Original Article

Immobilization Stress Inhibits Intimal Fibromuscular Proliferation in the Process of Arterial Remodeling in Rats

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We investigated role of β-endorphin (END), which is released by immobilization stress, on intimal fibromuscular proliferation in a rat model of arterial remodeling after intimal injury. The endothelium of the abdominal aorta of Wistar-Kyoto rats was denuded, and the rats were subjected to immobilization stress (6 h/d), which raised the serum concentration of END, and intraperitoneal administration of either END (20 ng/kg/d) or naltrexone (NAL: 4 mg/kg/d). The proliferative activity (PA) of medial smooth muscle cells (SMCs) and the intima/media area ratio (*R*) were determined at 3 and 14 d after denudation, respectively. PA and *R* were significantly reduced by immobilization (PA: 64.8%, *R*: 34.6%), and NAL treatment completely reversed the decreases in PA and *R*. On the other hand, END reduced both PA and *R* (PA: 21.7% and *R*: 24.9%), and NAL also reversed the decreases in PA and *R*. END (20 pg/mL) inhibited both the proliferation (79% at 96 h) and migration (26%) of SMCs cultured with 5% fetal bovine serum *in vitro*, and NAL (100 μg/mL) reversed the inhibition of both activities. Our results suggest that immobilization stress stimulates the release of endogenous END, which then prevents both proliferation and migration of medial SMCs after intimal injury. (*Hypertens Res* 2008; 31: 977–986)

***Key Words*: mental stress, endorphin, vascular remodeling**

Introduction

Mental stress is considered an independent risk factor for hypertension and ischemic heart disease (*1–4*). For example, the stress of being depressed or of having a type-A personality has been reported to aggravate the condition of ischemic heart disease, and anger, hostility, anxiety, or exhaustion might trigger acute coronary syndrome. In addition, the progression of atherosclerosis is accelerated by mental stress in animal models of atherosclerosis (*5–7*). However, the pathophysiological linkages between mental stress and the progression of arteriosclerosis are unclear.

Smooth muscle cells (SMCs) are the major parenchymal cells of arteries. They are the targets of structural remodeling during the process of arteriosclerosis. Medial SMCs proliferate and then migrate to the intima, resulting in the production of extracellular matrix. These processes, which comprise intimal fibromuscular proliferation, are activated not only in the early response to endothelial injury but also in the formation

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of a fibrous cap around the lipid core in complicated lesions (*8–10*). Particularly in the latter case, SMC proliferation has a direct influence on the solidity of the fibrous cap, which prevents the plaque from breaking (*11*). Therefore, investigations of the influences of mental stress on SMCs are important.

In the physiological responses to mental stress, both the sympathetic nervous system and the hypothalamo-pituitary-adrenal axis are activated. Catecholamines are released and act as activating hormones to fight the external stress (*12*). α- and β-Adrenergic blockades were reported to inhibit the formation of neointima after endothelial denudation of an artery in animal models (*13*, *14*), and the β-adrenergic blockade has a protective function against myocardial ischemic events, including plaque rupture, in humans (*14*). Besides these pathways, β-endorphin (END), an endogenous opioid, is also produced in the pituitary gland; it is released into the circulating blood to act as a relaxing factor against stress (*15–17*). However, little is known about the effects of endogenous opioids on the process of arteriosclerosis. We recently reported that the administration of END or Met-enkephalin prevents intimal hyperplasia after endothelial denudation in the rat aorta (*18*). Zagon *et al*. also reported growth-inhibitory effects of Met-enkephalin on SMCs (*19*).

In this study, we investigated the comprehensive influences of mental stress on intimal fibromuscular proliferation in a rat model of arterial remodeling after endothelial injury, in connection with the action of END. In addition to *in vivo* studies, we determined the direct effects of END on both the proliferation and migration of rat cultured arterial SMC.

Methods

Endothelial Denudation of Rat Aorta and Immobilization Stress in Rats

Male Wistar-Kyoto rats ($n=179$, 350 to 430 g body weight, Sankyo Laboratories, Toyama, Japan) were fed regular chow throughout the duration of the experiments. They were anesthetized with sodium pentobarbital (50 mg/kg i.p., Dainippon Seiyaku, Tokyo, Japan). The left carotid artery was dissected and cannulated with a 2F balloon catheter (Baxter, Deerfield, USA). The abdomen was opened and the catheter was advanced to the descending aorta. Endothelial denudation was performed from 2 mm distal from the divergence of the left renal artery to 2 mm above the bifurcation of the common iliac arteries in the descending aorta by scraping with an inflated balloon three times. The first inflation was performed with 0.37 mL air, and the volume of air for the other two inflations was adjusted according to the friction resistance sensed by the operator's hand during the first scraping. Just after the scraping, naltrexone (NAL) (2 mg/kg), END (10 ng/kg), or phentolamine (10 ng/kg) + propranolol (10 ng/kg) was injected intraperitoneally with up to 0.2 mL saline. The END dose was decided in order that the END serum concentration should be the same as that in the rats after immobilization stress (group [3]). The rats completely recovered from anesthesia within 2 h. Each agent was administered at the same dose again. The rats were maintained on chow and water ad libitum. Mental stress was induced by immobilizing the body and limbs in a restricted cage (rat holder KN-325-A; Natsume, Tokyo, Japan) for 6 h. Immobilization stress was induced following administration with the agents every day until the third day after denudation, and then every other day. The rats were divided into ten groups. 1) control: endothelial denudation only (group [1]), 2) denudation + NAL (group [2]), 3) denudation + immobilization (group [3]), 4) denudation + NAL + immobilization (group [4]), 5) denudation + END (group [5]), 6) denudation + NAL + END (group [6]), 7) denudation + phentolamine + propranolol (group [7]), 8) denudation + phentolamine + propranolol + NAL (group [8]), 9) denudation + phentolamine + propranolol + immobilization (group [9]), 10) denudation + phentolamine + propranolol + NAL + immobilization (group [10]). All experimental procedures were approved by the Animal Research Committee of Kanazawa Medical University.

