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Mechanical stretch enhances NF-κB-dependent gene expression and poly(ADP-ribose) synthesis in synovial cells

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Toshiaki Morisugi[1,*], Yasuharu Tanaka[2], Tetsuji Kawakami[1] and Tadaaki Kirita[1]

[1]Department of Oral and Maxillofacial Surgery, Nara Medical University, Shijio-cho 840, Kashihara, Nara 634-8521 and [2]Laboratory of Biometabolic Chemistry, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, Uehara 207, Nishihara, Okinawa 903-0215, Japan

*Toshiaki Morisugi, Department of Oral and Maxillofacial Surgery, Nara Medical University, Shijio-cho 840, Kashihara, Nara 634-8521, Japan. Tel: +81-744-223051, Fax: +81-744-257657, email: t_morisugi@hotmail.com

Temporomandibular joint disorders (TMD) show complex symptoms associated with inflammation, pain and degeneration of the peripheral tissues including synovium. Although it is believed that excessive mechanical stress on synovium causes development of TMD, the molecular mechanism by which mechanical stress triggers TMD has still remained unclear. In order to examine the effect of mechanical stress on synoviocytes, rabbit synovial cells were cyclically stretched *in vitro*. The stretch efficiently increased the gene expressions of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and NF-κB responsive reporter gene constructs. The interruption of NF-κB activating pathway by inhibitors resulted in the abrogation of those expressions, indicating the pivotal role of NF-κB in the mechanical stretch-mediated COX-2 and iNOS expressions. In parallel, the stretch remarkably increased NO production and poly(ADP-ribose) (PAR) synthesis, suggesting that excessive amounts of NO causes DNA injury and in turn activates PAR synthesis by poly(ADP-ribose) polymerase (PARP). The inhibition of PAR synthesis by a PARP inhibitor or a radical scavenger enhanced the mechanical stretch-induced gene expressions in a NF-κB-independent manner, implying an involvement of PARP in the gene expression. Taken together, these results demonstrate that mechanical stress on synovial cells not only induces gene expressions of COX-2 and iNOS but also affects PAR synthesis.

Keywords: COX-2/iNOS/mechanical stress/NF-κB/PARP/TMD.

Abbreviations: COX-2, cyclooxygenase-2; DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]- 1(2H)-isoquinolinone; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκB, inhibitor of NF-κB; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; NAC, N-acetylcysteine; NF-κB, nuclear factor κB; NO, nitric oxide; PAGE, polyacrylamide gel electrophoresis; PAR, poly(ADP-ribose); PARG, poly(ADP-ribose) glycohydrolase; PARP, poly(ADP-ribose) polymerase; PDTC, pyrrolidine dithiocarbamate; PG, prostaglandin; PVDF, polyvinylidene difluoride; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecylsulphate; SF, synovial fluid; SIN-1, 3-(4-morpholinyl)sydnonimine hydrochloride; TMD, temporomandibular joint disorders; TMJ, temporomandibular joint; TNFα, tumor necrosis factor α.

Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are induced in response to various stimuli and are closely related to inflammation and/or pain in tissues (*1*, *2*). COX-2 is involved in the metabolism of arachidonic acid, of which metabolites are prostaglandins (PGs) including PGE_2. PGE_2 elicits inflammation through its bindings to G-protein-coupled receptors (*3*). On the other hand, iNOS greatly synthesizes NO, of which bioactivities are elicited in either a cGMP-dependent or -independent fashion (*4*). In the latter fashion, NO reacts with cysteine groups in protein molecules (S-nitrosylation), resulting in altered function of the proteins. In addition, reaction of NO with superoxide anion (O_2–) yields peroxynitrite anion ($ONOO^-$), which is a potent cytotoxic molecule and reacts with tyrosine residues in protein molecules (nitration) (*4*).

Temporomandibular joint (TMJ) disorders (TMD) are characterized as a complicated disease of the TMJ, of which symptoms are closely associated with inflammation and pain in the peripheral tissues of the joint, such as articular synovium (*5*). Since persons who have habits of bruxism or clenching contract TMD easily, mechanical stresses have been presumed to be a strong trigger of the appearance of TMD (*6*). Indeed, a few studies using animal models have corroborated the presumption (*7*, *8*). In the synovial fluid (SF) of patients with TMD, a significantly increased level of proinflammatory mediators such as NO (*9*), PGE_2 (*10*, *11*), and a few cytokines including tumour necrosis factor α (TNFα) (*12*) were detected. Also, in patients with TMD, the increased expressions of COX-2 (*13*–*15*) and iNOS (*16*–*18*) in the articular synovial membrane have been observed. These reports showed that the increased level of COX-2 and iNOS in synovial cells are probably involved in the appearance and/or aggravation of TMD.

A transcription factor, nuclear factor κB (NF-κB), is well-known to up-regulate the expression of COX-2 and iNOS in various cells in humans (*19*, *20*). With

regard to the increased expression of iNOS and COX-2 in synovium from the patient with TMD, the involvement of NF-κB has been suggested (*21*, *22*). However, the trigger(s) that causes activation of NF-κB in synovial cells has remained unclear. Two groups independently proposed hypotheses that reactive oxygen species (ROS) generated by mechanical stress play pivotal roles in the occurrence of articular tissue damage observed in TMD (*23*, *24*). Generally, ROS induces impairment of various cellular molecules. Among the impairments, DNA strand breakages secondarily cause excessive poly(ADP-ribose) (PAR) synthesis, which is catalysed by poly(ADP-ribose)polymerase-1 (PARP-1) (*25*, *26*). On the other hand, PARP-1 has been reported to play roles on the NF-κB activation (*27*). Thus, if ROS are generated by mechanical stress, PARP-1 is greatly expected to play a key role on the COX-2 and iNOS expression in the synovium.

In this study, we demonstrate that mechanical stretch of synovial cells enhances PAR synthesis and induces COX-2 and iNOS expression *via* activation of NF-κB, and we show that the inhibition of PAR synthesis by either a PARP-inhibitor or a radical scavenger up-regulates the mechanical stretch-dependent gene expression.

Materials and Methods

Reagents and plasmids

Inhibitors of NF-κB-activation, BAY11-7085 and MG132 (Calbiochem. Com., USA), curcumin (Nacalai Tesque, Japan) and pyrrolidine dithiocarbamate (PDTC) (Wako Pure Chemicals, Japan) were purchased commercially. A potent PARP inhibitor, 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ) (Sigma-Aldrich) (*28*), a radical scavenger N-acetylcysteine (NAC) (Nacalai Tesque) (*29*) and collagen (Cellmatrix Type1-P) (Nitta Gelatin Inc., Japan) were purchased commercially.

Rabbit polyclonal anti-murine COX-2 antibody (Cayman Chemical, USA) and Rabbit polyclonal anti-murine iNOS antibody (BD Transduction Laboratories, USA) were purchased commercially. Rabbit polyclonal anti-PAR poly(ADP-ribose) antibody (*30*) and rabbit polyclonal anti-bovine PARP antibody (*31*) were prepared as previously described. Mouse monoclonal anti-human β actin and anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Applied Biological Materials (ABM) Inc., Canada and Applied Biosystems Inc., Japan, respectively. Horseradish peroxidase-conjugated goat polyclonal anti-rabbit IgG antibody and horseradish peroxidase-conjugated rabbit polyclonal anti-murine IgG antibody were purchased from DAKOCytomation. Recombinant murine TNFα was obtained from Pepro Tech EC Ltd. The reporter luciferase gene constructs such as pNF-κB-luciferase (pNF-κB-luc.) and pp53-TA-luciferase (p53-TA-luc.) used in this study were purchased from Clontech, Japan (Mercury Pathway Profiling Systems). As a negative control, pTA-luciferase, which lacks response elements for binding of transcription factors, was also obtained from Clontech.

