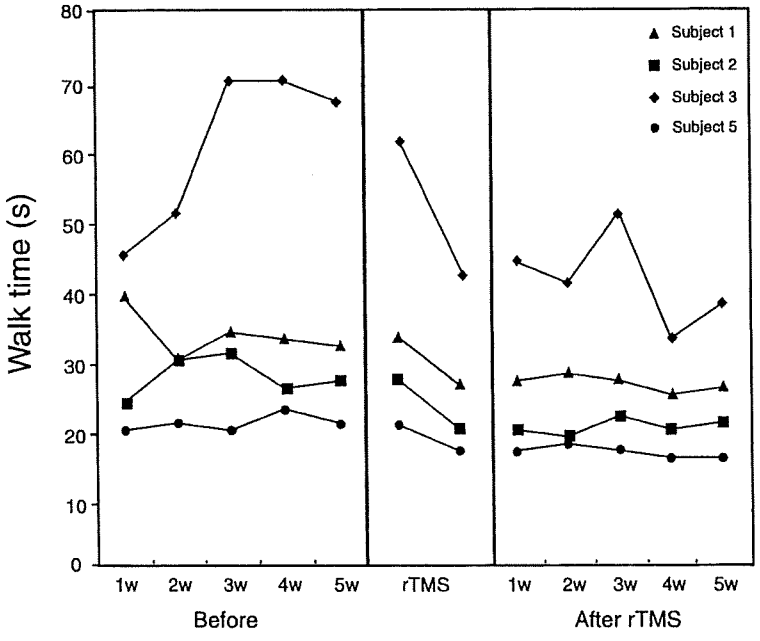

Fig. 4 Changes in 20 m walk time during the baseline,before rTMS and during follow up period, after rTMS. The straight lines in the central column represent the difference in the 20-m walk time measured pre-rTMS (mean of five measurements) and post-rTMS (mean of five measurements) in individual subjects.
In four subjects, the 20 m walk time was measured once a week for a total of five times on different days and the results were averaged. In all four subjects, the walk time after rTMS was significantly shorter.

Table 3 UPDRS before and after rTMS

Subject	1	2	3	4	5	6
rTMS	B / A	B / A	B / A	B / A	B / A	B / A
UPDRS						
(Mentation,Behavior,Mood total)	1 / 1	2 / 0	2 / 1	2 / 2	2 / 0	1 / 1
(ADL total)	21/18	9 / 7	9 / 7	13/10	10/ 5	10/ 8
Freezing When Walking	2 / 1	1 / 1	2 / 1	1 / 1	1 / 0	1 / 1
Walking	2 / 1	1 / 0	2 / 1	1 / 1	1 / 0	1 / 1
(Motor total)	35/31	14/11	29/24	28/25	18/12	19/11
Tremor at Rest						
(LUE)	2 / 2	1 / 1	1 / 1	1 / 1	0 / 0	2 / 2
(RUE)	2 / 2	2 / 2	1 / 1	1 / 1	2 / 2	0 / 0
(LLE)	1 / 1	1 / 1	0 / 0	1 / 1	0 / 0	1 / 1
(RLE)	1 / 1	1 / 1	0 / 0	1 / 1	2 / 1	0 / 0
Rigidity						
(LUE)	1 / 1	0 / 0	1 / 1	1 / 1	1 / 1	0 / 0
(RUE)	1 / 1	0 / 0	1 / 1	1 / 1	1 / 1	0 / 0
(LLE)	1 / 1	1 / 1	2 / 1	1 / 1	1 / 1	1 / 0
(RLE)	1 / 1	1 / 1	2 / 1	1 / 1	1 / 1	0 / 0
Rapid Alternating Movement (pronate and supinate hands)						
(Left)	1 / 1	0 / 0	1 / 1	1 / 1	0 / 0	1 / 1
(Right)	1 / 1	0 / 0	1 / 1	1 / 1	1 / 1	0 / 0
Posture	2 / 1	2 / 1	3 / 2	1 / 1	0 / 0	2 / 1
Gait	2 / 1	1 / 0	2 / 1	1 / 1	1 / 0	1 / 0
Body Bradykinesia/Hypokinesia	2 / 1	1 / 1	2 / 1	2 / 1	1 / 1	0 / 0

B / A, before / after; UPDRS, Unified Parkinson's Disease Rating Scale; LUE, Left Upper Extremity; RUE, Right Upper Extremity; LLE, Left Lower Extremity; RLE, Right Lower Extremity

"better body movement and smoother standing-up and movement", "more active", "more cheerful", and "more expressive". An increase in the amount of conversation, an increase in mutual understanding characteristics within daily living, and an improvement in responses to visitors were also noted, compared to the baseline period. Additionally, changes such as better hand usage while eating and better sleep were also observed.

DISCUSSION

(1) EEG

In the present study, continuous monitoring did not reveal any EEG abnormalities. While high-frequency rTMS has been reported to induce convulsions in healthy subject, low-frequency rTMS does not affect the EEG pattern [20, 21]. However, slow waves have been induced by low-frequency rTMS over the right prefrontal area [22]. Therefore, when performing magnetic stimulation for long periods of time, changes in EEG monitoring must be carefully observed [23, 24, 25] to confirm the safety of this procedure. No safety problems were noted in the present study.

(2) Changes in neuropsychological tests, assessments of ADL, UPDRS, and 20 m walk

Cognitive dysfunction occurs in Parkinson's disease at an early stage, and similarities between these patients and those with frontal lobe dysfunction have been emphasized [26, 27]. In particular, forms of cognitive dysfunction, such as reduced planning and problem solving, impaired set switching, impaired spatial working memory and visual cognitive dysfunction, have recently been reported. Furthermore, Laplane *et al.* [28] reported that the prefrontal area and basal ganglia play important roles in executive function. Cools *et al.* [29] reported that cognition and motor shifting aptitudes (ability to switch sets) are reduced in patients with Parkinson's diseases. Consequently, the core symptom for the cognitive dysfunction associated with Parkinson's disease appears to be executive dysfunction. Therefore, the present study utilized the WCST, which mainly examines concept and set switching and reaction flexibility, and the TMT-B, which is an attention-switching task. The long TMT-B execution times observed during the baseline period of this study may be one characteristic of the executive dysfunction associated with Parkinson's disease.

In addition, many subjects had discrepancies between performance and verbal tasks on the WAIS-R. In general, the total IQ in Parkinson's disease is normal, but the performance IQ is lower than the verbal IQ [30]. The results of this study also supported this finding. The WAIS-R is an intellectual function test that assesses posterior brain function [14]. By evaluating the WAIS-R, we were able to assess both anterior and posterior brain function.

In this study, the application of a low-frequency suprathreshold rTMS over bilateral dorsolateral prefrontal areas in Parkinson's disease improved not only executive function, but also motor function, subjective symptoms and objective findings. Although no significant changes in the WAIS-R scores before and after stimulation were found, the TMT-B execution time decreased and the scores in the WCST categories improved. These results suggest that rTMS specifically improves prefrontal function, one aspect of executive function.