Measurement of Serum END Concentration

Serum END concentration was measured in another 15 rats divided into three groups ($n=5$ each). Each group was subjected to the same procedures as in group [1], [3], or [5] above. Blood was sampled 6 h after recovery from anesthesia in group [1], just after the end of immobilization in group [3], and 6 h after administration of END in group [5]. The rats were anesthetized again, the chest was opened, and blood was collected from the right atrium. The serum concentration of END was measured by radioimmunoassay (RIA).

Assessment of Proliferation: Immunohistochemical Examination of Expression of Proliferating Cell Nuclear Antigen in Medial SMC Nuclei

To estimate the proliferative activity of medial SMCs in the acute phase after injury, 3 d after denudation another 8 rats from each group were anesthetized, the chest was opened, and the whole body was perfused with phosphate buffered saline (PBS) containing heparin (2 U/mL) *via* the left ventricle. The scraped region of the abdominal aorta was fixed with 100% methanol overnight. The specimen was embedded in paraffin, sectioned longitudinally at 3 μm thickness, and mounted on a silanized slide glass. Enzyme immunohistochemical staining of proliferating cell nuclear antigen (PCNA) was performed with mouse monoclonal anti-PCNA antibody (1:100, Dako, Glostrup, Denmark) using an LSAB kit (Dako). The specimen on the slide glass was deparaffinized and immersed in 100% ethanol. Endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in 99% methanol for 15 min. The slide was then briefly immersed in PBS three times. The specimen was covered with blocking reagent, and reacted with mono-

clonal anti-PCNA antibody (1:100, Dako) overnight at 4°C. After washing with PBS three times, it was reacted with biotinylated anti-mouse IgG for 10 min. Then it was reacted with streptavidin-conjugated horseradish peroxidase. Then, 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/mL) and H_2O_2 (0.03%) were added as substrates for peroxidase, and the reaction was performed for 15 min. The specimen was dehydrated with a series of ethanol, immersed in xylene, and mounted with balsam. Three parallel longitudinal sections (about 100 μm apart) for each specimen were analyzed. The number of PCNA-positive nuclei per denuded area of tunica media for each specimen was measured using an image processing system.

Assessment of Apoptosis: TUNEL Staining in Medial SMC Nuclei

To estimate the influence of immobilization stress on the apoptosis of medial SMC 3 d after denudation, terminal deoxynucleotidyl nick-end labeling (TUNEL) was performed using an *in situ* apoptosis detection kit (Takara, Otsu, Japan). The sections made from four embedded specimens of groups [1] and [3] were deparaffinized, immersed in distilled water, and treated with 20 μg/mL proteinase K (Wako Pure Chemical Industries, Osaka, Japan) for 15 min. Endogenous peroxidase was blocked with 3% H_2O_2 in distilled water for 5 min, and the slide was briefly immersed in PBS three times. The specimens were reacted with terminal deoxynucleotidyl transferase (TdT) enzyme in labeling-safe buffer for 60 min at 37°C, followed by washing with PBS three times. They were reacted with anti-FITC HRP conjugate for 30 min at 37°C followed by washing with PBS five times. Then, 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/mL) and H_2O_2 (0.03%) were added and the reaction was performed for 15 min. Counterstaining was performed with methyl green. The specimen was dehydrated with a series of ethanol, immersed in xylene, and mounted with balsam. The number of positively stained nuclei per total number of nuclei (apoptosis index) in the denuded area of tunica media for each specimen was measured.

Detection of Neointimal Proliferation

Fourteen days after denudation, 10 rats from each of groups [1] to [6] and 6 rats from each of groups [7] to [10] were anesthetized and fixed by perfusion with 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.4, at 100 mmHg. After post-fixation for 3 d, the aortic tissue was embedded in paraffin, sectioned longitudinally at 3 μm thickness, and stained with hematoxylin-eosin.

The image processing system was applied as follows. The denuded areas (3 d after intimal injury) and neointimal areas (14 d after intimal injury) were observed under a light microscope at ×16 magnification (OPTIPHOT-2, Nikon, Tokyo, Japan). Photos of the whole denuded areas were taken with a digital camera (HC-300Zi, Nikon) and transferred to a computer (PCG505CR/P; Sony, Tokyo, Japan). Using image processing software (Scion Image; Scion Corporation, Frederick, USA), the number of PCNA-positive nuclei, the area of the media after 3 d, and the ratio of intimal area to medial area after 14 d were measured.

Culture of Rat Aortic SMCs

Normal rat aortic medial SMCs were isolated by the explant method (*20*) and cultured at 37°C in a humidified incubator with CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, A Perbio Science Company, Logan, USA) and an antibiotic-antimycotic solution (final concentrations: 100 μg/mL streptomycin, 100 U/mL penicillin G, and 0.25 μg/mL amphotericin B; Gibco BRL). Cells were used between passages 5 and 8.

Growth of SMCs

An inoculum of 5×10^5 cells was seeded into each well of a 12-well culture dish (AGC Techno Glass, Chiba, Japan) with 1.0 mL DMEM supplemented with 10% FBS. After 48 h, the culture medium was replaced by serum-free DMEM containing 0.1% BSA supplemented with insulin and transferrin (both 10 μg/mL) and incubated for 24 h. Growth-arrested confluent cells were divided into five groups of four wells each according to the treating agents. Two wells were incubated for 48 h, and the other two for 96 h. The media of the five groups were replaced by DMEM with vehicle, vehicle, NAL (100 μg/mL), END (20 pg/mL) or NAL + END. Then, FBS was added in the second to fifth groups at a final concentration of 5%. After incubation for 48 and 96 h, the cells were trypsinized and suspended in 0.5 mL DMEM containing 10% FBS. The cell numbers were determined using a Burker counter (Kayagaki-Irika, Tokyo, Japan). All measurements were performed in duplicate. The experiments were performed four times and mean values were presented.