A NF-κB-responsive enhanced green fluorescent protein (EGFP) expression vector was constructed as described previously (*32*). Transfection reagents, TransIT® (Mirus, USA) and Lipofectamine™ 2000 (Invitrogen, USA), were purchased commercially.

Cell culture

A rabbit synovial cell line (HIG-82) was obtained from American Type Culture Collection (ATCC). HIG-82 cells were maintained in Ham's F-12 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS) (culture medium) at 37°C in a humidified CO_2-incubator under 5% CO_2 condition.

For the experiment of cell stretching, elastic silicon chambers (Scholar Tech Inc., Japan) were exposed with 0.3 mg/ml of collagen in 1 mM HCl at 37°C for 10 min in a CO_2-incubator. After the exposure, the collagen solution was removed, and the chambers were air-dried in a clean-bench. After drying, the chambers were stored at 4°C until use.

Cell stretching

HIG-82 cells were cultured in the collagen-coated elastic silicon chambers for 24 h, and then the chambers were set on a stretching apparatus driven by a computer-controlled motor (NS-350, Scholar Tech Inc., Japan). The chambers were uni-axially stretched under static or cyclic condition in a CO_2-incubator for 1 h. The extension degrees of the chambers were set at 120 or 140% under the static stretch condition. On the other hand, under the cyclic stretch condition, the cycle numbers of stretch were varied from 60 to 3,600 cycles/h under a fixed 120% extension condition, except as otherwise indicated. After the stretching, the cells were further cultured for 30 min under non-stretched conditions. In some experiments, the cells were pretreated with 5 mM NAC or 0.1 mM DPQ for 1 h prior to the stretching.

Under our experimental condition, the stretched cells were not detached from the surface of the silicon chamber during the culture.

In some experiments, the non-stretched cells were treated with TNFα (10 ng/ml) for 1 h to activate the expression of COX-2 and iNOS (a positive control).

Cell transfection

In order to examine the effect of stretch on NF-κB responsive gene expression, reporter assays were carried out. HIG-82 cells were seeded on the collagen-coated silicon chamber at a density of 1×10^5 cells/1.5 cm^2. At 24 h after the seeding, the cells were transfected with 50 ng of the luciferase gene constructs such as pNF-κB-Luc, using a transfection reagent, TransIT® (MIRUS), according to a previous method (*33*). At 24 h after the transfection, medium was changed to fresh culture medium, and the cells were subjected to the above stretch. In some experiments, the cells were pretreated with or without inhibitors of NF-κB activation for 1 h prior to the stretch.

Luciferase reporter assay

After the stretching of the cells transfected with the above reporter genes, the cells were harvested by treatment with trypsin/EDTA and collected by a low centrifugation as described previously (*33*). Briefly, the cells were lysed by a cell lysis buffer (LCβ-PGC-51, TOYO Ink Co., Japan) and centrifuged at 10,000*g* for min. The supernatant was collected and subjected to the assay for luciferase.

Luciferase assay was carried out using a luciferase reporter assay system (Promega) according to the manufacturer's instruction. The luciferase activity in the supernatant was measured by a luminometer (TR717 Microplate Luminometer, TROPIX). Luciferase activity was calibrated as specific enzyme activity (enzyme activity/μg protein).

Western blotting

HIG-82 cells were seeded at a density of 1×10^6 cells/10 cm^2-chamber and cultured for 24 h. The cultured cells were subjected to experiments of stretch as described in the above section. After the stretching, the cells were harvested, collected by a low centrifugation and lysed in 100 μl-aliquots of buffer D (10 mM Hepes–NaOH, pH 7.6, 0.42 M NaCl, 0.5 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA and 0.1% NP-40) containing 1/100 volume of the protease inhibitor cocktail (Nacalai Tesque Inc., Japan). The cell lysate was centrifuged at 14,000*g* for 20 min, and the resulting supernatant was collected. Proteins in the supernatant were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using 7.5 or 10% gel. After the electrophoresis, the proteins in the gel were transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane, and the membrane was subjected to western blot analyses according to a previous method (*33*). After blocking the membrane with 4% (W/V) skim milk in phosphate-buffered saline (PBS), the membrane was reacted with the following primary antibodies diluted with 4% (W/V) skim milk in PBS for 1.5 h: anti-COX-2 (1,000 × dilution), anti-iNOS (5,000 × dilution), anti-PAR (2,000 × dilution), anti-β actin (2,000 × dilution) and anti-GAPDH (5,000 × dilution).

After washing the membrane four times with PBS containing 0.02 % Tween 20 (PBST), it was incubated with horseradish peroxidase-conjugated secondary antibody (goat polyclonal anti-rabbit IgG antibody or rabbit polyclonal anti-mouse IgG antibody) 2,500 × diluted with PBS containing 4% skim milk for 1 h. The membrane was washed four times with PBST, and then the proteins bound to the specific antibody were detected as black bands on an X-ray film using ECL-Plus (GE Healthcare, UK). The visualized black bands were densitometrically quantified using software, ImageJ 1.38X (NIH).

Fluorescent microscopy

HIG-82 cells (1×10^5 cells/1.5 cm^2 chamber) were transfected with 500 ng of pNF-κB-EGFP vector using Lipofectamine2000 (Invitrogen). Twenty-four hours after the transfection, the cells were repetitively stretched under a condition at 120% extension and 600 cycles/h for 1 h. After the stretch, the cells were incubated for 30 min, and then harvested and collected by a low centrifugation. The cell pellet was resuspended in 1 ml of PBS and re-centrifuged. The cell pellet was resuspended in an appropriate volume of PBS, and a small aliquot of the cell suspension were loaded on an 8-well slide (MP Biomedicals). Pictures of cells were taken with a microscope connected to a digital camera system (Nikon ECLIPSE 80i, Japan) under fluorescence and bright field conditions.

Measurement of nitric oxide (NO)

For measurement of NO in medium, HIG-82 cells were cultured in a phenol-red free medium consisting of iso-volume of Ham's F12 and DMEM (Dulbecco's modified Eagle's medium) (GIBCO) supplemented with 10% FBS at a density of 1×10^6 cells/10 cm^2-chamber and cultured for 24 h. The cultured cells were stretched under various cycles as described in the above section. After the stretching, the 1 ml-aliquots of culture medium were taken out and centrifuged at 1,500*g* for 10 min to remove cells. The resultant supernatant was subjected to measurement of NO concentration. NO in the supernatant was measured using a 2,3-diaminonaphthalene kit according to the instruction's manual (Dojindo Laboratories, Tokyo, Japan) with slight modifications. Briefly, the reaction volume was scaled-up to 400 µl. After the reaction, the fluorescent intensities of the reaction solutions were measured under a condition with excitation at 360 nm and emission at 450 nm using SHIMADZU RF-1500 (Kyoto, Japan).

