

Rac1 mediates load-driven attenuation of mRNA expression of nerve growth factor beta in cartilage and chondrocytes

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Abstract

Objectives: To determine effect of gentle loads applied to the knee on mRNA expression of nerve growth factor, particularly, the active beta subunit (NGF β) in cartilage and chondrocyte. **Methods:** Cyclic compressive loads *in vivo* and fluid flow *in vitro* were used to determine the mRNA levels. Alteration of Rac1 GTPase as well as effect of salubrinal, a specific inhibitor of eIF2 α phosphatase was assessed using fluorescence resonance energy transfer (FRET)-based Rac1 biosensor. **Results:** Knee loading at 1 N reduced mRNA levels of NGF β and its low affinity receptor, p75 in cartilage and subchondral bone. In cartilage, knee loading at 1 N reduced the phosphorylation level of p38 MAPK (p38-p) and activity of Rac1 GTPase. Consistent with *in vivo* results, fluid flow at 5 and 10 dyn/cm² reduced mRNA levels of NGF β and p75 in C28/I2 human chondrocytes. SB203580, which decreases p38-p, reduced the mRNA levels of NGF β and p75. Silencing Rac1 by siRNA decreased the levels of p38-p and NGF β mRNA but not p75. Furthermore, administration of salubrinal reduced FRET-based activity of Rac1 as well as the mRNA levels of NGF β and p75. **Conclusions:** These results provide evidence that mechanical stimulation and salubrinal may attenuate pain perception-linked NGF β signaling through Rac1-mediated p38 MAPK.

Keywords: Knee Loading, Fluid Flow, Cartilage, Chondrocyte, NGF

Introduction

Mechanical stimuli play a critical role in homeostasis of musculoskeletal tissues including bone and articular cartilage. Physical exercises, for instance, enhance new bone formation, while gentle mobilization of synovial joints reduces activities of degenerative enzymes such as matrix metalloproteinases (MMPs)¹⁻³. Knee loading is a modality that applies gentle, lateral loads to the knee, providing a model system for under-

standing loading effects on preventing bone loss and cartilage degradation^{4,5}. Animal studies suggest that knee loading not only stimulates bone formation in the tibia and femur but also reduces activities of MMPs in articular cartilage^{6,7}. In this study, we employed knee loading to explore potential effects on neuronal signaling, in particular pain perception. Massage therapy is a unique form of mechanical stimuli for treating a variety of health conditions, including joint disorders as a regimen to relieve pain for those with osteoarthritis of the knee^{8,9}. Although a growing body of evidence supports the efficacy of massage therapy^{10,11}, little is known about the mechanism of its pain relieving action. As pointed out recently, the origin of chronic pain in osteoarthritic joint is less clear whether it is due primarily to damage of sensory nerves in bone and inflammation in synovium or due in part to innervation from mesenchyme into the aneural entity, cartilage¹².

In this study, we sought a surrogate target to block for relieving pain in the articular cartilage of joints. We considered nerve growth factor (NGF), particularly, β -subunit (NGF β), as well as its low affinity receptor, p75 as potential markers for pain perception in the joint¹³. The level of NGF β was reported to be low in normal chondrocytes, increased in mild osteoarthritic cartilage and further enhanced in severe os-

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teoarthritis cartilage¹⁴. Among three subunits, the active component of NGF protein is 118-amino-acid sequence of the β subunit. The most studied class of trophic factors that are involved in trophic function or survival of neuron is the neurotrophins. Other than NGF, three major neurotrophins have been isolated from mammals. Unlike tyrosine kinases receptors (Trk), each neurotrophin binds to p75 with similar affinity thus p75 is also called 'a common receptor'. In particular, the activation of the p75 receptor has been shown to promote neuronal cell death thus suggested a therapeutic target for neuropsychiatric disease¹⁵. The previous report also suggested that NGF and p75 causes the pathogenesis of discogenic pain in intervertebral discs¹⁶.