Migration of SMCs

SMC migration was measured by a modification of Boyden's chamber method with 24-well Biocoat GFR Matrigel Invasion chambers (Becton Dickinson, Franklin Lakes, USA) and polycarbonate filters with an 8 μm pore diameter. The filters in the upper insert were precoated with basement membrane gel matrix by submerging the filter at 30°C for 3 h in DMEM containing 0.1% BSA with diluted Matrigel at 250 μg/mL. They were then dried completely at room temperature. They were rehydrated in 200 μL DMEM containing 0.1% BSA for 1 hour just before the cells were seeded. Cultured SMCs maintained in serum-free DMEM supplemented with insulin and transferrin (both 1 μg/mL) for 24 h were trypsinized, suspended in DMEM plus 10% FBS to inactivate the trypsin,

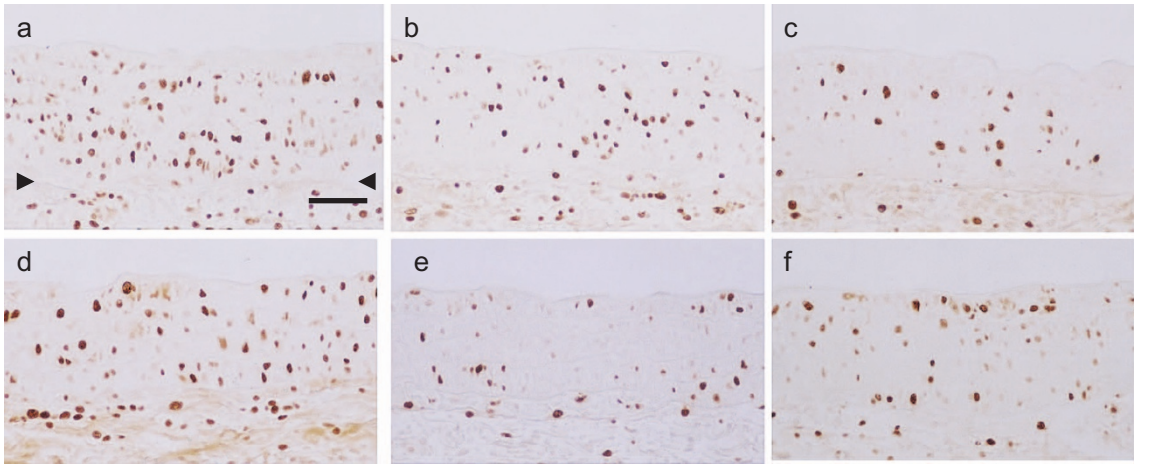

Fig. 1. *Typical examples of immunohistochemical staining for proliferating cell nuclear antigen in rat abdominal aorta 3 d after intimal injury. Arrowheads indicate the external elastic lamina. a: Artery after endothelial denudation only (group [1]). b: Artery of rat administered naltrexone (4 mg/kg) after endothelial denudation (group [2]). c: Artery of rat subjected to immobilization stress after endothelial denudation (group [3]). d: Artery of rat subjected to immobilization stress following administration of NAL after endothelial denudation (group [4]). e: Artery of rat administered β-endorphin (20 ng/kg) after endothelial denudation (group [5]). f: Artery of rat administered naltrexone and β-endorphin after endothelial denudation (group [6]). Bar = 50 μm.*

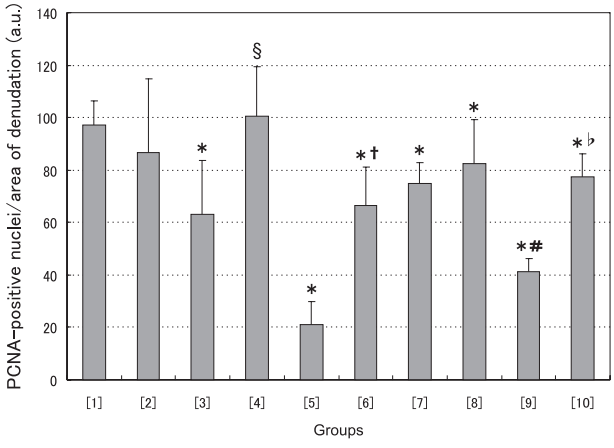

Fig. 2. *Influences of immobilization stress and administration of naltrexone (4 mg/kg) or β-endorphin (20 ng/kg) on proliferating cell nuclear antigen (PCNA) expression in medial smooth muscle cells of rat abdominal aorta 3 d after endothelial denudation. The number of PCNA-positive nuclei per area of denudation (arbitrary unit: a.u.) is shown. Data are expressed as mean ± SD (n = 6). *p < 0.05 compared with group [1]. §p < 0.05 compared with group [3]. †p < 0.05 compared with group [5]. #p < 0.05 compared with group [7]. ♭p < 0.05 compared with group [9].*

washed four times with serum-free DMEM, and suspended at a density of 1×10^6/mL in serum-free DMEM containing insulin (1 μg/mL), transferrin (1 μg/mL), and BSA (0.1%, wt/vol).

They were divided into five groups and seeded in the upper insert (each 200 μL). Vehicle, vehicle, NAL (100 μg/mL), END (20 ng/mL), or NAL + END was added, respectively. Then, FBS was added in the second to fifth groups at a final concentration of 5% as the chemoattractant in the lower compartment of each chamber (700 μL for each). The chambers were incubated at 37°C under 5% CO_2 in air for 9 h. The cells on the upper side of the filter were removed using cotton swabs, and the migratory cells on the underside were fixed with 10% formalin, then 70% ethanol, and stained with hematoxylin. The filters were removed and mounted on a slide glass with glycerin. The cells migrating to the bottom portion were counted under a microscope and expressed as a percentage of control (5% FBS group) to quantify migration. The data were averaged between the two wells. The experiments were performed six times independently.

Statistical Analysis

All results were expressed as means ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's multiple tests. Data analysis was performed on a microcomputer running SPSS version 8.0 (SPSS, Chicago, USA). A p value less than 0.05 was considered to indicate a significant difference.

