Activation of the renin-angiotensin system in mice aggravates mechanical loading-induced knee osteoarthritis

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Abstract

Epidemiological studies have shown an association between hypertension and knee osteoarthritis (OA). The purpose of this study was to investigate whether activation of the renin–angiotensin system (RAS) can aggravate mechanical loading-induced knee OA in mice. Eight-week-old male Tsukuba hypertensive mice (THM) and C57BL/6 mice were divided into four groups: i) running THM group, ii) running C57BL/6 mice group, iii) non-running THM group, and iv) non-running C57BL/6 mice group. Mice in the running group were forced to run (25 m/min, 30 min/day, 5 days/week) on a treadmill. All mice in the four groups (n=10 in each group) were euthanized after 0, 2, 4, 6, or 8 weeks of running or natural breeding. Cartilage degeneration in the left knees was histologically evaluated using the modified Mankin score. Expression of Col X, MMP-13, angiotensin type 1 receptor (AT1R), and AT2R was examined immunohistochemically. To study the effects of stimulation of the AT1R in chondrocytes by mechanical loading and/or Angiotensin II (AngII) on transduction of intracellular signals, phosphorylation levels of JNK and Src were measured in bovine articular chondrocytes cultured in three-dimensional agarose scaffolds. After 4 weeks, the mean Mankin score for the lateral femoral condylar cartilage was significantly higher in the THM running group than in the C57BL/6 running group and non-running groups. AT1R and AT2R expression was not detected at 0 weeks in any group but was noted after 4 weeks in the THM running group. AT1R expression was also noted at 8 weeks in the C57BL/6 running group. The expression levels of AT1R, COL X, and MMP-13 in chondrocytes were significantly higher in the THM running group than in the control groups. Positive significant correlations were noted between the Mankin score and the rate of AT1R-immunopositive cells, between the rates of AT1R- and Col X-positive cells, and between the rates of AT1R- and AT2R-positive cells. The phosphorylation level of JNK was increased by cyclic compression loading or addition of AngII to the cultured chondrocytes and was reversed by pretreatment with an AT1R blocker. A synergistic effect on JNK phosphorylation was observed between compression loading and AngII addition. Transgene activation of renin and angiotensinogen aggravated mechanical load-induced knee OA in mice. These findings suggest that AT1R expression in chondrocytes is associated with early knee OA and plays a role in the progression of cartilage degeneration. The RAS may be a common molecular mechanism involved in the pathogenesis of hypertension and knee OA.

Introduction

Since Tigerstedt and Bergman first identified renin in 1898,[1] the renin–angiotensin system (RAS) has been investigated extensively and is now known to be an important regulatory system involved in maintenance of blood pressure and fluid homeostasis.[2] Abnormal activation of the RAS induces hypertension, and RAS inhibitors such as angiotensin-converting enzyme (ACE) inhibitors and angiotensin II (AngII) receptor blockers (ARB) are used for treatment of hypertension.[3] AngII, the final ligand of the RAS, is thought to act mainly through the AngII type 1 receptor (AT1R). Although the functions of the AngII type 2 receptor (AT2R) are not understood clearly, these two receptors appear to have opposite functions.[4,5] Recently, the RAS has been reported to operate locally by exerting distinct biological actions in each organ; this aspect of the RAS is referred to as "local RAS".[6] In bone tissue, osteoblasts and osteoclasts express AT1R in cell cultures,[7] and AT2R blockade increases bone mass.[8] However, there is little information about the function of the local RAS in articular chondrocytes and cartilage.

Some epidemiological studies have shown an association between hypertension and knee osteoarthritis (OA) and have suggested that hypertension is an independent risk factor for knee OA.[9–11] A Japanese epidemiological study (the Research on Osteoarthritis/Osteoporosis Against Disability Study) has also shown that hypertension is associated with the development and progression of knee OA.[12] However, it is unclear how hypertension is involved in the pathophysiology of knee OA.

Recently, RAS components, including AT1R, AT2R, ACE1, and angiotensinogen (ANG), have been reported to be expressed in hypertrophic chondrocytes in the epiphyseal plates of adult mice but not in normal articular chondrocytes.[13] It has also been demonstrated *in vitro* that the RAS can modulate hypertrophic differentiation of chondrocytes.[14] Furthermore, activation of AT1R by AngII has well-known potent proinflammatory functions in many tissues.[15] Considering these findings and that hypertrophic differentiation of chondrocytes is considered to be a key process of cartilage degeneration, we hypothesized that the local RAS may be involved in degeneration of articular cartilage through the AT1R.[16,17]

The purpose of this study was to investigate whether activation of the local RAS is involved in the progression of mechanical loading-induced knee OA. We used Tsukuba hypertensive mice (THM), which are transgenic mice that carry the human renin and ANG genes.[18–20] We histologically evaluated the development and progression of knee OA in the THM induced by mechanical loading and immunohistochemically investigated the changes in the expression of AT1R, AT2R, type X collagen

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(Col X) and matrix metalloproteinase-13 (MMP-13) with the development and progression of OA, because MMP-13 (a representative proteolytic enzyme for type II collagen) and Col X (a standard biomarker of hypertrophic differentiation of chondrocytes) are known to be expressed in the process of the cartilage degeneration.[16,17] Because AT1R belongs to the G-protein coupling receptor family, we also measured phosphorylation levels of c-Jun N-terminal kinase (JNK) as an indicator of G-protein-dependent pathway and those of Src as an indicator of G-protein-independent pathway in bovine articular chondrocytes cultured in three-dimensional agarose scaffolds after application of mechanical loading and/or AngII addition.

Materials and Methods

In vivo study

Animals

Eight-week-old C57BL/6 male mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and used as control animals. Eight-week-old male THM were used as the study animals and were supplied by the RIKEN Bio Resource Center (Rikagaku Kenkyusyo, Tsukuba, Japan) with the authorization of A. Fukamizu.[18–20] The THM are hypertensive transgenic mice that carry the human renin and human ANG gene on a C57BL/6 mouse genetic background. In the THM, human ANG is cleaved to AngI by human renin. AngI is molecularly identical in humans and mice. Therefore, the THM produce large amounts of AngII, and the serum concentrations in this mouse are four to five times higher than in wild-type mice. As a result, blood pressure in the THM is 30 to 40 mmHg higher than in wild-type mice.[18] All mice were reared in a standard environment. All experiments were conducted according to the guidelines of the Animal Welfare Committee of Kindai University Hospital (Approval No: KAME-24-039).