Although cartilage is not an innervated tissue, the expression of NGF and its receptors is developmentally regulated¹⁷. A basal expression level of NGF β is high in embryos undergoing skeletal morphogenesis and low in mature cartilage. It is reported that its expression is linked to pain perception and increased in arthritic joints¹⁸. Herein we addressed a question: Does mechanical stimulation downregulate expression of NGF β in cartilage and chondrocytes? Since load-driven downregulation of MMPs is in part mediated by p38 mitogen activated protein kinase (MAPK) signaling and GTPases, we hypothesized that gentle mechanical loading reduces the mRNA levels of NGF β and p75 through p38 MAPK.

In testing our hypothesis, knee loading was applied to mice to determine a potential loading modality effective for downregulation of NGF and receptor genes in the cartilage. *In vitro* fluid flow experiments were also conducted using C28/I2 chondrocyte cells. We determined the mRNA levels of NGF β and p75 using quantitative PCR and the phosphorylation level of p38. Focusing on the role of Rac1 GTPase, its expression and activity were determined using immunoprecipitation of an active form of Rac1, RNA interference with siRNA specific to Rac1, and a fluorescence resonance energy transfer (FRET) technique with a Rac1 biosensor. Since a synthetic chemical agent, salubrinal, is reported to protect against neurotoxicity in the central nervous system, we examined its effect on expression of NGF.

Materials and methods

Animals

Experimental procedures were approved by the Indiana University Animal Care and Use Committee and were in compliance with the Guiding Principles in the Care and Use of Animals endorsed by the American Physiological Society. Three mice were housed per cage, and they were fed with mouse chow and water *ad libitum*. Two groups of C57/BL/6 female mice (~12 weeks, Harlan Laboratories) were used, in which the group I was for examining effects of time (1 h and 3 h), and the group II for testing effects of loading amplitude (1 N and 3 N). Six unloaded knees served as control.

Knee loading

Cyclic compression was applied to the mouse right knee using a custom-made piezoelectric loading device¹⁹. The

mouse was mask-anesthetized using 2% isoflurane, and lateral loads to the knee were applied for 5 min at 5 Hz with a peak-to-peak force of 1 and 3 N. The left hindlimb was used as sham-loaded control, with the left knee placed under the loading rod in the same procedure without applying a voltage signal to the loader. The femoral articular cartilage and subchondral bone tissue were harvested from loaded and non-loaded mice 1 h after the loading bout.

In vitro experiments

Human chondrocytes, C28/I2 cells²⁰, were cultured in DMEM (Lonza) containing 10% FBS (Hyclone) and antibiotics (Life Technologies). For mechanical stimulation, cells were grown on glass slides treated with 1% rat-tail collagen (BD Biosciences). Twenty four hours before flow application, cells were incubated in DMEM containing 0.5% FBS. The slides were loaded into a Streamer Gold flow device chamber (Flexcell International). For inhibition of p38 phosphorylation, cells were incubated with 10, 20 and 40 μ M SB203580 (Calbiochem) for 15 min. Salubrinal, an agent acting as an inhibitor of eIF2 α phosphatase (Tocris Bioscience), was administered at 2, 10, and 50 μ M.

Rac1 activity assay

The activity level of Rac1 was detected with a Rac1 activation assay kit (Millipore) using the procedure provided by the manufacturer. In brief, the protein sample was lysed in a magnesium lysis/wash buffer containing 10 μ g/ml leupeptin and 10 μ g/ml aprotinin. The cell lysate was incubated with a Rac1 assay reagent (agarose beads) to precipitate Rac1-GTP. The level of Rac1-GTP was detected with anti-Rac1 antibody using a standard Western blot procedure.