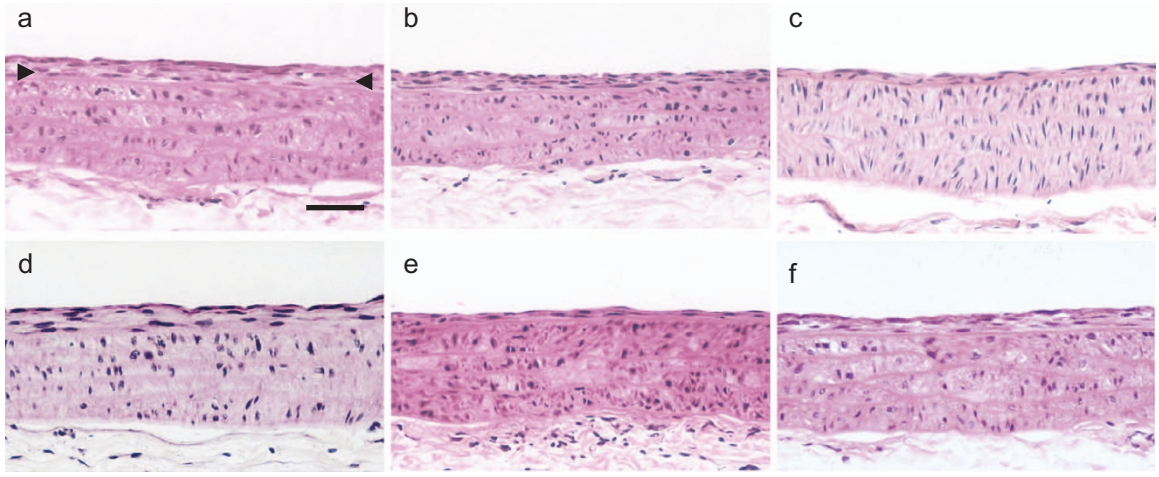

Fig. 3. *Typical examples of neointima formation of rat abdominal aorta 14 d after intimal injury (hematoxylin and eosin staining). Arrowheads indicate the internal elastic lamina. a: Artery after endothelial denudation only (group [1]). b: Artery of rat administered naltrexone (4 mg/kg) after endothelial denudation (group [2]). c: Artery of rat subjected to immobilization stress after endothelial denudation (group [3]). d: Artery of rat subjected to immobilization stress following administration of naltrexone after endothelial denudation (group [4]). e: Artery of rat administered β-endorphin (20 ng/kg) after endothelial denudation (group [5]). f: Artery of rat administered naltrexone and β-endorphin after endothelial denudation (group [6]). Bar = 50 μm.*

Results

Serum END Concentration

The serum END concentrations in the rats in groups [3] and [5] were 20.0±1.4 and 18.7±2.7 pg/mL, respectively, which were significantly higher than that in group [1] (10±0.6 pg/mL) ($p<0.05$).

PCNA Immunoreactivity

Figure 1 shows the immunohistochemical staining of PCNA 3 d after endothelial denudation. In the control group [1], there were abundant labeled nuclei scattered in the tunica media of the aorta (Fig. 1a), indicating that a considerable number of medial SMCs were in a proliferative state. Figure 2 shows the number of labeled nuclei per area of the media (PA) in each group. PA was 97.1±3.3 (number/pixel: arbitrary unit [a.u.]) in group [1]. PA was significantly reduced by immobilization (62.9±7.31: $p<0.01$: [3] *vs*. [1]), indicating that immobilization stress had an inhibitory effect on SMC proliferative activity. NAL completely reversed this effect (100.6±6.70: $p<0.01$: [4] *vs*. [3], not significant: [4] *vs*. [1]), suggesting that the inhibitory effect of immobilization stress on SMC proliferative activity occurred through a μ-subtype opioid receptor–mediated mechanism. NAL itself had no significant influence (86.8±9.9: not significant: [2] *vs*. [1]). Moreover, END significantly reduced PA (21.1±3.05: $p<0.01$: [5] *vs*. [1]), and NAL partially restored it (66.6±5.09: not significant: [6] *vs*. [1]). The pharmacological blockade of sympathetic activity significantly reduced PA (74.6±2.96: $p<0.01$: [7] *vs*. [1]), and even under this blockade, immobilization significantly reduced PA (41.3±1.79: $p<0.01$: [9] *vs*. [7]). NAL also completely reversed the decrease in PA induced by immobilization (77.5±3.08: $p<0.01$: [10] *vs*. [9], not significant: [10] *vs*. [7]).

Apoptosis in Media

Three days after endothelial denudation, we observed a few TUNEL-positive nuclei in the medial SMC layers. The percentage of apoptosis was 2.2 in the total nuclei (*1*), which was significantly increased by immobilization (7.3±4.6 *vs*. 2.2±1.6: $p<0.05$: [3] *vs*. [1]), indicating that immobilization stress exaggerated apoptosis of the medial SMCs.

Neointima Formation

Fourteen days after endothelial denudation, neointima was observed on the luminal side of the tunica media bounded by the internal elastic lamina (Fig. 3a). In sham-operated rats ($n=3$), only the endothelial cell layer was observed in the intima (data not shown). Figure 4 shows the area ratio of the intima to media (*R*) 14 d thereafter. *R* was 0.185±0.012 in control rats. *R* was significantly reduced by immobilization (0.064±0.008: $p<0.01$: [3] *vs*. [1]), indicating that immobilization stress had an inhibitory effect on SMC proliferation and migration, and prevented the formation of neointima. This effect was completely reversed by NAL (0.161±0.022: not significant: [4] *vs*. [1]), suggesting that the inhibitory effect of immobilization stress on the formation of neointima was through a μ-opioid

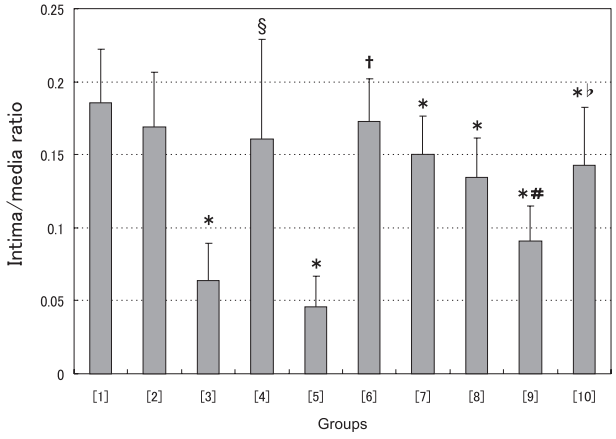

Fig. 4. *Influences of immobilization stress and administration of naltrexone (4 mg/kg) or β-endorphin (20 ng/kg) on neointima formation in rat abdominal aorta 14 d after endothelial denudation. The ratio of intimal to medial area is shown. Data are expressed as mean±SD (n=10 in groups [1] to [6], n=6 in groups [7] to [10]). *p<0.05 compared with group [1]. §p<0.05 compared with group [3]. †p<0.05 compared with group [5]. #p<0.05 compared with group [7]. bp<0.05 compared with group [9].*