Forced running to induce mouse knee OA

The THM and C57BL/6 mice were divided into four groups: i) running THM group, ii) running C57BL/6 mice group, iii) non-running THM group, and iv) non-running C57BL/6 mice group. Total number of mice used in the study was fifty in each group. The mice of each groups were divided into five subgroups (euthanatized at 0, 2, 4, 6 and 8 weeks, n=10 in each subgroup). We used electronic stimulation to force mice in the running mice groups to run on a treadmill (Melquest, Toyama, Japan).

The running protocol was as follows. Mice were forced to run 25 m/min for 30 min/day on 5 days/week. The total distance run was 7.5 km after 2 weeks, 15.0 km after 4 weeks, 22.5 km after 6 weeks and 30.0 km after 8 weeks. This protocol was devised based on a mouse running protocol to induce knee OA using a treadmill proposed by Lapveteläinen *et al*.[21] In the course of this forced running (for a maximum of 8 weeks), two THM died after 6 weeks of running, and two THM and one C57/BL6 mouse died after 7 weeks of running from unknown causes. Mice of the non-running THM and C57BL/6 group remained in their cage.

Measurement of mouse body weight

Each mouse was weighed after 0, 2, 4, 6, and 8 weeks of running or natural breeding, using an electronic balance (GF-2000; A&D Company Ltd., Tokyo, Japan).

Histopathological evaluation of knee OA

The left knee joints were resected after euthanasia using pentobarbital after 0, 2, 4, 6, and 8 weeks of running or natural breeding. The resected knees were fixed in a 10% formalin neutral buffer solution for 24 h, and the tissues were decalcified in 10% ethylenediaminetetraacetic acid solution for 3 weeks. The fixed and decalcified samples were embedded in paraffin and then sliced into serial frontal sections of 3-μm thickness. Sections were deparaffinized in xylene and rehydrated in a descending graded series of alcohol. The frontal sections, including the full length of the anterior cruciate ligament, were selected and then stained with Safranin-O and fast green; the lateral and medial femoral condylar cartilage were evaluated using the modified Mankin score.[22] The scoring system includes four components: i) cartilage structure, ii) cartilage cell appearance, iii) Safranin O stainability, and iv) tidemark integrity.

Immunohistochemistry

Some series of sections were stained for immunohistochemical evaluation (n=8). The expressions of AT1R, AT2R, Col X and MMP-13 were evaluated after 0, 4, and 8 weeks of running or natural breeding. The numbers of cells positive for AT1R, AT2R, Col X, and MMP-13 were counted in articular cartilage after 0, 4, and 8 weeks of running or natural breeding. We observed and counted immunopositive cells in the entire lateral femoral condylar cartilage of each section. Positive cell rates are expressed as the ratio of the number of stained cells to all chondrocytes.

Endogenous peroxidase was devitalized by 3% H_2O_2 for 20 min. After blocking with normal bovine serum for 60 min at room temperature, the sections were incubated overnight with primary antibodies (15 h at 4°C). Sections for control staining were incubated with normal rabbit serum and normal goat serum (15 h at 4°C) instead of primary antibodies. The primary antibodies were as follows: anti-AT1R goat polyclonal antibody (1:200 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, #31181), anti-AT2R rabbit polyclonal antibody (1:200 in PBS; Santa Cruz Biotechnology, #9040), anti-Col X rabbit monoclonal antibody (1:100 in PBS; LSL, Tokyo, Japan, #0092), and anti-MMP-13 rabbit polyclonal antibody (1:100 in PBS; Abcam, Cambridge, UK, #39012). The sections were incubated with the secondary antibody for 1 h at room temperature. Horseradish peroxidase-conjugated bovine anti-goat IgG antibody (1:1000 in PBS; Santa Cruz Biotechnology, #2350) was used as the secondary antibody for AT1R staining. Horseradish peroxidase-conjugated bovine anti-rabbit IgG antibody (1:1000 in PBS; Santa Cruz Biotechnology, #2370) was used as the secondary antibody for AT2R, Col X, and MMP-13 staining. The staining was visualized with a diaminobenzidine chromogen kit (DAB Chromogen; Dako, Glostrup, Denmark) and counterstained lightly with Mayer's hemalum solution. These stained samples were observed using a light microscope (BZ-9000; Keyence, Osaka, Japan). To investigate whether the expression of AT1R and Col X increased with the development and progression of articular degeneration, we examined the correlations between the modified Mankin score and the immunological positive cell rates of AT1R and Col X. Correlations between the immunological positive cell rate of AT1R and Col X, and between the rates of AT1R and AT2R were also investigated in the running THM group.

In vitro study

The downstream signals of AT1R include G-protein-dependent and G-protein-independent pathways.[23] We evaluated changes in the phosphorylation levels of JNK and Src as indicators of G-protein-dependent and the G-protein-independent pathways, respectively.

Cell culture

Chondrocytes were isolated from articular cartilage of the metatarsophalangeal joints of a 10-month-old cow by digestion with 0.08% collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 6 h at 37°C. After filtration, cells were seeded at a density of 2×10^4 cells/mL in 100-mm plates and cultured until confluent, as previously described.[13] The cells were then embedded in agarose hydrogels at a cell density of 2×10^6 cells/mL, as described pre-

viously.[24,25] The cell–agarose constructs were maintained in culture for 8 days at 37°C in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) at 37°C in a humidified hypoxic atmosphere (5% O_2 and 5% CO_2).

Application of dynamic compression loading

The cell–agarose constructs were allocated into six groups (groups A–F). Group A (Control) cell–agarose constructs were cultured without adding any agents or dynamic compressive loading. Group B (Compression) constructs were treated with intermittent compressive loading according to the protocol described in the next paragraph. Group C (Compression + ARB) constructs were treated with 10 mM olmesartan (a selective AT1R blocker) for 12 h before application of the loading. Group D (AngII) constructs were treated with 1 μM AngII for 30 min. Group E (Compression + AngII) constructs were treated with 1 μM AngII for 30 min before application of the loading. Group F (Compression + AngII + ARB) constructs were treated with 10 μM olmesartan for 12 h and with 1 μM AngII for 30 min before application of the loading.

The cell–agarose constructs allocated to groups B, C, E, and F were submitted to cyclic compressive loading using the FX-4000C™ Flexer-cell® Compression Plus™ System (FlexcellÒ International Corp, Burlington, NC, USA) following the protocol described previously.[25] The loads comprised pulses of 20 kPa (2 s on, 1 s off) superimposed on 20 kPa static offset pressure for 30 min.