Silencing Rac1 GTPase using siRNA

To evaluate the role of Rac1 GTPase in regulation of p38 MAPK and NGF, cells were treated with siRNA specific to Rac1 GTPase (Invitrogen). The targeted Rac1 sequence was 5'-AGGGUCUAGCCAUGGCCUAAGGAGAU-3', while a negative siRNA (StealthTM siRNA negative control High, Invitrogen) was used as a nonspecific control. C28/I2 cells were transiently transfected with Rac1 siRNA or control siRNA in Opti-MEM I medium with Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. Six hours later, the medium was replaced by regular culture medium. The efficiency of silencing Rac1 was assessed with immunoblotting 48 h after the transfection.

Real-time PCR

The mRNA expressions of NGF β and p75 were determined using quantitative real-time PCR with the following primers: human NGF β -TCAGCATCCCTTGACTG (forward), TGCTCCTGTGAGTCCTGTTG (backward); human p75-GTGGGACAGAGTCTGGGTGT (forward), AAGGAGGGGAGGTGATAGGA (backward); human GAPDH- GCACCGTCAAGGCT GAGAAC (forward), ATGGTGGTGAAGAC

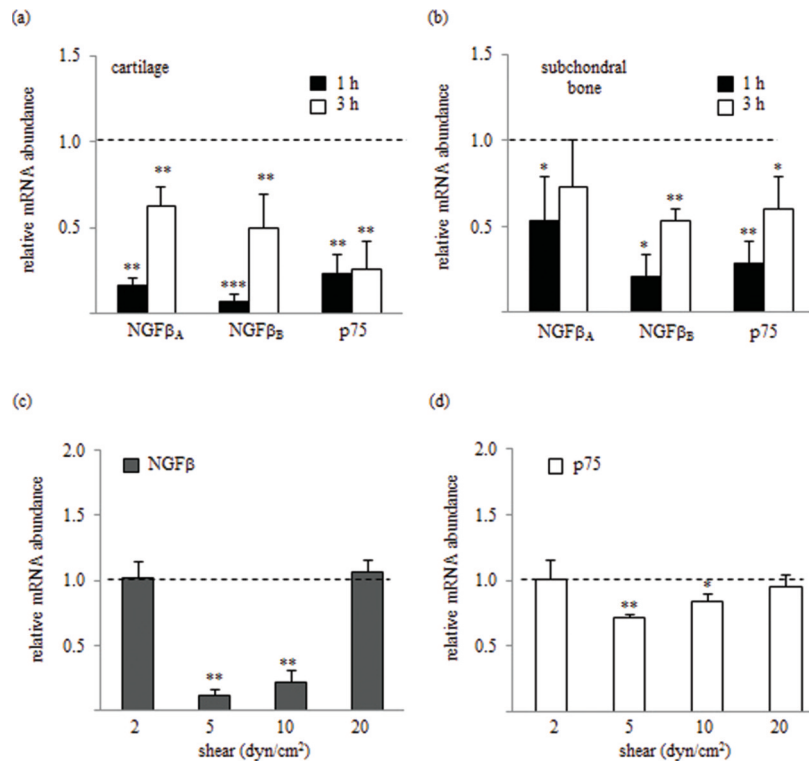


Figure 1. Effects of mechanical stimulation on the mRNA levels of NGF β and p75. **(A)** Effects of knee loading on the mRNA levels of NGF β -A, NGF β -B and p75 in the articular cartilage. **(B)** Effects of knee loading on the mRNA levels of NGF β -A, NGF β -B and p75 in subchondral bone. **(C)** Effects of shear stress on the levels of NGF β mRNA in C28/I2 cells. **(D)** Effects of shear stress on the levels of p75 mRNA in C28/I2 cells. The dash line represents the level of the control group.