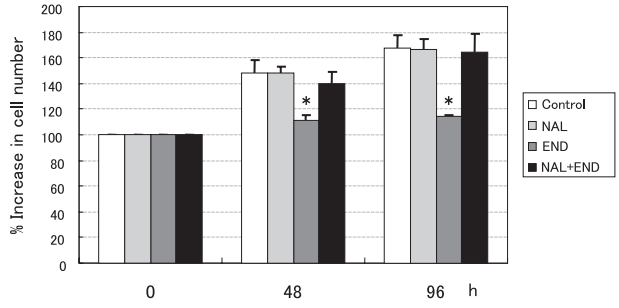

Fig. 5. *Effect of β-endorphin (END) on growth of rat cultured smooth muscle cells. The cells were divided into four groups (5% fetal bovine serum [FBS] as control, 5% FBS with naltrexone [NAL], 5% FBS with END, 5% FBS with NAL + END). The cell number at 0 h was 4.4×10^5 per well. The stimulation of growth induced by 5% FBS is shown as the percentage of the number at 0 h. Each experiment was performed in duplicate, and four independent experiments were performed. Data are expressed as mean±SD (n=4). *p<0.05 compared with control.*

receptor–mediated mechanism. NAL itself had no significant influence (0.169±0.012: not significant: [2] *vs*. [1]). Moreover, END itself reduced *R* (0.046±0.007: $p<0.01$: [5] *vs*. [1]), and *R* was also completely restored by NAL (0.172±0.009: not significant: [6] *vs*. [1]). The pharmacological blockade of sympathetic activity had little effect (0.178±0.021: not significant: [7] *vs*. [1]). Even under this blockade, immobilization significantly reduced *R* (0.091±0.010: $p<0.01$: [9] *vs*. [7]) and NAL also completely reversed the immobilization-induced decrease in *R* (0.143±0.002: $p<0.05$: [10] *vs*. [9], not significant: [10] *vs*. [7]).

Growth of SMCs

To investigate END's effect on SMC growth, we determined the increase in the number of cultured SMCs. In the group without FBS, after both 48 h and 96 h, the cell number was the same as that at 0 h. Figure 5 shows the percentage increases in cell number in the other four groups. In the control group, 5% FBS significantly increased the cell number (148±10% after 48 h and 167±11% after 96 h). NAL did not have a significant effect on the increase in cell number by FBS (148±5% after 48 h and 166±8% after 96 h). On the other hand, END significantly prevented the increase (111±4% after 48 h and 114±1% after 96 h, both $p<0.05$ compared with control). Moreover, NAL reversed the prevention by END (140±9% after 48 h and 164±15% after 96 h, both $p<0.05$ compared with the END group and not significant compared with control).

Migration of SMCs

To investigate END's effect on SMC migration, we determined the number of migrated cells by a modified Boyden's chamber method. Without FBS, very few SMCs migrated, whereas 5% FBS significantly stimulated migration after 9 h (control). Figure 6 shows the migrated cell numbers in the four groups expressed as percentages of control. The migration induced by 5% FBS was not significantly influenced by NAL (99.5±1.7%), but it was significantly prevented by END (74.3±2.0% of control, $p<0.01$). Moreover, NAL reversed the prevention by END (105.8±7.0% of control).

Discussion

In this study, we investigated immobilization stress's influence on fibromuscular proliferation of the arterial wall after endothelial denudation in rats. Immobilization stress inhibited both the proliferation of arterial SMCs in the acute phase of intimal injury and neointima formation in the chronic phase. These effects of stress were antagonized by NAL, a preferential μ-opioid receptor antagonist. Moreover, immobilization raised the serum concentration of END, and exogenously administered END mimicked the effects of immobilization stress. An *in vitro* study also suggested that END has a direct inhibitory action on both the proliferation and migration of SMCs.

Medial SMCs play a major role in neointimal hyperplasia after endothelial denudation. This process requires two characteristic features of their differentiation status: proliferation and migration. Some medial SMCs undergo DNA synthesis and migrate to the intima, where they continue the prolifera-

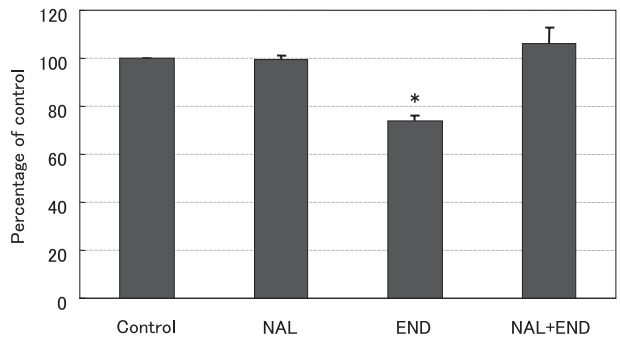

Fig. 6. *Effect of β-endorphin (END) on migration of rat cultured smooth muscle cells. The cells were divided into four groups (5% fetal bovine serum [FBS] as control, 5% FBS with naltrexone [NAL], 5% FBS with END, 5% FBS with NAL + END). The cell number in the upper well was 2×10^5/mL. In each group, the migration to the underside induced by 5% FBS is shown as a percentage of control. Each experiment was performed in duplicate, and six independent experiments were performed. Data are expressed as mean±SD ($n = 6$). *$p < 0.05$ compared with control.*

tion process. Another population of medial SMCs just migrates without DNA synthesis (*21*, *22*). Therefore, the results of the present study suggest that SMCs have functional μ-opioid receptors whose signal transduction pathway interferes with both proliferation and migration. Although SMCs have been reported to possess μ-opioid receptors, their function was to modify SMC contraction through an increase in the intracellular Ca^{2+} concentration (*23*, *24*). In other cell types, END modifies the cell biology through μ-opioid receptors. END is shown to inhibit the proliferation of embryonic stem cells and to induce differentiation to neural progenitor cells through the G-protein–coupled extracellular-regulated kinase pathway (*25*). END also suppresses the migration of cytotoxic T cells through the G-protein–coupled adenylate cyclase pathway (*26*). Therefore, there is a possibility that END inhibited the proliferation and migration of SMCs through μ-opioid receptor–mediated subcellular signal transduction systems.