Assay for poly(ADP-ribose) glycohydrolase

To examine the changes in poly(ADP-ribose) glycohydrolase (PARG) activity in stretched cells, the cell lysate prepared using buffer D (described in 'western blotting') was subjected to a PARG assay system with slight modifications (*34*). Briefly, the assay mixture consists of 50 mM potassium phosphate buffer, pH 7.2, 50 mM KCl and 20 µM free PAR polymers, which was prepared as described previously (*35*). Appropriate aliquots of the cell lysate (corresponding to 10 µg proteins) were added to the PARG assay mixture in a total volume of 0.4 ml. After incubation at 37°C for 20 min, the reactions were terminated by the addition of 40 µl of 8.5 M KOH and 0.85 M benzamidine hydrochloride, respectively. After heating at 110°C for 10 min and then cooling, the fluorescence intensity of the reaction product which are fluorescent derivatives of ADP-ribose was measured under a condition with excitation at 360 nm and emission at 445 nm using SHIMADZU RF-1500 (Kyoto, Japan).

As a positive control, 40 ng of purified human recombinant PARG (human rPARG) was used in place of the cell lysate. The preparation method for the rPARG will be described otherwise. Briefly, a plasmid carrying human PARG cDNA (MGC clone No. 6064831, Invitrogen) was purified, and the inserted PARG cDNA was amplified and then re-inserted into a pCold I vector (Takara Bio Co., Kyoto, Japan) to reconstruct fusion cDNA encoding histidine-tagged rPARG. The pCold I vector carrying the cDNA was purified and transfected into *Escherichia coli* BL21. The induction of rPARG expression in the *E. coli* and the preparation of cell lysis were carried out according to the manufacturer's instructions (Takara). The rPARG in the cell lysate was purified by Ni–NTA agarose column chromatography (QIAGEN).

Measurement of protein concentration

The protein concentration was determined using a protein assay kit (Micro BCA Protein Assay Reagent, PIERCE Co., USA) with bovine serum albumin as a standard.

Statistics

All values were expressed as mean ± SE. The significance of group comparisons was determined by paired Student's *t*-test. The values <0.05 were considered to be significant. All analyses were conducted with JSTAT (version 4.9) software.

Results

Mechanical stretch induces expression of COX-2 and iNOS in HIG-82 cells

Since the increased expression of COX-2 and iNOS in synovial tissues were observed in patients with TMD (*13–18*), we examined whether mechanical stress enhances the expression of both proteins in the synovial cells *in vitro*. A synovial fibroblast cell line, HIG-82 cells, were cultured on an elastic silicon membrane-chamber and stretched by a cyclic stretching apparatus. As shown in Fig. 1A and B, when the cells were cyclically stretched up to 120% of the control for 1 h, COX-2 was increased maximally to ~8-fold under the condition of 1,200 cycles/h. However, the expression was lowered at 2,400 cycles/h. On the other hand, the increase of COX-2 in the statically stretched cells was slight, in spite of more severe extension (140%). The expression of iNOS in the stretched HIG-82 cells was also examined (Fig. 1C). Although a detectable level of iNOS protein was constitutively expressed in the un-stretched cells, the iNOS expression was increased to ~2-fold under the condition of 600 or 1,200 cycles/h (Fig. 1D). In contrast, the static stretch under the conditions of 140% extension or more of repetitive stretch under the conditions at 120% extension and 2,400 cycles/h was less effective on iNOS expression as well as COX-2 expression. These results suggest that cyclic stretch enhances expression of COX-2 and iNOS in synovial cells.

Mechanical stretch of HIG-82 cells activates NF-κB

The expression of human COX-2 is regulated by multiple transcription factors (*36*, *37*). Similarly, iNOS expression is regulated by some transcription factors, though the related transcription factors are slightly different among species (*38–40*). Among the transcription factors, NF-κB is able to up-regulate the expression of both genes. Therefore, we examined whether or not the mechanical stretch substantially up-regulates the expression of a reporter luciferase gene with NF-κB responsive element. HIG-82 cells were transfected with pNF-κB-luc and then statically or cyclically stretched under the conditions of 120 or 140% extension for 1 h. The luciferase activity of the statically 140% stretched cell was increased to ~2.5-fold, compared with the un-stretched cells (Fig. 2A). The increase in the activity was comparable to that of the cells treated with TNFα under non-stretching conditions. However, the static 120% stretching was less effective (Fig. 2A). On the other hand, the luciferase activity of the cyclically 120% stretched cells were proportionally increased, depending on the cycle number

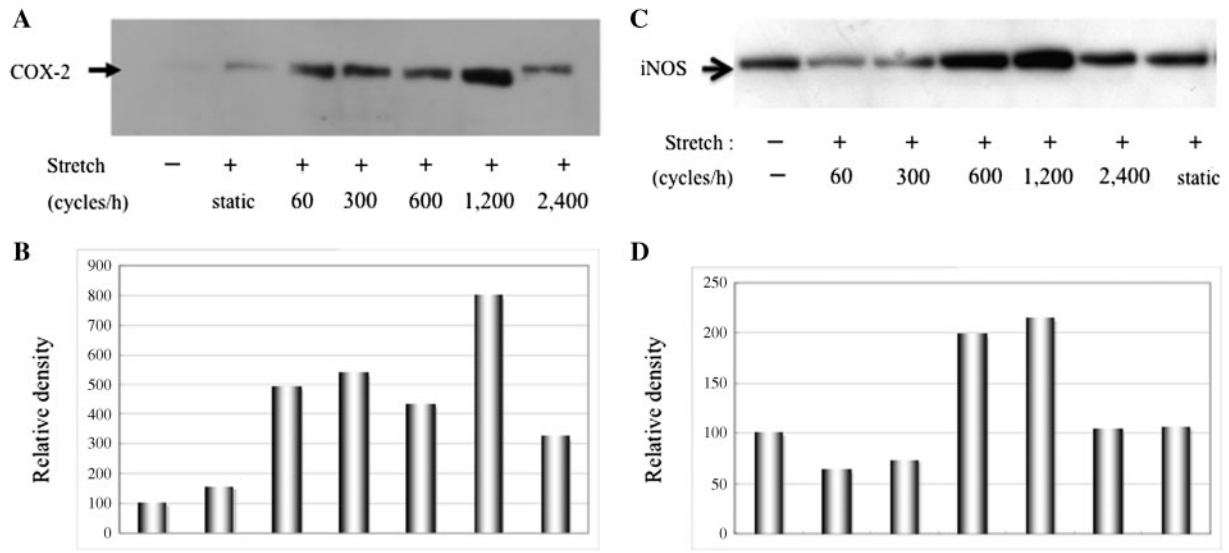

Fig. 1 Western blotting of COX-2 and iNOS in synovial HIG-82 cells. Rabbit synovial HIG-82 cells were seeded at a density of 1×10^6 cells/10 cm^2-chamber and cultured for 24 h and then uni-axially stretched under static or cyclic condition in a CO_2-incubator for 1 h as described in 'Materials and Methods' section. In the static condition, the extension degree of the chamber was set at 140%. On the other hand, under the cyclic stretch condition, the cycle numbers of stretch were varied from 60 to 2,400 cycles/h at a fixed 120% extension degree. After stretching, the cells were further cultured for 30 min under non-stretched conditions. After the culture, the cells were harvested, collected by a low centrifugation, and lysed in 100 µl-aliquots of the buffer D containing the protease inhibitors. The cell lysates were centrifuged, and the resulting supernatant was subjected to SDS–PAGE and then western blot analyses as described in 'Materials and Methods' section. (A) Proteins (20 µg) extracted from the cells were separated by SDS–PAGE and subjected to immunoblot analysis using an anti-COX-2 antibody as described in 'Materials and Methods' section. The symbols (−) and (+) mean un-stretch and stretch, respectively. (B) The densitometrical analysis of the immuno-stained bands (A) was carried out using software, Image J. The data shown represent relative density compared with negative control (un-stretched cells). (C) Like in (A), the proteins (50 µg) were separated by SDS–PAGE and subjected to immunoblot analysis using an anti-iNOS antibody as described in 'Materials and Methods' section. The symbols (−) and (+) mean un-stretch and stretch, respectively. (D) The densitometrical analysis of the immuno-stained bands (C) was carried out as described in (B).