GCCAGT (backward); mouse NGF β -A- CCAGT-GAAATTAGGCTCCCTG (forward), CCTTGGCAAACCTTTATTGG (backward); mouse NGF β -B- TGATCGGCGTACAGGCAGA (forward), GCTGAAGTTTAGTCCAGTGGG (backward); mouse p75- CTAGGGGTGTCCTTTGGAGGT (forward), CAGGGTTCACACACGGTCT (backward); mouse GAPDH- TGCACCACCAACTGCTTAG (forward), GGATGCAGAGAAGATGTTTC (backward). Total RNA was extracted using an RNeasy Plus mini kit with Qiazol (Qiagen) for cartilage and β -mercaptoethanol-based reagent for chondrocyte as starting buffer, respectively. Reverse transcription was performed, and real-time PCR was carried out using ABI 7500 with SYBR green PCR kits (Applied Biosystems). The mRNA level of GAPDH was used as an internal control. Data were further normalized with respect to unloaded controls in the *in vivo* and *in vitro* experiments²¹.

Western blot analysis

Samples isolated from cartilage were dissociated with a mortar and pestle in a lysis buffer containing the following: 20 μ l phosphatase inhibitor, 5 μ l proteinase inhibitor, 5 μ l phenylmethanesulfonylfluoride, and 5 μ l sodium orthovanadate (Calbiochem) in 500 μ l RIPA (Santa Cruz). BSA assay (Thermo scientific) was conducted for quantification of proteins extracted from tissues

and 1:10 dilution of the initial extract in RIPA is applied to be in the working range (125-2,000 μ g/ml). Samples isolated from C28/I2 chondrocytes were transferred to the RIPA lysis buffer containing inhibitors for proteases and phosphatases. Isolated proteins in the working range (125-2,000 μ g/ml) were fractionated using 10% SDS gels and electro-transferred to Immobilon-P membranes (Millipore). Immunoblots were carried out using antibodies specific to p38 MAPK, phospho p38 MAPK, and β -actin (Sigma). After incubation with secondary antibodies conjugated with HRP, signals were detected with ECL chemiluminescence. Images were captured using an image analyzer (LAS-3000, Fuji Photo Film) and analyzed using Multi Gauge V 3.0 software.

Fluorescence resonance energy transfer (FRET)

To visualize Rac1 activity in response to salubrinal, FRET imaging was conducted using a cyan fluorescent protein (CFP)-yellow fluorescent protein (YFP) Rac1 biosensor. The filter sets (Semrock) were chosen for CFP excitation at 438 \pm 24 nm (center wavelength \pm bandwidth), CFP emission at 483 \pm 32 nm, and YFP emission at 542 \pm 27 nm. Time-lapse images were acquired at an interval of 5 min using a fluorescence microscope (Nikon). The level of Rac1 activity was determined by computing an emission ratio of YFP/CFP for individual cells using NIS-Elements software (Nikon).