Protein extraction and analysis of phosphorylation status

The phosphorylation status of specific signal transduction proteins was analyzed using the Bio-Plex bead suspension system (Bio-Rad Corp., Hercules, CA).[26] This is a multiplexing system that allows the assay of multiple proteins in a single sample. The cell–agarose constructs were frozen in liquid nitrogen and freeze-dried. For protein extraction, 200 mL of Laemmli buffer (250 mM Tris-HCl, 20% glycerol, and 10% SDS) was added to each freeze-dried construct, and the mixture was boiled immediately for 5 min. The lysates were left at room temperature to gel before being transferred to paper filter Mini-Spin columns and centrifuged at 12,000 *g* for 1 h at room temperature. The exudates were adjusted to 500 μg/mL for use in an assay for two phosphorylated proteins (phosphorylated JNK and phosphorylated Src). The bead–antibody complexes were vortexed and added to the wells of a 96-well filter plate. Tissue lysates (50 μL) and positive control samples were added to the wells in duplicate, and the plate was incubated for 15 h with constant agitation. The plate was then vacuum filtered and washed three times. Detection antibodies were added to the wells, and the plate was incubated for 30 min at room temperature and then vacuum filtered and washed again. Streptavidin–phycoerythrin was added to each well for 10 min, after which the plate was vacuum filtered and rinsed, and resuspension buffer was added. The 96-well plate was placed in the Bio-Plex reader, and the samples were analyzed.

Statistical analysis

All data are presented as mean ± standard deviation. The scores for each group were compared using Student's unpaired *t*-test. P-values <0.05 were considered to be significant. All data were analyzed using StatView 5.0 statistical software (SAS Institute Japan Corp., Tokyo, Japan). Pearson's correlational analysis was performed to analyze relationships between the mean Mankin scores and the positive cell rates for Col X, AT1R, and AT2R.

Results

In vivo study

Weight changes in THM and C57BL/6 mice

There was a trend for a lower mean body weight in THM than in C57BL/6 mice. However, the mean body weights of the THM and C57BL/6 mice did not differ significantly after each running and breeding period (n = 10 in each group) (Table 1).

Histopathological evaluation of knee OA

First, we examined the lateral femoral condylar cartilage (Figure 1A). In the running THM group, reduction in Safranin O staining was observed after 4 weeks of running, and cartilage degeneration and hypocellularity were observed after 8 weeks. In the running C57BL/6 mice group, a slight reduction in Safranin O staining was observed after 8 weeks of running. In the non-running THM and C57BL/6 mice groups, the lateral femoral condylar cartilage showed little change in OA at 8 weeks (Figure 1B).

In the running THM group, the mean modified Mankin scores were 0.3±0.2, 0.9±0.7, 2.1±1.3, 4.8±1.5, and 7.0±2.8 after 0, 2, 4, 6, and 8 weeks of running, respectively. In the running C57BL/6 mice group, the mean scores were 0.2±0.1, 0.5±0.4, 0.6±0.4, 1.0±0.6, and 1.7±0.8, respectively. In the non-running THM group, the mean scores were 0.2±0.2, 0.2±0.3, 0.4±0.4, 0.4±0.4, and 0.6±0.4, respectively. In the non-running C57BL/6 mice group, the mean scores were 0.2±0.2, 0.2±0.2, 0.3±0.2, 0.2±0.3, and 0.4±0.4, respectively (Figure 1C). The mean scores were significantly higher in the running THM group than in the running C57BL/6 mice group after 4, 6, and 8 weeks of running (P=0.026, P=0.015 and P=0.001, respectively, n=10 in each group). The mean scores were also significantly higher in the running THM group than in the non-running THM group after 4, 6, and 8 weeks of running or natural breeding (P=0.012, P=0.005 and P=0.001, respectively, n=10 in each group).

Immunohistochemical analysis

In all groups, AT1R expression was not detected at 0 weeks of running or natural breeding. In the running THM group, AT1R expression was detected after 4 weeks of running, and the expression level increased significantly to 8 weeks. In the running C57BL/6 mice group, AT1R expression was detected only at 8 weeks. In the non-running THM and C57BL/6 mice groups, AT1R expression was barely detectable at any time (Figure 2A). In the running THM group, the AT1R-immunopositive cell rates were 1.7%±2.4%, 28.1%±6.1%, and 58.0% ±10.1% at 0, 4, and 8 weeks of running, respectively. In the running C57BL/6 mice group, the respective rates were 0.0%±0.0%, 3.3%±1.5%, and 18.2%±8.6%. In the non-running THM group, the respective rates were 2.1%±1.5%, 3.3%±1.8%, and 9.2%±5.6%. In the non-running C57BL/6 mice group, the respective rates were 0.0%±0.0%, 1.2%±1.3%, and 3.0%±3.0%. The AT1R-immunopositive

Table 1. Mean body weights (grams) at each breeding period (weeks). Data are presented as mean ± standard deviation.

Breeding periods		0	2	4	6	8
Running	THM	22.95±1.21	23.87±1.97	23.92±2.04	24.91±2.85	25.04±1.96
	C57BL/6	23.97±1.52	24.65±1.48	24.91±1.46	25.69±2.32	25.72±2.60
	P-value	0.43	0.52	0.49	0.51	0.62
Non-running	THM	22.62±1.36	24.05±2.35	24.02±2.49	25.16±1.95	25.33±1.71
	C57BL/6	23.37±1.54	24.96±2.65	24.69±1.30	25.70±2.91	26.15±2.76
	P-value	0.64	0.62	0.58	0.62	0.53

cell rates were significantly higher in the running THM group than in the running C57BL/6 mice group after 4 and 8 weeks of running (P=0.025 and P=0.001, respectively; n=8 in each group). The positive cell rates did not differ significantly between the running C57BL/6 mice group, the non-running C57BL/6 mice group, and the non-running THM group (Figure 2B).