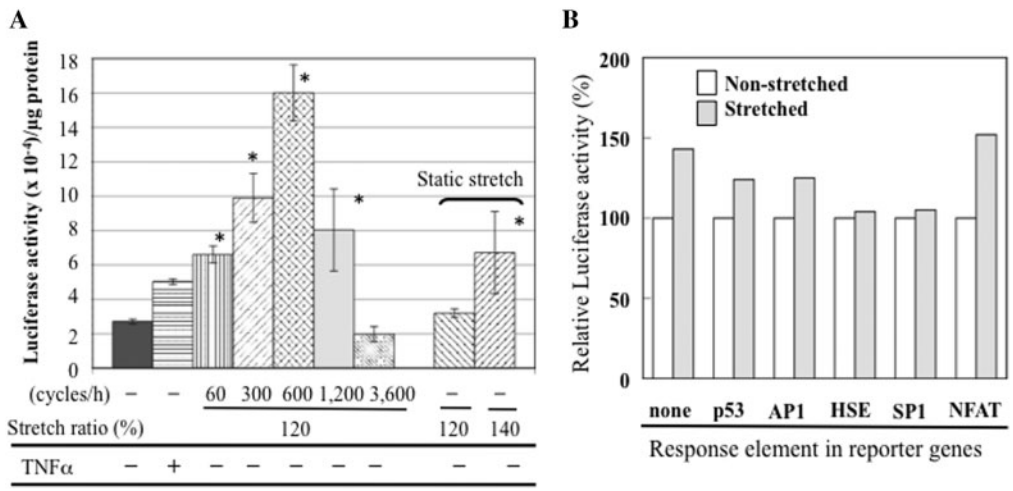

Fig. 2 The effect of mechanical stretch on the expression of luciferase gene constructs. In order to examine the effect of stretch on NF-κB responsive gene expression, reporter assays were carried out. HIG-82 cells transfected with luciferase gene constructs containing various response elements were mechanically stretched and subjected to the reporter assay as described in 'Materials and Methods' section. (A) HIG-82 cells transfected with pNF-κB-luc. were statically (120 and 140%-extension) or repetitively stretched under 60–3,600 cycles/h (120%-extension) for 1 h. As a positive and a negative control, un-stretched cells were treated with (+) or without TNFα (10 ng/ml) (−). After the treatments, relative luciferase activity was determined as described in 'Materials and Methods' section. The data shown represent the average values of three independent experiments with standard error bar. Asterisks denote that those values are significantly different ($P<0.05$) from control. (B) HIG-82 cells transfected with various luciferase gene constructs containing different response elements were stretched or un-stretched under a condition at 600 cycles/h and 120% extension. The p53, AP1, HSE, SP1 and NFAT show reporter luciferase gene constructs with response elements for binding of p53, activator protein-1, heat shock factor, specificity protein-1 and nuclear factor of activator T cells, respectively. None means pTA-luc that lacks response elements. The luciferase activities of non-stretched cells were set at 100%, respectively. The data shown represent the average values of two independent experiments.

of stretch, and at 600 cycles/h of the stretch, the enzyme activity attained a maximum (5.8-fold of the un-stretched cell) (Fig. 2A). These results indicate that the cyclic stretch effectively induces the activation of NF-κB. An increase in the cycle number (1,200 and 3,600 cycles/h), however, was weakly effective on the induction of the expression, implying severe damage of the cells by excessive extension.

In contrast to NF-κB dependent up-regulation of the luciferase gene, the cyclic stretch at 600 cycles/h was less effective on the expression of reporter luciferase genes constructed with other response elements for several other transcription factors, p53, AP1 (activator protein-1), HSF (heat shock factor), Sp1 (specificity protein-1) and nuclear factor of activated T (NFAT) cells (Fig. 2B). Similarly, the increase in expression of the luciferase gene construct without response elements was slight under the same conditions (Fig. 2B). Among the transcription factors tested here, the response elements for SP1 and NFAT exist in the COX-2 gene promoter (*36*, *37*), and a response element for AP-1 exists in human and rat iNOS gene promoter, but not in mouse promoter (*39*, *40*). These results suggest that cyclic stretch preferentially induces NF-κB-dependent gene expression.

In order to further confirm that cyclic stretch can induce NF-κB activation, HIG-82 cells transfected with NF-κB-responsive EGFP vector (pNF-κB-EGFP) was cyclically stretched under a condition of 600 cycles/h and 120% extension for 1 h and then observed under fluorescent microscopy (Fig. 3). As shown in Fig. 3B′, a part of the stretched cells expressed green fluorescent protein, indicating that cyclic stretch induces NF-κB-dependent gene expression in synovial cells.

Taken together, these results suggest that cyclic stretch significantly stimulates the expression of NF-κB-responsive gene in synovial cells *via* activation of NF-κB.

Inhibitors of NF-κB activation attenuate stretching-induced expression of NF-κB responsive luciferase gene

To examine whether the mechanical stretch induces the activation of NF-κB, the pNF-κB-luc-transfected HIG-82 cells were pretreated with several inhibitors of NF-κB activating pathways for 1 h prior to the stretching (Fig. 4). It has been reported that there are at least two types of pathways to activate NF-κB (*41*). A classical NF-κB activating pathway (canonical pathway) is induced by a variety of mediators such as TNFα and IL-1β.Another one (non-canonical pathway), which is mainly involved in secondary lymphoid organ development and adaptive immunity, is induced by mediators such as B-cell activating factor (BAFF) and lymphotoxin β (LTβ) (*41*). Since the canonical pathway includes processes of IKK (IκB kinase)-mediated dissociation of NF-κB/IκB (inhibitor of NF-κB) complex and degradation of IκB by proteasome (*41*), several types of inhibitors were used: Curcumin (Cur 20 or 40 μM) and BAY11-7085 (BAY; 2 μM) are known as IKK inhibitors (*42*, *43*). The proteasome inhibitor MG132 (MG; 2 μM) inhibits NF-κB activation by preventing IκB degradation (*44*). PDTC (PD; 5 μM) inhibits the IκB-ubiquitin ligase activity and/or nuclear translocation of NF-κB (*45*).