There have been no reports that immobilization stress influenced the function of SMCs. We can consider many pathways in which immobilization stress might affect the function of SMCs. First, stress is recognized in the central nervous system, and the responses are transmitted by several hormones and cytokines, which are accepted by several types of cells. Catecholamines are released from nerve endings to the peripheral tissue and from adrenal medulla to circulating blood. They can directly stimulate the proliferation of arterial SMCs (*27*). They also increase blood pressure and heart rate, thereby increasing shear stress and producing endothelial damage. Adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) (*28*, *29*) and vascular cell adhesion molecule-1 (VCAM-1), are up-regulated (*30–32*). Circulating inflammatory cells (monocytes and lymphocytes) become able to bind to the surface of the endothelium and translocate into the subendothelial space. Catecholamines activate macrophages originating from the invading monocytes and produce proinflammatory cytokines (*e.g.*, interleukin [IL]-1, IL-6, and tumor necrosis factor-α [TNF-α]) and other cytokines (*e.g.*, interferon-γ [INF-γ]), both of which are capable of influencing the functions of SMCs, leading to the deterioration of arteriosclerosis (*33*, *34*). Therefore, catecholamines indirectly stimulate the proliferation of arterial SMCs. Immobilization stress also stimulates the release of corticosterone and END. The influences of these stress hormones on the fibromuscular proliferation of arterial smooth muscles have never been examined. The results of our study showed the "total" effects of catecholamines and these stress hormones, whose functional directions are different.

The macrophages in atherosclerotic plaque are activated through those mechanisms and bring about plaque rupture in acute coronary syndrome. The fibrous cap surrounding the plaque consists of fibromuscular tissue, which is strengthened by SMCs. Therefore, inhibition of SMC proliferation through mental stress might also have a deteriorating influence on the solidity of the fibrous cap (*11*). The significance of fibromuscular proliferation during the process of arteriosclerosis in humans has effects on both sides. One effect is aggravating: neointimal formation leads to a decrease in the arterial inner diameter and induces ischemia of organs. This process is clearly reflected in restenosis after percutaneous transluminal coronary angioplasty. Another effect is protective: thickening of the fibrous cap surrounding the lipid core in complicated lesions, which protects the plaque from rupture (*35*).

Therefore, according to our finding that opioids inhibit neointima formation, opioids have either protective or aggravating effects on arteriosclerotic lesions.

We discuss the possibilities that released END may influence the function of the other cell types and indirectly modify the activities of medial SMCs. Psychoneuro-immunological studies have shown that mental stress can modify the functions of monocytes and lymphocytes (*36*, *37*). Mental stress stimulates the release of endogenous opioids and catecholamines, which activate nuclear factor-κB (NF-κB) of these cells, produce nitric oxide, free radicals, and proinflammatory cytokines, and upregulate both chemotaxic and phagocytotic functions (*38–42*). Also, in splenic lymphocytes, immobilization stress inhibits proliferation and induces apoptosis through a μ-opioid receptor–mediated mechanism (*43*). Therefore, we cannot completely exclude the possibility that the inhibition of SMC proliferation observed in our experiments *in vivo* was mediated by μ-opioid receptors on mononuclear cells (*44*). In this case, two mechanisms can be considered. One is that the mononuclear cells invaded the subintimal regions and released cytokines that stimulated the proliferation and migration of medial SMCs (*34*, *35*). The other is that mononuclear cells themselves transdifferentiated

into myofibroblasts and became components of the neointima (*45*, *46*). There is also evidence that the circulating END becomes the pathogenesis of stress-induced endothelial dysfunction. In this case μ-opioid receptors on human monocytes and endothelial cells mediated both the stimulation of endothelin-1 release and the suppression of nitric oxide release (*47*). In our experiments, endothelial denudation was complete and reendothelialization was not observed until at least 48 h after denudation, which was examined by the Evans-blue extraction method (data not shown) (*48*, *49*). Therefore, the influences on SMCs could not be through the opioid receptor on endothelial cells.

Recent studies revealed that apoptosis is induced by balloon distension injury and may contribute to medial SMC loss (*50*). In our experiments, only 2.2% of medial SMCs underwent apoptosis by endothelial denudation. Immobilization stress after denudation significantly exaggerated apoptosis, but the amount was only 7.3%. Therefore, the exaggeration of apoptosis was not the major etiology of immobilization stress's anti-proliferating activity, as assessed by PCNA immunohistochemistry.

Moreover, recent studies on the origin of neointimal SMCs suggest that adventitial myofibroblasts (*51*) or circulating progenitor cells originating from bone marrow (*52*) migrate into the intima and differentiate after endothelial denudation. In our study, we cannot exclude the possibility that these cell types influence, because it has not been clarified whether such cells have μ-opioid receptors. However, during our acute phase examination of the proliferating activities in arterial wall using PCNA immunohistochemistry, positive cells were positioned in the media layers and the cell linings were maintained. If the progenitor cells had invaded and proliferated, the media structure should have been disarranged. Moreover, on the third day after denudation, the progenitor cells had not fully been recruited in the media (*53*, *54*). Thus, the PCNA-positive cells were thought to be pre-existing medial SMCs. On the other hand, in neointima formation during the chronic phase, it cannot be denied that END's inhibitory effect was due to the inhibition of progenitor cell activities.

Neointima formation after intimal injury, as shown in this study, can be considered a "wound healing" of arteries. There are many recent reports that psychological stress delays wound healing of the skin (*55*). Stress is known to inhibit IL-1 production, which decreases the infiltration of polymorphonuclear leukocytes (*56*). Although the types of target cell and mediator(s) may be different from those in our system, the phenomenon has a similar meaning in the living body.