In all groups, AT2R expression was not detected at 0 weeks of running or natural breeding. In the running THM group, slight AT2R expression was detected after 4 weeks of running, and the expression level increased at 8 weeks of running. In the other groups, slight AT2R expression was detected at 4 and 8 weeks (Figure 2C). In the running THM group, the AT2R-immunoposi-

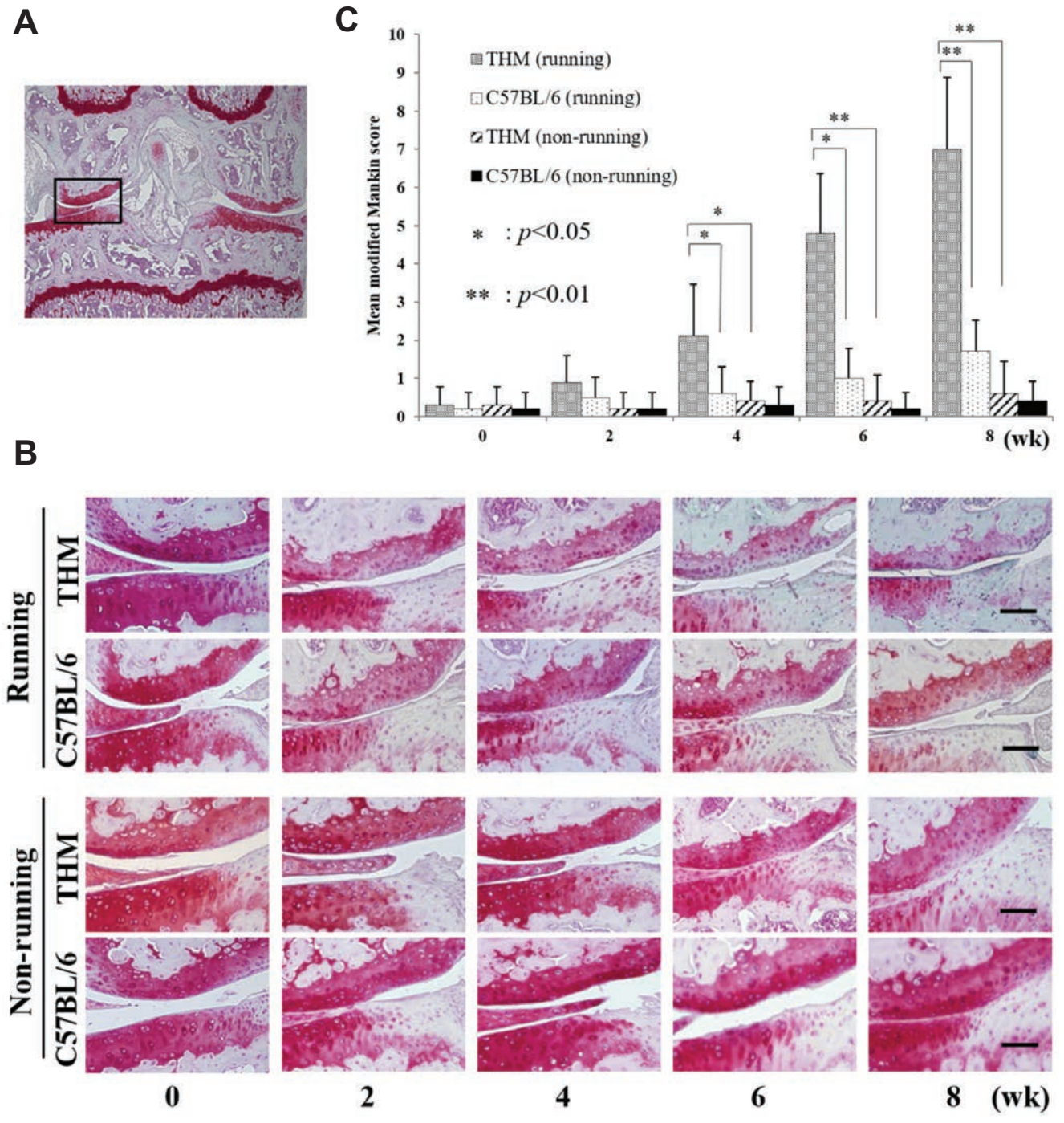

Figure 1. Histopathological evaluation of the lateral femoral condylar cartilage in the frontal knee sections. The femoral articular cartilage stained with Safranin-O and Fast green was evaluated in the indicated box area (A). Representative histological samples after 0, 2, 4, 6 and 8 weeks of running or natural breeding are shown (B). Changes in mean modified Mankin scores of the lateral femoral condylar cartilage in THM (running), C57Bl/6 mice (running), THM (non-running) and C57Bl/6 mice (non-running) (C). The mean scores of the THM of running group were significantly higher than those of other groups after 4, 6 and 8 weeks of running or natural breeding. n=10 in each group. Scale bar: 100 μm.

tive cell rates were 0.0%±0.0%, 7.3%±4.0%, and 27.0%±10.4%, respectively. In the non-running C57BL/6 mice group, the respective rates were 0.0%±0.0%, 0.3%±1.0%, and 6.7%±4.5%. In the non-running THM group, the respective rates were 0.0%±0.0%, 5.5%±4.4%, and 6.2%±4.6%. In the non-running C57BL/6 mice group, the respective rates were 0.0%±0.0%, 0.0%±0.0%, and 2.6%±2.0%. The rate was significantly higher in the running THM group than in the running C57BL/6 mice group after 8 weeks of running (P=0.015, n=8) (Figure 2D).

In all groups, Col X expression was not detected at 0 weeks of running or natural breeding. In the running THM group, Col X expression was detected at 4 weeks (Figure 3A). The Col X-immunopositive cell rates were 1.3%±1.6%, 16.7%±7.5%, and 36.6%±15.4% after 0, 4, and 8 weeks of running, respectively. The expression level was significantly higher at 8 weeks than at 4 weeks (P=0.016, n=8). Col X expression was significantly higher in the running THM group than in the running C57BL/6 mice group after 4 and 8 weeks of running (P=0.023 and P=0.012, respectively, n=8) (Figure 3B).