When the HIG-82 cells pretreated with these inhibitors were stretched under a condition with 120% extension and 600 cycles/h for 1 h, the NF-κB responsive luciferase gene expression was strongly attenuated (Fig. 4). On the other hand, the pretreatment of

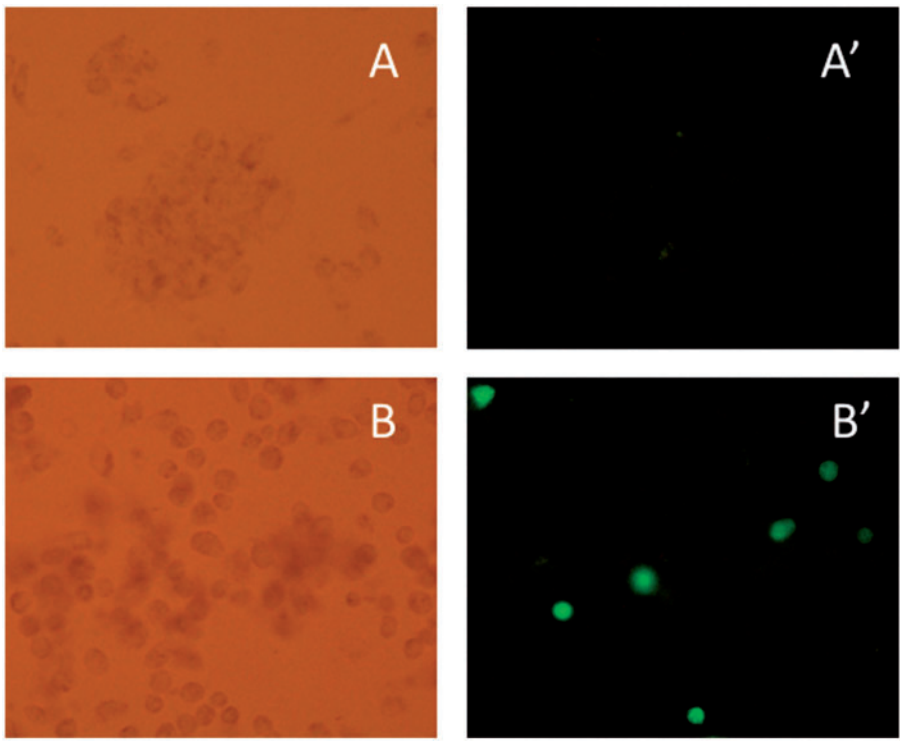

Fig. 3 The mechanical stretch-induced expression of NF-κB-responsive EGFP gene in synovial cells. HIG-82 cells transfected with NF-κB-responsive EGFP plasmid was cyclically stretched (B and B′) or un-stretched (A and A′) under a condition with 600 cycles/h and 120% stretch, and then observed by fluorescent microscope (200× magnification). The photographs on the left panels are bright field images (A and B) and those on the right panels are fluorescent images (A′ and B′).

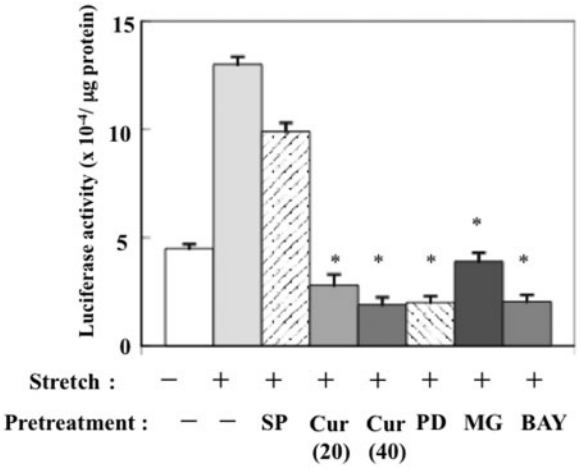

Fig. 4 Suppression of mechanical stretch-induced luciferase gene expression by inhibitors of NF-κB activation. HIG-82 cells transfected with pNF-κB-luc were pretreated with or without several inhibitors of NF-κB activation pathways for 1 h prior to stretching. After the pretreatments, the cells were cyclically stretched under a condition with 120% extension and 600 cycles/h for 1 h (+ and − mean stretch and un-stretch, respectively). The data shown represent the average values of three independent experiments with standard error bar. Asterisks denote that those values are significantly different ($P<0.05$) from a control (stretched cells without inhibitors). The abbreviations used are: SP, SP600125 (5 μM); Cur, curcumin (20 or 40 μM); PD, PDTC (5 μM); MG, MG132 (2 μM); BAY, BAY11-7085 (2 μM).

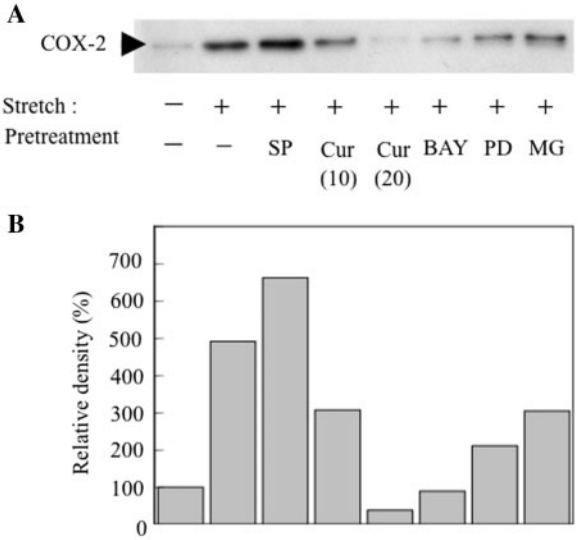

Fig. 5 Suppression of mechanical stretch-induced COX-2 expression by inhibitors of NF-κB activation. (A) HIG-82 cells were pretreated with several inhibitors of NF-κB activation for 1 h prior to stretching. After the pretreatments, the cells were cyclically stretched under a condition with 120% extension and 600 cycles/h for 1 h. Proteins (20 μg) prepared from cells were separated by SDS–PAGE and subjected to immunoblot analysis using an anti-COX-2 antibody as described in 'Materials and Methods' section. The abbreviations used are: SP, SP600125 (5 μM) Cur, curcumin (10 or 20 μM); BAY, BAY11-7085 (2 μM); PD, PDTC (5 μM); MG, MG132 (2 μM). The symbols (−) and (+) mean un-stretch and stretch, respectively. (B) The densitometrical analysis of the immuno-stained bands (A) was carried out as described in Fig. 1. The data shown represent relative density compared with negative control (un-stretched cells).

SP600125 (SP; 5 μM), which is a potent and selective JNK (c-Jun N-terminal kinase) inhibitor (*46*), was less effective (Fig. 4).

These results suggest that cyclic stretch substantially affects NF-κB activating pathway, which is probably the canonical pathway.

The inhibitors of NF-κB activation suppress stretch-induced expression of COX-2

Next, to confirm that the mechanical stretch-induced expression of COX-2 is practically dependent on the activation of NF-κB, HIG-82 cells were pretreated with four types of inhibitors of NF-κB activating pathway and then subjected to cyclic stretch under the same conditions described in the above section. As a result, the pretreatment of these inhibitors counteracted the effect of mechanical stretch on the expression of COX-2 in HIG-82 cells (Fig. 5). Especially, 20 μM curcumin (Cur) decreased the amount of COX-2 below the level of control (un-stretched cells), whereas the JNK inhibitor SP600125 (SP) did not. These results indicate that cyclic stretch induces the expression of COX-2 *via* activation of NF-κB.

Mechanical stretch enhances nitric oxide (NO) synthesis in synovial cells

Two groups independently proposed a hypothesis that reactive oxygen species (ROS) are generated in articular tissues by mechanical stress, resulting in occurrence of damage in the tissue (*23*, *24*). In our result, iNOS that synthesizes NO was increased in the stretched cells (Fig. 1C). Therefore, the increase of NO in culture media after cell stretching was examined (Fig. 6). As shown in this figure, NO was increased, correlating with cycle number. At 1,200 cycles/h, NO in the culture medium was increased to ~3.5-fold of that of un-stretched cells, suggesting that *de novo* synthesized iNOS is closely related to the increase in NO in synovial cells. On the other hand, the pretreatment of the cells with 2 μM BAY11-7085 limited the increase to ~1.4-fold of that of un-stretched cells (data not shown).