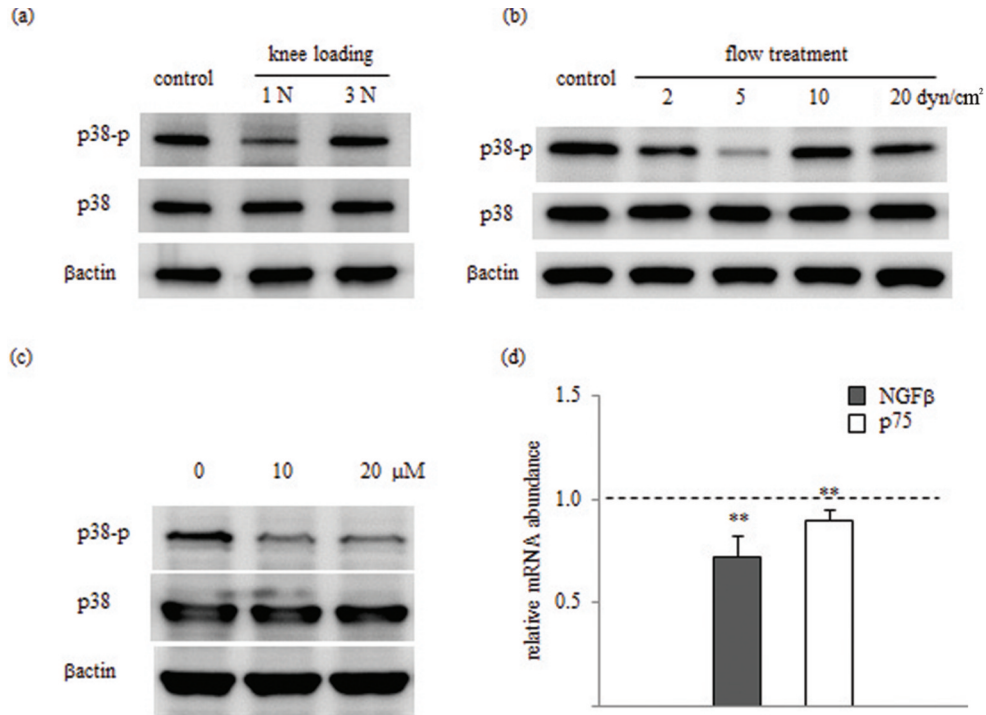


Figure 2. p38 MAPK signaling. (A) Phosphorylation of p38 in mouse cartilage in response to knee loading. (B) Phosphorylation of p38 in C28/I2 cells in response to fluid flow at 2-20 dyn/cm². (C) Effects of SB203580 on phosphorylation of p38 in C28/I2 cells. (D) Effects of SB203580 on the mRNA levels of NGFβ and p75 in C28/I2 cells.

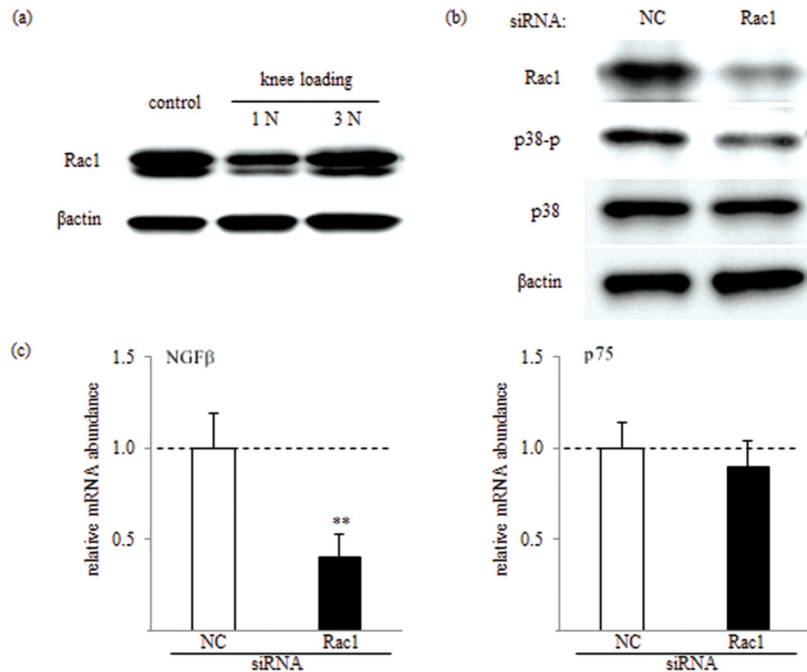


Figure 3. Involvement of Rac1 GTPase. (A) Activity of Rac1 in mouse cartilage in response to knee loading. (B) Effects of RNA interference with Rac1 siRNA on phosphorylation of p38 in C28/I2 cells. (C) Effects of Rac1 siRNA on the mRNA levels of NGFβ and p75 in C28/I2 cells. The dash lines represent the level of the control group. NC: non-specific control siRNA.