Administration of the opioid antagonist NAL after intimal injury *in vivo* did not enhance SMC proliferation. If endogenous opioids play a physiological role, it can be expected that NAL would inhibit their effects and restore SMC proliferative activity. One of the reasons for the contradictory results may be that the concentration of END in serum has a threshold of inhibitory action. We found a serum END concentration of 10.0±0.6 pg/mL (n=3) in anesthetized rats, which did not significantly change during 3 d after endothelial denudation. On the other hand, the concentration measured in rats during END administration using an osmotic pump was 18.7±2.7 pg/mL (n=3). Moreover, the concentration measured in rats just after 6 h of immobilization was 20.0±1.4 pg/mL (n=4). Therefore, the threshold may be approximately between 13 and 15 pg/mL.

References

1. Kaushik RM, Mahajan SK, Rajesh V, Kaushik R: Stress profile in essential hypertension. *Hypertens Res* 2004; **27**: 619–624.
2. Harada K, Karube Y, Saruhara H, Takeda K, Kuwajima I: Workplace hypertension is associated with obesity and family history of hypertension. *Hypertens Res* 2006; **29**: 969–976.
3. Hemingway H, Marmot M: Evidence based cardiology: psychosocial factors in the aetiology and prognosis of coronary heart disease. Systematic review of prospective cohort studies. *BMJ* 1999; **318**: 1460–1467.
4. Linden W, Stossel C, Maurice J: Psychosocial interventions for patients with coronary artery disease. A meta-analysis. *Arch Intern Med* 1996; **156**: 745–752.
5. Kaplan JR, Manuck SB, Clarkson TB, Lusso FM, Taub DM, Miller EW: Social stress and atherosclerosis in normocholesterolemic monkeys. *Science* 1983; **220**: 733–735.
6. McCabe PM, Gonzales JA, Zaias J, *et al*: Social environment influences the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Circulation* 2002; **105**: 354–359.
7. Caligiuri G, Levy B, Pernow J, Thoren P, Hansson GK: Myocardial infarction mediated by endothelin receptor signaling in hypercholesterolemic mice. *Proc Natl Acad Sci U S A* 1999; **96**: 6920–6924.
8. Ross R: The pathogenesis of atherosclerosis: a perspective for 1990s. *Nature* 1993; **362**: 801–809.
9. Fuster V, Badimon L, Badimon JJ, Chesebro JH: The pathogenesis of coronary artery disease and the acute coronary syndromes. *N Engl J Med* 1992; **326**: 242–250.
10. Kishi K, Muramatsu M, Jin D, *et al*: The effects of chymase on matrix metalloproteinase-2 activation in neointimal hyperplasia after balloon injury in dogs. *Hypertens Res* 2007; **30**: 77–83.
11. Falk E: Why do plaques rupture? *Circulation* 1992; **86** (6 Suppl): III30–III42.
12. Murakami E, Matsuzaki K, Sumimoto T, Mukai M, Kazatani Y, Kodama K: Clinical significance of pressor responses to laboratory stressor testing in hypertension. *Hypertens Res* 1996; **19**: 133–137.
13. Vashisht R, Sian M, Franks PJ, O'Malley MK: Long-term reduction of intimal hyperplasia by the selective alpha-1 adrenergic antagonist doxazosin. *Br J Surg* 1992; **79**: 1285–1288.
14. Ablad B, Bjorkman JA, Gustafsson D, Hansson G, Ostlund-Lindqvist AM, Pettersson K: The role of sympathetic activity in atherogenesis: effects of β-blockade. *Am Heart J* 1988; **116**: 322–327.
15. Lenz HJ: Regulation of duodenal bicarbonate secretion dur-