Mechanical stretch enhances PAR synthesis in synovial cells

It is well known that reactive oxygen and reactive nitrogen species (ROS/RNS) such as hydroxyl radical and peroxynitrite cause DNA injury, and in turn PARP-1 is activated by its binding to the generated DNA nick, resulting in excessive synthesis of PAR, which is covalently bound to acceptor proteins (*47*, *48*). Thus, if ROS/RNS are generated during the mechanical stretch, PAR synthesis is probably increased in the stretched cells. In order to examine the effect of cyclic stretch on PAR synthesis, HIG-82 cells were stretched under various cycles. As shown in Fig. 7A, the amount of PAR significantly began to increase at 60 cycles/h and attained a maximal level at 300 cycles/h, indicating an excessive accumulation of PAR during the cyclic stretch. The immunological

signal of PAR was mainly detected as a broad-band located at the position of about 100 kDa or more, suggesting that main poly(ADP-ribosyl)ated protein is PARP-1 itself, that is PARP-1 associated with heterogeneous lengths of ADP-ribose [auto-poly(ADP- ribosyl)ation] (*49*). The amount of PAR in the cells is controlled by PAR-synthesizing enzyme PARP and PAR-degrading enzyme PARG (*50*). In order to examine whether or not the accumulation of PAR in the stretched cells is due to lowering of PARG activity, the enzyme activity of the stretched cells were compared with that of non-stretched cells (Fig. 7B). Although the PARG activities in the cells stretched at 600 and 1,200 cycles/h were slightly decreased, the cyclic stretch hardly affected PARG activity, suggesting that the excessive PAR accumulation is due to *de novo* PAR synthesis caused by cell stretching.

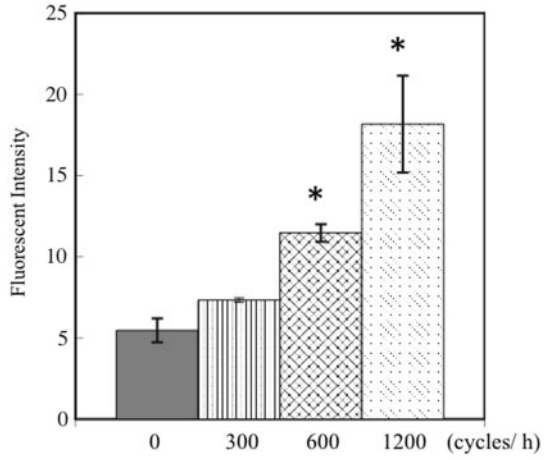

Fig. 6 Mechanical stretch-induced NO generation in synovial HIG-82 cells. Synovial HIG-82 cells (1×10^6 cells/10 cm^2) were stretched at 120%-extension and the indicated cycles for 1 h and then further cultured for 0.5 h under non-stretch condition. The amounts of nitric oxide (NO) in culture medium were measured as described in 'Materials and Methods' section. The NO amount was assessed as fluorescent intensity of the reaction products. The data shown represent the average values of three independent experiments with standard error bar. Asterisks denote that those values are significantly different ($P<0.05$) from control (un-stretched cells).

The amounts of PAR in the stretched cells at more than 600 cycles/h were less than that of the stretched cells at 300 cycles/h (Fig. 7A). It may be due to consumption of NAD^+ and ATP by rapid metabolism of PAR (*50*). On the other hand, the static stretch of the cells under 120% extension was hardly effective on the increase in PAR as well as COX-2 expression (Fig. 7A).

In contrast to the effect on PAR synthesis, cell stretching effectively enhanced COX-2 expression in a broad range from 60 to 1,200 cycles/h (Fig. 7A). β-Actin as a negative control showed slight increases under the conditions with the cyclic stretch.

These results demonstrate that cell stretch induces excessive PAR synthesis, especially automodification of PARP-1.

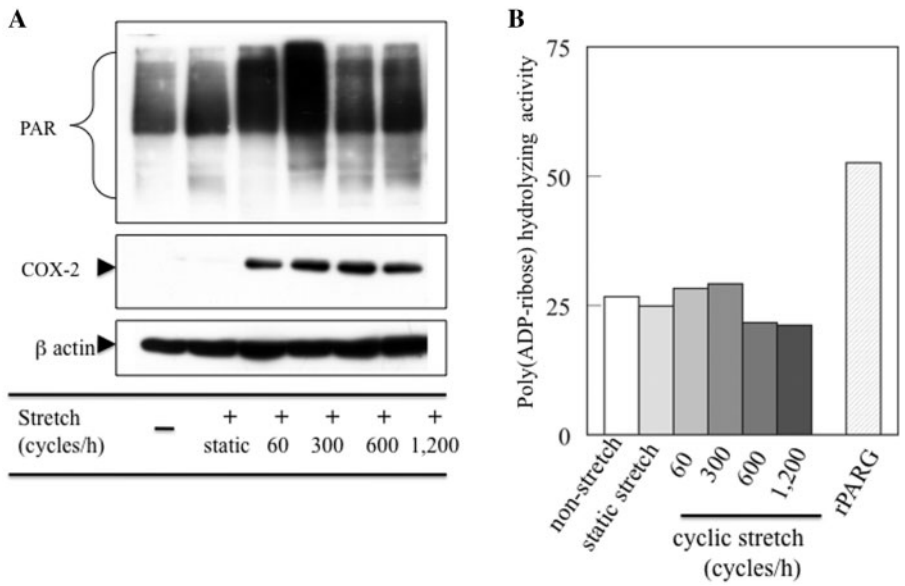

Fig. 7 Stimulation of poly(ADP-ribose) synthesis in mechanically stretched synovial cells. Synovial HIG-82 cells (1×10^6 cells/10 cm^2) were cultured for 24 h and then uni-axially stretched under static or cyclic condition in a CO_2-incubator for 1 h as described in Fig. 1. In the static condition, the extension degree of the chamber was set at 120%. On the other hand, under the cyclic stretch condition, the cycle numbers of stretch were varied from 60 to 1,200 cycles/h at a fixed 120% extension degree. After the culture, the cells were harvested and lysed in 100 μl-aliquots of the buffer D containing the protease inhibitors. The cell lysates were centrifuged, and the resulting supernatant was subjected to western blot analyses (A) or assay for PARG activity (B) as described in 'Materials and Methods' section. (A) Proteins (20 μg) extracted from the cells were subjected to SDS–PAGE and immuno-blotting using anti-PAR, anti-COX-2, and anti-β actin antibodies, respectively. The arrows indicate the positions of molecular weight markers with 145.3, 95.5 and 69.6 kDa, respectively. (B) The extracted proteins (10 μg) were subjected to assay for PARG activity as described in 'Materials and Methods' section. The intensity of the fluorescent products derived from free ADP-ribose was measured under a condition with excitation at 360 nm and emission at 445 nm. The PARG activity was assessed as the intensity of fluorescent ADP-ribose derivatives. As a positive control, 40 ng of purified human rPARG was used in place of the cell extracts.