In all groups, the expression of MMP-13 was not detected at 0 week of running or natural breeding. In the running THM group, slight MMP-13 expression was

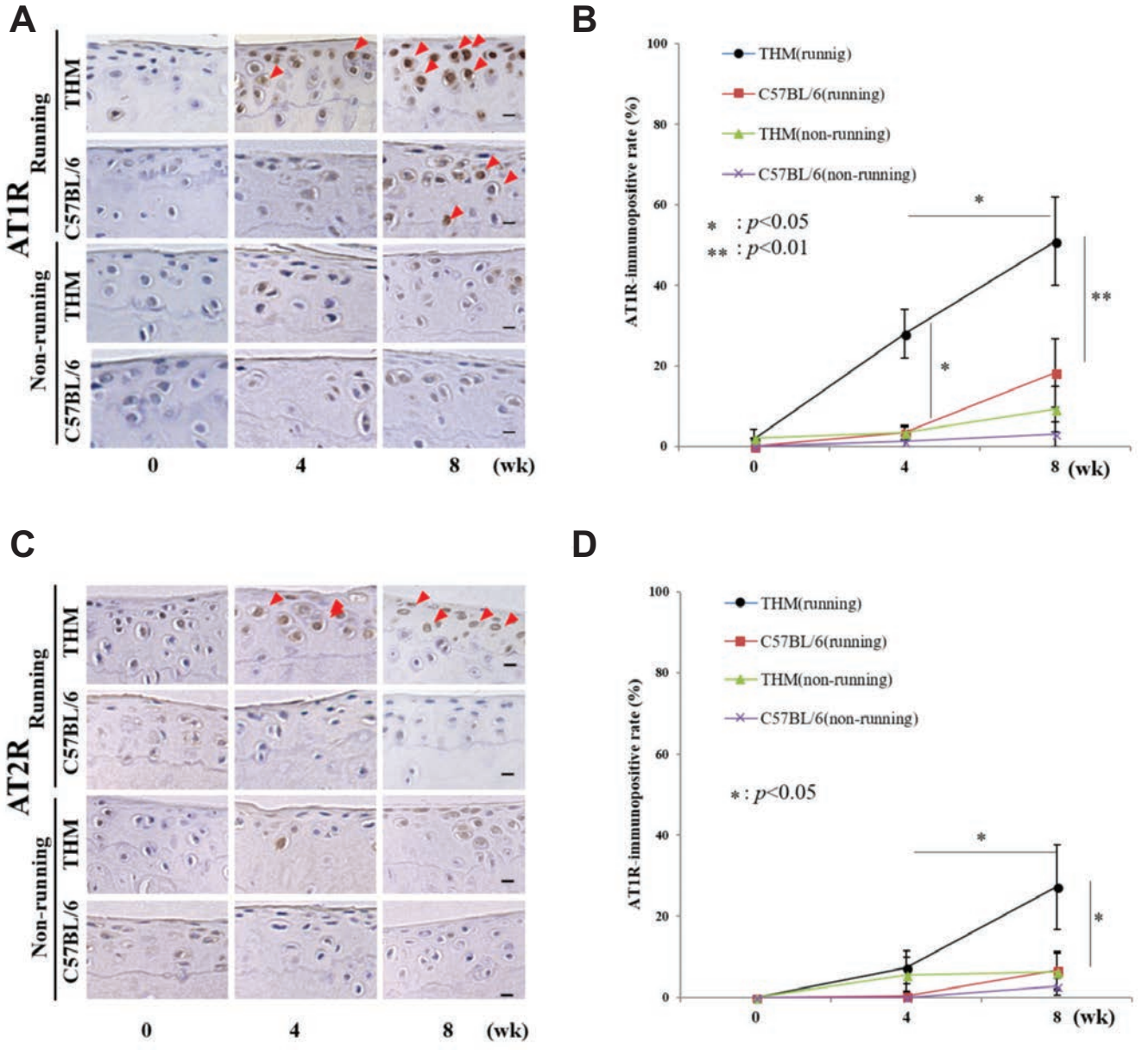

Figure 2. Expression of AT1R (A) and AT2R (C) in articular cartilage of running and non-running group after 0, 4 and 8 weeks of running or natural breeding (arrows). Changes in immunopositive rates of AT1R (B) and AT2R (D) with running periods or natural breeding periods. In the THM of running group, the AT1R-immunopositive rate were significantly higher than those of the C57BL/6 mice of running group after 4 and 8 weeks of running. In the THM of running group, the AT2R-immunopositive rate was significantly higher than those of the C57BL/6 mice of running group after 8 weeks of running. n=8 in each group. Scale bar: 10 μm.

detected at 4 weeks of running (Figure 3C). The MMP-13-immunopositive cell rates were 2.4%±1.6%, 13.1%±6.7%, and 32.1%±8.6% after 0, 4m and 8 weeks of running, respectively. The expression level was significantly higher at 8 weeks than at 4 weeks (P=0.018, n=8). The MMP-13 expression rate was significantly higher in the running THM group than in the running C57BL/6 mice group after 4 and 8 weeks of running (P=0.032 and P=0.015, respectively, n=8) (Figure 3D).

Pearson's correlational analysis in the running THM group (n=24; n=8 each at 0, 4, and 8 weeks), showed the following significant positive correlations: i) between the Mankin score and the AT1R-immunopositive cell rate (r^2=0.823, P<0.0001; Figure 4A); ii) between the Mankin score and the Col X-immunopositive rate of in the running THM group (r^2= 0.625, P<0.0001; Figure 4B); iii) between the AT1R-positive rate and Col X-positive rate (r^2=0.654, P<0.0001; Figure 4C); and iv) between the AT1R-positive rate and AT2R-positive rate (r^2= 0.589, P<0.0001; Figure 4D).

In vitro study

Compared with the control, the JNK phosphorylation level increased significantly to 2.4-, 2.2-, and 8.1-fold in Group B (Compression), Group D (AngII), and Group E (Compression with AngII), respectively (P=0.028, 0.001, and 0.002, respectively; n=6 in each group) (Figure 5A). These increases were significantly suppressed by pretreatment with the selective ARB (Groups C and F, P=0.041 and 0.029, respectively; n=6 in each group). Compared