Statistical analysis

The *in vivo* experiment was conducted with $n=6$ per group. The *in vitro* experiment was independently carried out three times. Statistical significance was calculated using independent t test for two group comparison, and one-way ANOVA followed by Dunnett's *post hoc* test for more than two groups. Data are reported with S.E., and the asterisks (*, **, and ***) denote $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

Results

Mechanical stimulation downregulates NGF signaling

Compared to the non-loaded controls, knee loading at 1 N significantly reduced the mRNA levels of NGF β and p75 in the cartilage and subchondral bone. Such load-induced downregulation of NGF β and p75 mRNA persisted 3 h following the application of loading except for the mRNA level of NGF β variant A in subchondral bone (Figure 1a and 1b). Fluid shear at 5 and 10 dyn/cm² also downregulated the mRNA levels of NGF β and p75 in C28/I2 cells as compared to the no-flow controls (Figure 1c and 1d).

Phosphorylation of p38 is involved in loading effects on NGF signaling

Knee loading at 1 N but not at 3 N decreased the phosphorylation level of p38 (p-p38) in the cartilage (Figure 2a). Consistent with this reduction of p-p38 at relatively low amplitude *in vivo*, fluid flow at 5 dyn/cm² *in vitro* also reduced p-p38. At 2, 10, and 20 dyn/cm², however, flow-driven reduction of p-p38 was not observed, leading to biphasic response over different flow shear (Figure 2b). Administration of 10 and 20 μ M SB203580 suppressed p38 phosphorylation within 15 min in C28/I2 cells (Figure 2c). As compared to the controls, SB203580 (10 μ M) significantly reduced the level of NGF β mRNA ($p=0.0014$) and p75 mRNA ($p=0.007$) in 60 min in C28/I2 cells (Figure 2d).

Knee loading suppresses Rac1 and siRNA for Rac1 downregulates NGF

We further investigated whether Rac1 is involved in regulation of NGF mRNA through p38 MAPK. In the mouse cartilage, knee loading suppressed Rac1 GTPase at 1 N but not at 3 N (Figure 3a). Silencing of Rac1 GTPase by siRNA abolished production of Rac1 protein as compared to the negative control siRNA. The cells transfected with Rac1 siRNA showed a reduced level of p38 phosphorylation (Figure 3b). Subsequently, gene expression of NGF β was significantly downregulated by 2-fold ($p=0.0048$) in human C28/I2 chondrocytes transfected with Rac1 siRNA, while mRNA level of p75 did not show statistical difference (Figure 3c).

Salubrinal induces attenuation of NGF mRNA expression and Rac1 in chondrocytes

Salubrinal administration (10 μ M) led to downregulation of the mRNA levels of NGF β ($p=0.002$) and p75 ($p=0.019$) as compared to the vehicle control, 24 h following treatment to

human C28/I2 cells. Salubrinal at 50 μ M also gave rise to a reduction of mRNA levels for NGF β ($p=0.001$) but not p75 (Figure 4a). In the FRET-based assay, administration of salubrinal at 10 μ M significantly decreased activity of Rac1 GTPase in C28/I2 cells within 15 min and sustained its decrease more than 60 min (Figure 4b).

Discussion

It is reported that the expression of NGF β is elevated in osteoarthritic cartilage¹⁴. In this study, we demonstrate that knee loading at 1 N reduces the mRNA levels of NGF β and p75 in the articular cartilage and subchondral bone of the mice, and shear stress at 5 and 10 dyn/cm² on C28/I2 chondrocytes downregulates their mRNA levels. Pain of the knee joint is multi-factorial and inflammatory synovium and subchondral bone collapse are suggested to play a role in osteoarthritic joints¹⁴. Alternatively, it is hypothesized that the invasion of articular cartilage by vascularized mesenchymal tissues followed by the innervation of sensory nerves is associated with severity of injury as well as chronic pain²². Nerves exist in trabecular bone of the epiphysis²³, and they grow in response to NGF β ²⁴. Although healthy cartilage does not consist of vascular or neural tissues, arthritic cartilage loses its ability to remain aneural and avascular²⁵⁻²⁷. It has been reported that dynamic loading to cartilage evokes stimulation of matrix synthesis²⁸, as well as regulation of enzymatic activities of matrix metalloproteinases²⁹. In addition to the reported regulatory role in matrix homeostasis, the result herein points out that mechanical stimuli at moderate amplitudes regulate transcription of NGF β and its receptor in cartilage and chondrocytes.