The inhibitors of PARP and the free radical scavenger enhance the mechanical stretch-induced expression of COX-2 and iNOS

Since the cyclic stretch induces both PAR synthesis and NF-κB-dependent gene expression, the correlation between the PAR synthesis and the expression of COX-2 and iNOS in the stretched HIG-82 cells were examined. The cells were pretreated with 0.1 mM DPQ, which is a potent PARP inhibitor (*28*) and has no antioxidant activity (*51*), prior to the cell stretching (Fig. 8A). As shown in this figure, DPQ suppressed almost completely the cyclic stretch-induced PAR synthesis, indicating that the stretch-induced PAR synthesis is due to excessive activation of PARP. Like DPQ, pretreatment of the cells with 5 mM NAC, a radical scavenger (*29*), significantly reduced the cyclic stretch-induced PAR synthesis (Fig. 8A), suggesting that mechanical stress generates radical(s) and that the radical(s) is involved in the PAR synthesis presumably through DNA injury (*47*, *48*). On the other hand, the pretreatment of the cells with DPQ or NAC did not suppress the cyclic stretch-dependent COX-2 and iNOS expression (Fig. 8A). NAC rather enhanced the expressions of COX-2 and iNOS, whereas the stimulatory effect of DPQ was slight. In order to examine whether the enhanced expressions are due to the reinforced NF-κB activation *via* the inhibition of PAR synthesis, the reporter gene assay using pNF-κB-luc was carried out (Fig. 8B). The pretreatment with DPQ or NAC, however, did not enhance the stretch-induced and NF-κB-dependent expression of luciferase. This result suggest that the inhibition of PAR synthesis did not contribute to the NF-κB activation and that the inhibition enhanced mechanical stretch-mediated COX-2 and iNOS expressions in a NF-κB-independent manner.

Discussion

In this study, we demonstrated that the cyclic stretch affects two cellular events, NF-κB-dependent expression and PAR synthesis in rabbit synovial cells.

COX-2 and iNOS are inducible enzymes up-regulated by various proinflammatory stimuli and are involved in the synthesis of inflammatory mediators PGE_2 and NO, respectively, which become causes of pain and inflammation in various tissues (*2*). Several lines of evidence suggest that NO is involved in the pathogenesis of TMD including synovitis (*52*): the NO level in synovial fluid (SF) isolated from inflamed human TMJ was significantly higher than that of healthy TMJ (*9*), and the increased expression of iNOS was observed in synovial tissue of human TMJ with internal derangement (ID) (*16–18*). In this study, the cyclic stretch-induced iNOS expression of rabbit synovial cells was accompanied by the increase of NO in the culture media (Figs 1C and 6). The NO level in the medium of the stretched cells was elevated to ~2.2- and 3.5-fold under stretch at 600 and 1,200 cycles/h, respectively (Fig. 6), suggesting that excessive cyclic stretch on the synovial tissue is a powerful trigger for appearance of an inflammatory mediator. Like iNOS, there are some reports showing strong correlation between the expression of COX-2 in synovial tissue and onset of TMD with ID in patients (*13–15*). However, it has not been elucidated how these enzymes are induced in the early stage of degeneration of TMJ tissues. In this study, the expression of COX-2 and iNOS of synovial cells were

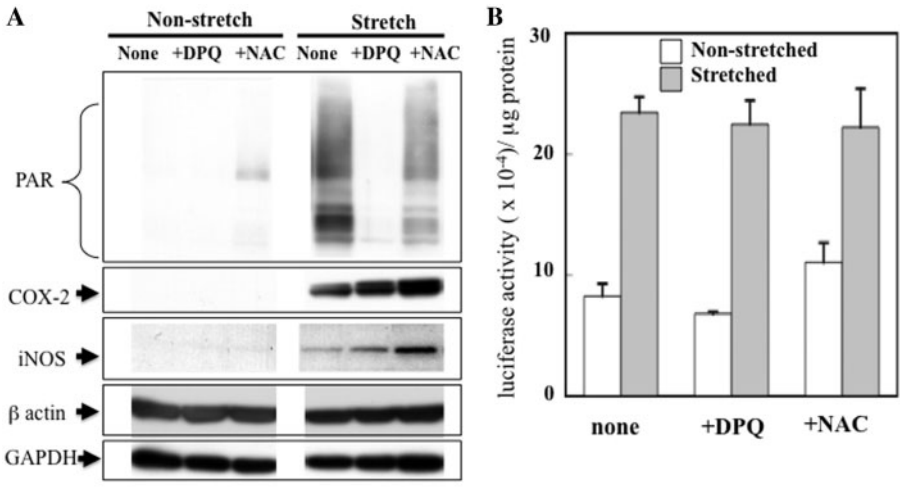

Fig. 8 Effect of a PARP inhibitor and a radical scavenger on stretch-induced PAR synthesis, expression of iNOS and COX-2, and pNF-κB luciferase. (A) Synovial HIG-82 cells (1×10^6 cells) were cultured in a 10 cm^2-chamber for 24 h and then pretreated with or without 0.1 mM DPQ or 5 mM NAC for 1 h. After the pretreatments, the cells were stretched under a condition with 1,200 cycles/h and 120% extension. After the culture, the cells were harvested and lysed in 100 μl-aliquots of the buffer D containing the protease inhibitors. The cell lysates were centrifuged, and the resulting supernatant was subjected to western blot analyses as described in 'Materials and Methods' section. The arrows indicate the positions of molecular weight markers with 145.3, 95.5 and 69.6 kDa, respectively. (B) The synovial cells (1×10^5 cells) were cultured in a 1.5 cm^2-chamber for 24 h and then transfected with pNF-κB luc as described in Fig. 2. After 24 h, the cells were pretreated with or without 0.1 mM DPQ or 5 mM NAC for 1 h and then stretched under the same condition as in (A). After the treatments, the luciferase activity was determined as described in the 'Materials and Methods' section. The data shown represent the average values of three independent experiments with standard error bar.

maximally increased to ~8- and 2-fold, respectively, under a condition of cyclic stretch at 1,200 cycles/h (Fig. 1), suggesting that in response to mechanical stress, articular synovial tissues can initiate iNOS and COX-2 expression and that they are involved in the inflammation process in TMJ. Two groups independently demonstrated the importance of NF-κB for the induction of inflammation in TMJ *via* the expression of iNOS and COX-2 (*21*, *22*). Although multiple transcription factors are independently involved in the expression of human COX-2 and iNOS, NF-κB up-regulates both the expressions (*19*, *20*). Our present study showed the cyclic stretch-dependent expression of exogenous NF-κB-responsive genes as well as endogenous COX-2 and iNOS genes (Figs 1, 2A and 3), supporting the importance of NF-κB in induction of inflammation of articular synovial tissues. NF-κB consists of two different subunits, and there are at least two types of NF-κB. One is a heterodimer composed of either p65 or RelA and p50 [p65(RelA)/p50], which is distributed in various cells and activated through dissociation from an inhibitory protein IκB (canonical pathway). Another is a heterodimer composed of RelB and p100 (RelB/p100), which is mainly localized in immunocompetent cells such as macrophages and activated by degradation of the p100 subunit to p52 (non-canonical pathway) (*41*). In the canonical pathway, the inactive form of NF-κB, p65(RelA)/p50/IκB complex, is sequestered in cytosol. In response to stimuli such as IL-1β and TNFα, IKK (IκB kinase) is activated and phosphorylates IκB. The phosphorylated IκB is dissociated from the p65(RelA)/p50 and in turn the heterodimer translocates to the nucleus (*41*). In our previous study with the same synovial cells, like TNFα, cyclic stretch induced translocation of p65(RelA)/p50 into the nucleus and enhanced COX-2 expression (*33*). In addition, all of the three inhibitors, which target different steps in the canonical pathway, strongly suppressed the mechanical stretch-induced increase in the pNF-κB luciferase expression (Fig. 4) and the endogenous COX-2 expression (Fig. 5). Previous reports showed that in endothelial cells, mechanical stress could cause activation of AP-1 together with NF-κB (*53*, *54*). It has also been shown that AP-1 promotes COX-2 gene expression through the binding of AP-1 to CRE (cyclic AMP response element) in human cells stimulated by PMA or IL-1β (*55*, *56*). AP-1 is phosphorylated by JNK and thereby acquires a potent *trans*-activating activity (*57*). In our results using AP-1 responsive luciferase gene, however, the cyclic stretch was less effective on the AP-1-dependent luciferase expression in the synovial cells (Fig. 2B). In addition, a JNK inhibitor, SP600125 did not suppress the cyclic stretch-induced COX-2 expression in the cells (Fig. 5). This result suggests that AP-1 is not involved in the cyclic stretch-induced COX-2 expression in synovial cells. It has been shown that JNK is also involved in the activation of NF-κB through accumulation of β-TrCP (β-transducin repeat-containing protein), resulting in activation of p65(RelA)/p50 (*58*). However, the JNK inhibitor was less inhibitory to the cyclic stretch-induced NF-κB responsive luciferase expression (Fig. 4). Thus, although a possibility that other transcription factors are involved in COX-2 and iNOS expression cannot be completely excluded, these results suggest that cyclic stretch induces NF-κB-dependent gene expression *via* the canonical pathway.