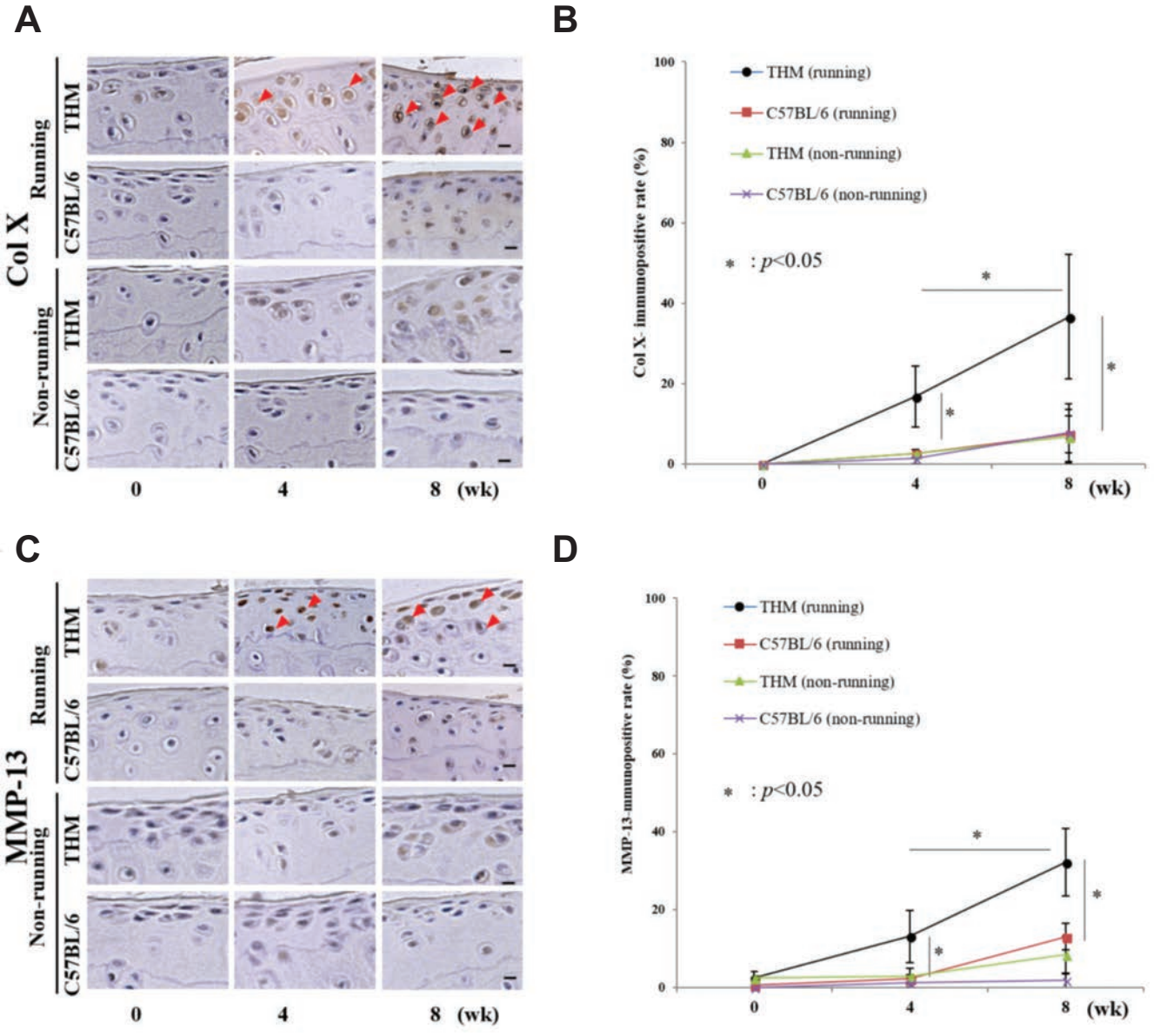

Figure 3. Expression of Col X (A) and MMP-13 (C) in articular cartilage of running and non-running group after 0, 4 and 8 weeks of running or natural breeding (arrows). Changes in immunopositive rate of Col X (B) and MMP-13 (D) with running periods or natural breeding periods. In the THM of running group, the Col X and MMP-13-immunopositive rate were significantly higher than those of other groups after 8 weeks of running or natural breeding. Scale bar: 10 μm.

with the control, the Src phosphorylation level increased significantly to 2.1-, 2.5-, and 2.4-fold in Group B (Compression), Group D (AngII), and Group E (Compression with AngII), respectively (P=0.015, 0.0001, and 0.001, respectively; n=6 in each group) (Figure 5B). However, these increases were not suppressed by pre-treatment with the ARB (Groups C and F, n=6 in each group).

Discussion

Body weight did not differ significantly between the THM and running C57BL/6 mice groups during the study, which indicates that the amount of mechanical loading to the knees generated by the forced running did not differ between these two strains of mice. It is well known that excessive mechanical loading on a joint can induce degeneration of articular cartilage and is one of the most important risk factors for OA development.[17] Destabilization of the medial meniscus[27] and transection of the anterior cruciate ligament[28] are the most common methods for surgical induction of mouse knee OA. In the absence of these surgical interventions, strenuous forced running can induce mild knee OA in rats[29,30]