Knee loading induces not only pressure alterations but also pressure driven fluid flow to chondrocytes. Unlike well-studied effects of normal stress on chondrocytes, it has been recently suggested that a consequence of compressive loading is production of hydrostatic pressure as well as fluid flow to cartilage³⁰. In osteoarthritis, chondrocytes are exposed to flow shear due primarily to synovial fluid and high amplitude of fluid flow reproduces the hallmarks of osteoarthritis *in vitro*³¹. The frequency of 5 Hz might not be representative of massage to humans by hands but more pertinent to those by vibrator for foot massage. The levels of loading *in vivo* have been optimized in our lab to produce anabolic response in the bone and cartilage. In the current study, 1 N is relatively optimal than 3 N in suppressing mRNA expression of NGF and p75. The culture model was derived from the previous report³⁰⁻³¹. Unlike the long duration exposure applied in the *in vitro* OA model, we found 0.5 h exposure at 5 and 10 dyn/cm² led to comparable effects in cells as in our *in vivo* compressive loading (1 N) from tissues.

Our immunoblot results were limited to p38, showing biphasic effects, as the lower load/fluid shear levels applied were inhibitory. As shown in wider force range, however, the mRNA data on load- and shear-driven alteration of NGF and p75 were more convincingly demonstrated the same biphasic responses. A more thorough study is warranted to address load-driven suppression of NGF at protein level.