ing stress by corticotropin-releasing factor and β-endorphin. *Proc Natl Acad Sci U S A* 1989; **86**: 1417–1420.
16. Iglesias T, Montero S, Otero MJ, Parra L, Fuentes JA: Preproenkephalin RNA increases in the hypothalamus of rats stressed by social deprivation. *Cell Mol Neurobiol* 1992; **12**: 547–555.
17. Fontana F, Bernardi P, Pich EM, *et al*: Opioid peptide modulation of circulatory and endocrine response to mental stress in humans. *Peptides* 1997; **18**: 169–175.
18. Kawanishi K, Iwai K, Matsumoto M: Endogenous opioids prevent intimal hyperplasia after endothelial injury in rat aorta. *J Kanazawa Med Univ* 2001; **26**: 162–169.
19. Zagon IS, Essis FM Jr, Verderame MF, Healy DA, Atnip RG, Mclaughlin PJ: Opioid growth factor inhibits intimal hyperplasia in balloon-injured rat carotid artery. *J Vasc Surg* 2003; **37**: 636–643.
20. Ross R: The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J Cell Biol* 1971; **50**: 172–186.
21. Clowes AW, Reidy MA, Clowes MM: Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest* 1983; **49**: 327–333.
22. Casscells W: Migration of smooth muscle and endothelial cells: critical events in restenosis. *Circulation* 1992; **86**: 723–729.
23. Parra L, Perez-Vizcaino F, Alsasua A, Martin MI, Tamargo J: μ- and δ-Opioid receptor–mediated contractile effects on rat aortic vascular smooth muscle. *Eur J Pharmacol* 1995; **277**: 99–105.
24. Kai L, Wang ZF, Shi YL, Liu LM, Hu DY: Opioid receptor antagonists increase $[Ca^{++}]_i$ in rat arterial smooth muscle cells in hemorrhagic shock. *Acta Pharmacol Sin* 2004; **25**: 395–400.
25. Kim E, Clark AL, Kiss A, *et al*: μ- and κ-Opioids induce the differentiation of embryo stem cells to neural progenitors. *J Biol Chem* 2006; **281**: 33749–33760.
26. Entschladen F, Bastian P, Niggemann B, Zaenker KS, Lang K: Inhibition of cell migration *via* G protein–coupled receptors to opioid peptides and angiotensin. *Ann N Y Acad Sci* 2004; **1028**: 320–328.
27. Zhang H, Faber JE: Trophic effect of norepinephrine on arterial intima-media and adventitia is augmented by injury and mediated by different alpha 1–adrenoceptor subtypes. *Circ Res* 2001; **89**: 815–822.
28. Rehman J, Millis PJ, Carter SM, Chou J, Thomas J, Maisel AS: Dynamic exercise leads to an increase in circulating ICAM-1: further evidence for adrenergic modulation of cell adhesion. *Brain Behav Immun* 1997; **11**: 343–351.
29. Mills PJ, Dimsdale JE: The effects of acute psychologic stress on cellular adhesion molecules. *J Psychosom Res* 1996; **41**: 49–53.
30. O'Malley T, Ludlam CA, Riemermsa RA, Fox KA: Early increase in levels of soluble inter-cellular adhesion molecule-1 (sICAM-1); potential risk factor for the acute coronary syndromes. *Eur Heart J* 2001; **22**: 1226–1234.
31. Mizia-Stec K, Zahorska-Markiewicz Z, Mandecki T, Janowska J, Szulc A, Jastrzebska-Maj E: Serum levels of adhesion molecules in patients with coronary artery disease. *Int J Cardiol* 2002; **83**: 143–150.
32. Soeki T, Tamura Y, Shinohara H, Sakabe K, Onose Y, Fukuda N: Increased soluble platelet/endothelial cell adhesion molecule-1 in the early stages of acute coronary syndromes. *Int J Cardiol* 2003; **90**: 261–268.
33. Libby P: Molecular bases of the acute coronary syndromes. *Circulation* 1995; **91**: 2844–2850.
34. Ross R: Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999; **340**: 115–126.
35. Weissberg PL, Clesham GJ, Bennett MR: Is vascular smooth muscle cell proliferation beneficial? *Lancet* 1996; **347**: 305–307.
36. Black PH, Garbutt LD: Stress, inflammation and cardiovascular disease. *J Psychosomatic Res* 2002; **52**: 1–23.
37. Gidron Y, Gilutz H, Berger R, Huleihel M: Molecular and cellular interface between behavior and acute coronary syndromes. *Cardiovasc Res* 2002; **56**: 15–21.
38. Fricchione GL, Bilfinger TV, Hartman A, Liu Y, Stefano GB: Neuroimmunologic implications in coronary artery disease. *Adv Neuroimmunol* 1996; **6**: 131–142.
39. Persoons JH, Schornagel K, Breve J, Berkenbosch F, Kraal G: Acute stress affects cytokines and nitric oxide production by alveolar macrophages differently. *Am J Respir Crit Care Med* 1995; **152**: 619–624.
40. Shapira L, Frolov I, Halabi A, Ben-Nathan D: Experimental stress suppresses recruitment of macrophages but enhanced their *P. gingivalis* LPS-stimulated secretion of nitric oxide. *J Periodontol* 2000; **71**: 476–478.
41. de Wazieres B, Harraga S, Spehner V, *et al*: Effect of an auditory stress on peritoneal and alveolar cells in C57 BL/6J mice of advanced age. *Luminescence* 2000; **15**: 233–237.
42. Bierhaus A, Wolf J, Andrassy M, *et al*: A mechanism converting psychosocial stress into mononuclear cell activation. *Proc Natl Acad Sci U S A* 2003; **100**: 1920–1925.
43. Wang J, Charboneau R, Barke RA, Loh HH, Roy S: μ-Opioid receptor mediates chronic restraint stress–induced lymphocyte apoptosis. *J Immunol* 2002; **169**: 3630–3636.
44. Roy S, Loh HH: Effects of opioids on the immune system. *Neurochem Res* 1996; **21**: 1375–1386.
45. Hancock WW, Adams DH, Wyner LR, Sayegh MH, Karnovsky MJ: $CD4^+$ mononuclear cells induce cytokine expression, vascular smooth muscle cell proliferation, and arterial occlusion after endothelial injury. *Am J Pathol* 1994; **145**: 1008–1014.
46. Bayes-Genis A, Campbell JH, Carlson PJ, Holmes DR Jr, Schwartz RS: Macrophages, myofibroblasts and neointimal hyperplasia after coronary artery injury and repair. *Atherosclerosis* 2002; **163**: 89–98.
47. Wilbert-Lampen U, Trapp A, Barth S, Plasse A, Leistner D: Effects of beta-endorphin on endothelial/monocytic endothelin-1 and nitric oxide release mediated by mu1-opioid receptors: a potential link between stress and endothelial dysfunction? *Endothelium* 2007; **14**: 65–71.
48. Lindner V, Reidy MA, Fingerle J: Regrowth of arterial endothelium. Denudation with minimal trauma leads to complete endothelial cell growth. *Lab Invest* 1989; **61**: 556–563.
49. Chen D, Asahara T, Krasinski K, *et al*: Antibody blockade of thrombospondin accelerates reendothelialization and reduces neointima formation in balloon-induced rat carotid artery. *Circulation* 1999; **100**: 849–854.

50. Holm AM, Anderson CB, Hauns S, Hansen PR: ACE-inhibition promotes apoptosis after balloon injury of rat carotid arteries. *Cardiovasc Res* 2000; **45**: 777–782.
51. Shi Y, O'Brien Jr JE, Fard A, Mannion JD, Wang D, Zalewski A: Adventitial myofibroblasts contribute to neointimal formation in injured porcine coronary arteries. *Circulation* 1996; **94**: 1655–1664.
52. Religa P, Bojakowski K, Maksymowicz M, *et al*: Smooth-muscle progenitor cells of bone marrow origin contribute to the development of neointimal thickenings in rat aortic allografts and injured rat carotid arteries. *Transplantation* 2002; **74**: 1310–1315.
53. Zernecke A, Schober A, Bot I, *et al*: SDF-1α/CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. *Circ Res* 2005; **96**: 784–791.
54. Massberg S, Konrad I, Schurzinger K, *et al*: Platelet secrete stromal cell-derived factor 1α and recruit bone marrow–derived progenitor cells to arterial thrombi *in vivo*. *J Exp Med* 2006; **203**: 1221–1233.
55. Kiecolt-Glaser JK, Page GG, Marucha PT, MacCallum RC, Glaser R: Psychological influences on surgical recovery. Perspectives from psychoneuroimmunology. *Am Psychologist* 1998; **53**: 1209–1218.
56. Kiecolt-Glaser JK, Marucha PT, Malarkey WB, Mercado AM, Glaser R: Slowing of wound healing by psychological stress. *Lancet* 1995; **346**: 1194–1196.