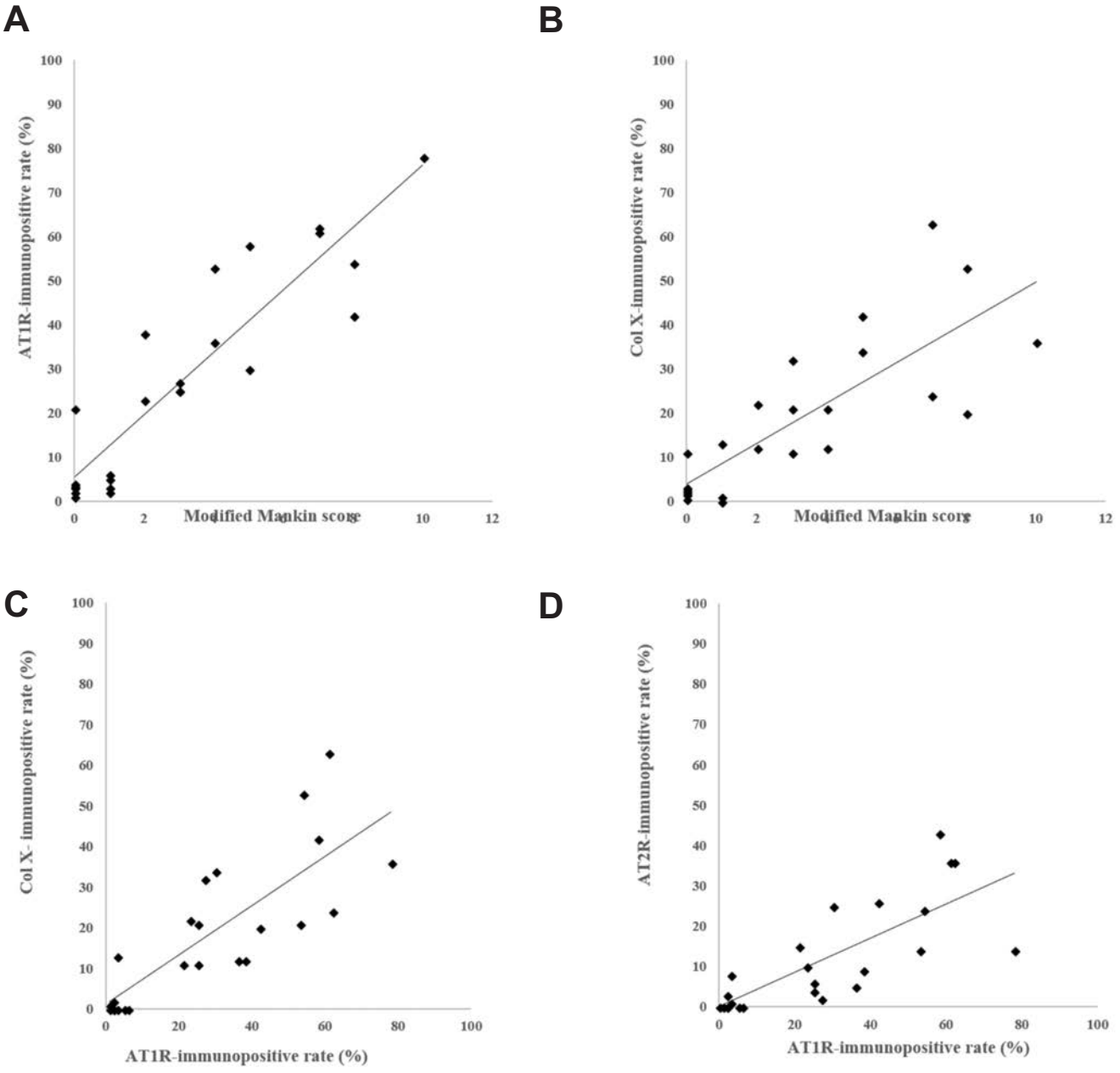

Figure 4. Pearson's correlational analysis in the running THM group (n=24; n=8 each at 0, 4, and 8 weeks). A) between the Mankin score and the AT1R-immunopositive rate (r^2=0.823, P<0.0001); B) between the Mankin score and the Col X-immunopositive rate (r^2= 0.625, P<0.0001); C) between the AT1R- and Col X-immunopositive rate (r^2=0.654, P<0.0001); D) between the AT1R- and AT2R-immunopositive rate (r^2= 0.589, P<0.0001).

and mice.[21,31] In the present study, we used forced running to induce mouse knee OA and examined differences in cartilage degeneration between the THM and the C57BL/6 mice. In the course of this forced running (for a maximum of 8 weeks), two THM and one C57BL/6 mouse died at 7 weeks of running, and two THM died at 6 weeks of running from unknown causes; the deaths of these animals suggest that the exercise intensity of this running protocol was strenuous for the mice.

In the present study, apparent changes in OA were observed in the lateral knee compartment of THM in the running group at 4 and 8 weeks, and slight changes in OA were noted in the medial compartment even at 8 weeks (*data not shown*). Strenuous running has been reported to induce knee OA in rats, mainly in the lateral compartment, probably because of greater loading to the lateral than to the medial compartment.[29] In mice, the mechanical loading caused by forced running also seems to be higher in the lateral than in the medial knee compartment.

The articular cartilage in the running THM showed apparent changes in OA at 8 weeks of running, whereas that in the running C57BL/6 mice showed slight changes in OA at 8 weeks. The non-running THM and C57BL/6 mice groups showed little changes in OA at 8 weeks of natural breeding. The mean modified Mankin scores were significantly higher in the running THM group than in the other groups. The expression levels of Col X (a marker of hypertrophic chondrocytes)[16] and MMP-13 (a proteolytic enzyme in cartilage matrix) were detected in the THM after 4 and 8 weeks of running, but these markers were only slightly detectable in the C57BL/6