Previously, Milam *et al*. (*23*) proposed a model to explain how mechanical stress causes disorders of TMJ. They speculated that excessive jaw movements generate free radicals, and that the generated free radicals directly and/or indirectly affect various cellular molecules in TMJ, resulting in impairment of the articular tissues. Indeed, since reactive oxygen species (ROS) in synovial fluid (SF) were significantly elevated in patients with TMD (*59*), it is conceivable that ROS affect the articular synovium (*60*). Matrix metalloproteinase-3 (MMP-3), of which expression is up-regulated by NF-κB (*61*), is an effector to degrade TMJ tissues (*62*). With respect to this, our recent study has demonstrated that nitrated MMP-2 and -3 were detected in SF isolated from TMD patients with ID, implying an involvement of peroxynitrite in degeneration of TMJ, and that MMP-3 activity of SF was enhanced by treatment with a donor of peroxynitrite SIN-1 *in vitro* (*63*). Since peroxynitrite is generated from a reaction of NO with superoxide anion (*4*), ROS probably plays a pivotal role in development of TMD. Among the effects of ROS on various cellular events, DNA injury by peroxynitrite and hydroxyradical is extremely toxic for cells (*64–66*). Peroxynitrite- and/or hydroxyradical-mediated DNA injuries result in occurrence of DNA strand breaks, and in turn the strand break activates PARP-1, which binds to DNA nick and initiates poly(ADP-ribosyl)ation of various proteins, especially PARP-1 itself [autopoly(ADP-ribosyl)ation] (*25*, *26*). In the present study, the remarkable enhancement of PAR synthesis, that is mainly autopoly(ADP-ribosyl)ation, were observed in the cyclic stretched cells, implying DNA injury occurred in the stretched synovial cells (Fig. 7A). A potent PARP inhibitor, DPQ strongly suppressed the PAR synthesis (Fig. 8A). Since a radical scavenger, NAC could suppress the cyclic stretch-dependent PAR synthesis, ROS is probably involved in the occurrence of DNA injury (Fig. 8A). Thus, cyclic stretch-induced accumulation of ROS/RNS including NO may trigger activation of PARP-1.

Is the PARP-1 activation closely related to the function of NF-κB? Several lines of evidence have shown that the PARP-1 molecule itself and/or autopoly(ADP-ribosyl)ation is essential for NF-κB-dependent transcription (*67–69*). These studies have shown that PARP-1 is a co-activator of NF-κB (*67*) or a regulator of NF-κB activation cascade in mouse myocardiocytes in a successive process of ischemia and reperfusion (*68*). Alternatively, autopoly(ADP-ribosyl)ation is a regulator of NF-κB-dependent transcription in mouse microglia cells stimulated by lipopolysaccharide (*69*). In addition, the evidence that TNFα-induced MMP-3 expression in synovial cells isolated from patients with rheumatoid arthritis was partially suppressed by treatments with a PARP inhibitor or PARP knock down by siRNA suggest the involvement of PARP-1 in NF-κB-dependent

transcription (*70*). On the other hand, another group has emphasized the dual roles of PARP-1 (*71*). Under physiological conditions, PARP-1 is an inhibitory factor of NF-κB *via* its association with the NF-κB p50 subunit, whereas under a condition exposed to oxidative stress, damaged DNA-dependent autopoly(ADP-ribosyl)ation occurs and leads to dissociation of NF-κB p50 from the automodified PARP-1, resulting in association of the p50 subunit with p65 subunit and thereby NF-κB activation. However, a previous study has also shown NFκB[p65(RelA)/p50] can be poly(ADP-ribosyl)ated and thereby lower DNA binding activity of NF-κB (*30*). Moreover, PARP-1-defect increased the amount of active form of NF-κB in the nucleus in mouse lymphocytic leukemia L1210 cells, suggesting negative regulation of NF-κB by PARP-1 (*30*). Thus, the role of PARP-1 in the function of NF-κB is still controversial and remains to be elucidated. In our present study, the stretch-induced NF-κB-dependent luciferase expression was unchanged by pretreatment of synovial cells with DPQ or NAC (Fig. 8B), nevertheless cyclic stretch-induced COX-2 and iNOS expression were augmented by the pretreatment (Fig. 8A). This result indicates that inhibition of poly(ADP-ribosyl)ation does not affect the NF-κB-dependent gene expression caused by cyclic stretch. Why could be COX-2 and iNOS proteins increased without augmentation of NF-κB *trans*-activating activity? One possibility is that another common transcription factor, NF-IL-6 (nuclear factor for IL-6) (*36–40*), cooperatively up-regulates expression of both under the condition of suppression of PAR synthesis. Another possibility is that like NF-κB, PARP-1 may function as a *trans*-activator of these genes. Interestingly, PARP-1 also plays a role as a novel *trans*-activator of iNOS promoter in mouse mesangial cells and IL-1β-induced iNOS gene transcription was lowered by knock down of PARP-1 expression (*72*). It has also demonstrated that NO nitrosylates PARP-1 and negatively regulates its *trans*-activating activity, resulting in suppression of iNOS gene transcription. In addition, they observed recovery of iNOS promoter activity in the presence of a NO scavenger (*72*). Consistent with the report, the increase of iNOS protein in NAC-pretreated and stretched synovial cells was more remarkable than that of the DPQ-pretreated cells or COX-2 protein of the stretched cells pretreated with NAC or DPQ (Fig. 8A). Thus, at least the augmentation of iNOS protein in the stretched cells pretreated with NAC may be a result of cessation of the NO-dependent negative feedback regulation of PARP-1. Taken together, these results imply that further studies will be required to elucidate the relationship between PARP-1 and expressions of COX-2 and iNOS. However, it is clear that mechanical stretch is involved in the activations of PARP-1 and NF-κB.

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Conflict of interest
None declared.

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