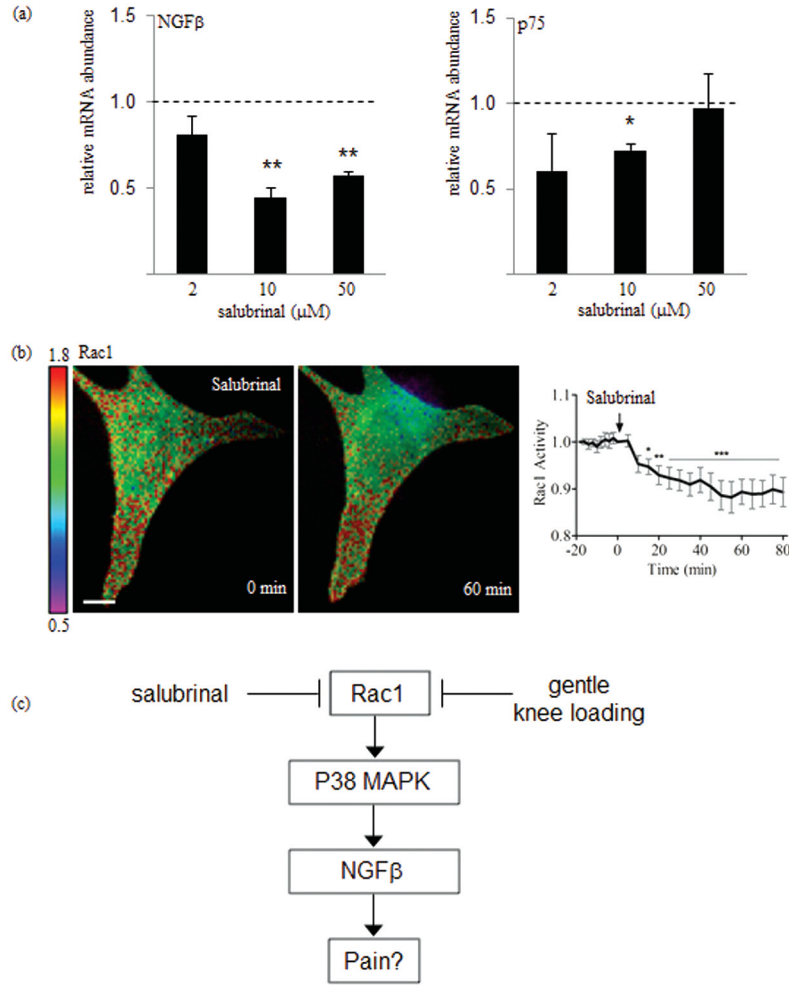


Figure 4. Effects of salubrinal. (A) mRNA levels of NGF β and p75 by 10 μ M salubrinal in C28/I2 cells. n=6. (B) Reduction in Rac1 activity in C28/I2 cells by FRET. The arrow indicates the commencement time (t=0) for salubrinal administration at 10 μ M to C28/I2 cells. The color bar represents emission ratio of YFP/CFP, an index of Rac1 activation. Ratio images were scaled according to the corresponding color bar. Scale bar=10 μ m. (C) Proposed mechanism of Rac1-mediated regulation of NGF β through p38 MAPK. In osteoarthritic joints, patients accompanying chronic pain are manifested with an elevated level of NGF β and p75.

In this study, we focused on the role of Rac1 in load-driven downregulation of NGF β . Rac1 has been shown to activate p38 MAPK³², and laminar fluid shear has been reported to induce transient alteration of p-p38 and Rac1³³. Consistent with those previous studies, we observed that Rac1 siRNA in C28/I2 cells reduced p-p38. In our siRNA experiment, however, load-driven downregulation of p75 was not affected by RNA interference. It is thus possible that other GTPases such as RhoA and cdc42 are involved in the regulation of p75, and possibly of NGF β . For instance, we have previously observed that the activity of RhoA alters in response to fluid flow in a flow-intensity dependent manner³⁴. Further analysis is necessary to determine a regulatory mechanism of p-p38 in cartilage and chondrocytes. Although TrkA is the high affinity receptor for NGF β , our pilot data using real time qPCR indicated that the basal expression of TrkA in human C28/I2 cells is 6 fold

lower than that of p75 (not shown). Thus, the current study was focused on p75 rather than Trk.

The *in vitro* result also reveals that salubrinal, a specific inhibitor for dephosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α), can attenuate transcription of NGF β and activity of Rac1. Our result suggests that suppression of both NGF β and p75 mRNA expression is achievable at 10 μ M but not necessarily at the higher dose at 50 μ M. In the central nervous system, salubrinal has been shown to protect against excitotoxic neuronal injury induced by Kainic acid³⁵. Kainic acid-induced brain injury is a long-standing animal model of seizure and is known to stimulate NGF expression in the hippocampus³⁶. However, any effect of salubrinal on the peripheral nervous system remains undetermined. Our *in vitro* results suggest that both gentle mechanical loading and salubrinal share the Rac1-mediated signaling pathway for mRNA expression of NGF β (Figure 4c).

In myocardial remodeling, it is reported that deficiency of Rac1 reduces stress to the endoplasmic reticulum³⁷. Since the elevated phosphorylation level of eIF2 α by salubrinal also suppresses stress to the endoplasmic reticulum, the observed linkage of salubrinal to Rac1 is consistent with downregulation of NGF β .

In response to administration of 10 μ M salubrinal, the response of the Rac1 biosensor in the FRET analysis presents variations among cells. Approximately 40% of the cells (7 out of 17 cells) exhibited a clear decrease in the activity of Rac1, while the others did not show a significant change. The observation clearly indicate that the result with PCR and Western blotting can only present the average response, and the degree of pain reception may not be necessarily represented by the response of a whole population of cells.

In summary, this study demonstrates that knee loading at 1 N and fluid flow at 5 dyn/cm² can attenuate mRNA expression of NGF β mediated by Rac1. The result suggests that gentle knee loading analogous to massage therapy is beneficial not only to enhancing bone formation and accelerating wound healing but also to preventing NGF β -induced nerve growth and pain perception in cartilage. Further analysis targeted to the loading effects on pain perception may warrant our basic understanding of the role of mechanical stimulation and massage therapy in relieving pains associated with joint diseases such as osteoarthritis.

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