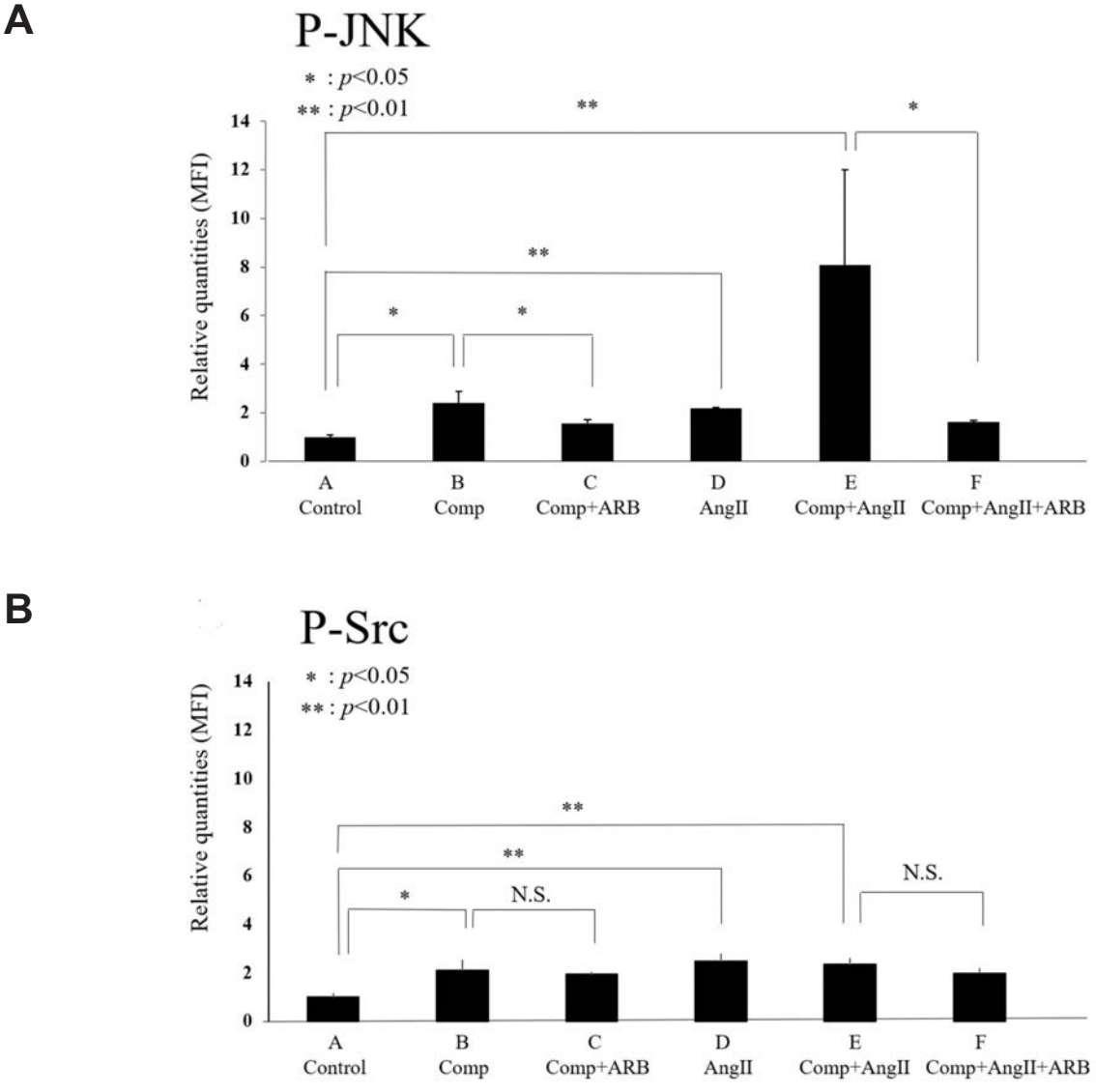

Figure 5. Changes in the phosphorylation level of JNK (A) and Src (B). Application of cyclic compression loads, and addition of Ang II significantly upregulated the phosphorylation of JNK and Src. The synergistic effect is observed through the JNK pathway, but not observed through the Src pathway. The upregulation of JNK phosphorylation was significantly downregulated by the addition of ARB (Olmesartan). The upregulation of Src phosphorylation was not downregulated by the addition of ARB (Olmesartan). n=6 in each.

mice after 8 weeks of running. Cartilage degeneration in OA is known to be accompanied by the hypertrophic differentiation of chondrocytes accompanied by activation of MMP-13 and expression of Col X.[16,17] These results suggest that THM were more susceptible to the development and progression of mechanically induced knee OA than were the other groups.

Immunostaining for AT1R and AT2R was positive in articular cartilage of the running THM group at 4 and 8 weeks. By contrast, in the running C57BL/6 mice group, AT1R staining was slightly detectable only after 8 weeks. In the non-running THM and C57BL/6 mice groups, AT1R staining was not detected at 8 weeks. These findings suggest that accumulation of mechanical stresses induced by to the forced running induced AT1R expression in articular chondrocytes. Previous studies have shown that mechanical loading upregulates AT1R expression in cardiac myocytes[32] and AT2R expression in the thoracic aorta.[33] In the same way, AT1R expression may be upregulated in chondrocytes by mechanical loading.

The high concentration of AngII in serum and cartilage of the THM may stimulate the induction of AT1R in chondrocytes. AngII can upregulate the expression of the local AT1R and AT2R. For example, AngII promotes expression of the AT1R in the rostral ventrolateral medulla,[34] retinal pigment epithelium,[35] and adrenal gland,[36] and promotes expression of the AT2R in the brain cortex.[37] The serum concentration of AngII is 4 to 5 times higher in THM than in the C57BL/6 mice.[38,39] Because nutrition of articular cartilage is provided by diffusion from vessels in subchondral bone and bone marrow and percolation through synovial fluid,[40] it is possible that the high serum AngII concentration upregulated the AT1R in chondrocytes in articular cartilage. Together, the mechanical stresses induced by forced running and higher serum and cartilage concentrations of AngII in the THM might have resulted in synergistic upregulation of AT1R expression. Increased expression of the AT1R in hypertrophied heart has been reported in THM.[41]

We also investigated the relationships between the progression of cartilage degeneration and changes in the expression of Col X, AT1R, and AT2R. We found a significant relationship between the Mankin score and the AT1R-immunopositive cell rate in chondrocytes in the running THM group and a strong relationship between Mankin score and Col X-immunopositive rates in chondrocytes in this group. A significant positive relationship was also observed between the AT1R-and Col X- immunopositive rates. These results suggest that there is a cause-and-effect relationship between the expression of AT1R and Col X. That is, in the running THM group, excessive mechanical stress to the knee may have induced AT1R expression in chondrocytes and stimulated Col X expression through signal transduction involving, at least in part, the AT1R. The moderate but significant relationship between the AT1R- and AT2R-immunopositive rates is interesting. Given that the AT1R and AT2R generally have opposite functions,[4,5] it is possible that the AT2R may have a role by providing negative feedback for AT1R function in cartilage degeneration through AT2R upregulation associated with increased AT1R expression.

We conducted *in vitro* experiments to determine whether mechanical loading of articular chondrocytes could transduce intracellular signals through the AT1R in chondrocytes. By measuring the phosphorylation of JNK (a signaling protein in the G protein-dependent pathway), we found evidence that cyclic compressive loading of chondrocytes transduced intracellular signals through the AT1R. In addition, mechanical loading and the ligand AngII synergistically activated the AT1R. These findings support the *in vivo* results in the present study and suggest that the AT1R may have a mechanosensing function in chondrocytes.

There are some limitations of the present study. First, the effect of osteoporosis on knee OA was not evaluated in THM. Y. Asaba *et al.* reported that activation of the RAS in THM induced high-turnover osteoporosis with accelerated bone resorption, which occurred independently of the development of hypertension.[42] Additional studies of subchondral osteoporosis in THM are needed because subchondral osteoporosis is observed in the early stage of OA,[43] and microfractures caused by subchondral fragility may cause acceleration of OA progression.[44,45] Second, differences in the subchondral circulation between THM and C57BL/6 mice were not evaluated. It has been suggested that reduced peripheral circulation associated with hypertension may cause subchondral ischemia, which could reduce nutrient and gas exchange between articular cartilage and subchondral bone.[46–48] Third, we did not investigate effects of other angiotensin metabolites such as angiotensin (Ang) 1-7 or Ang1-9 on cartilage degeneration in this study. Because several studies have reported that the ACE2/Ang1-7/Mas receptor system has an antagonistic function to the ACE/AngII/AT1R system,[49] those metabolites may have a role to antagonize the local RAS function on hypertrophic differentiation of chondrocytes. Further studies are needed on this topic. Finally, we did not examine whether the downstream signal transductions of the AT1R promote hypertrophic differentiation of chondrocytes, although we showed *in vitro* that application of cyclic compressive loading and addition of AngII transduced intracellular signals through the AT1R. This topic is currently under investigation in our laboratory.

In conclusion, transgenes of renin and ANG aggravated mouse knee OA induced by strenuous forced running in THM. Activation of a local RAS in articular cartilage may play a role in the development and progression of knee OA. The results of this study suggest that the RAS is a common molecular mechanism involved in the pathogenesis of hypertension and knee OA and may be a therapeutic target for both diseases.

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