Olmesartan inhibits cultured rat aortic smooth muscle cell death induced by cyclic mechanical stretch through the inhibition of the c-Jun N-terminal kinase and p38 signaling pathways

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Running title: Olmesartan reduces VSMC death by stretch

Abstract

Acute aortic dissection (AAD) is a life-threating disease; however, there is almost no effective pharmacotherapy for it. An increase in c-Jun N-terminal kinase (JNK) phosphorylation and smooth muscle cell (SMC) apoptosis is observed tissues in patients with AAD. Therefore, we hypothesized that an acute rise in blood pressure leads to SMC death through phosphorylation of JNK or p38, which may cause AAD. We investigated the influence of cyclic mechanical stretch, which mimics an acute increase in blood pressure, on cultured rat aortic SMCs (RASMCs) and examined the changes in JNK and p38 phosphorylation. Further, we investigated the effect of olmesartan, an angiotensin II receptor blocker, on stretch-induced RASMC death. We found that mechanical stretch induced RASMC death in a time-dependent manner, which correlated with the phosphorylation of JNK and p38. Olmesartan inhibited RASMC death and the phosphorylation of JNK and p38. JNK and p38 inhibitors reversed stretch-induced RASMC death. These results suggest that acute mechanical stretch causes JNK and p38 phosphorylation, which may result in SMC death leading to aortic dissection. Olmesartan may be used for pharmacotherapy to prevent aortic dissection, independent of its blood pressure-lowering effect, through its inhibition of JNK and p38 phosphorylation.

Keywords: stretch, c-Jun N-terminal kinase, p38, acute aortic dissection, olmesartan

Introduction

Acute aortic dissection (AAD) is a disease associated with high morbidity and mortality (1-3). AAD begins with a sudden initial tear in the aortic media, and this tear allows pulsatile blood to enter the media and cause separation of the medial layer along the effective length of the vessel (4-6). However, the molecular mechanisms by which the tear occurs are poorly understood (1, 7). Hypertension is present in 75% of individuals with aortic dissection, and is known as a primary risk factor for cardiovascular disease (1, 2). Thus, it may be also related to the onset of AAD (8). When surgical treatment is inapplicable, there is no effective treatment for AAD other than the reduction of blood pressure (9). Therefore, the development of nonsurgical pharmacotherapy for AAD is required.

Mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38, are a family of serine-threonine protein kinases that are activated in response to a variety of extracellular stimuli (10). ERK1/2 mediates cell proliferation and differentiation, which is activated by various cell growth factors. On the other hand, JNK and p38 are associated with stress responses, cell apoptosis, and growth suppression, which are activated by stress or cytokines (11). It was reported that AAD tissue showed a high level of phosphorylated JNK, and that apoptosis occurred in the medial smooth muscle cell (SMC) layers (12, 13). In addition, phosphorylation of p38 was induced by stretch stimuli in SMCs (12). These

findings led us to assume that apoptosis of SMCs in AAD tissue may be related to JNK and p38 phosphorylation.

Angiotensin II has been shown to induce cellular hypertrophy in vascular SMCs by acting through the G protein-coupled AT1 receptor, which results in various cardiovascular diseases and activates ERK1/2, JNK, and p38 (14, 15). In recent years, much focus has been placed on the role of G protein-coupled receptors, including the angiotensin II receptor, because they can be activated without agonist stimulation (16). The angiotensin II receptor also causes initiation of an intra-cellular signaling cascade in response to mechanical stretch without agonist stimulation. A specific type of angiotensin II receptor blocker (ARB) inhibits both agonist-induced and stretch-induced activation (17). Olmesartan is known as a potent ARB and works as an inverse agonist (18). We previously reported that olmesartan inhibits SMC migration through the inhibition of JNK activation (19). Therefore, we hypothesized that olmesartan may inhibit stretch-induced SMC death through the inhibition of the JNK- or p38-mediated intracellular signaling cascades.

In this study, we investigated cultured rat aortic smooth muscle cell (RASMC) death induced by cyclic mechanical stretch, which mimics an acute increase in blood pressure, and examined the effect of olmesartan on this event. We also investigated the changes in stretch-induced intracellular signaling including JNK and p38 and examined the effect of olmesartan on these changes.

Materials and methods

The study design was approved by the animal care and use committee of Nara Medical University based on the Guidelines for the Use of Laboratory Animals of Nara Medical University (No. 11011) and this study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

Cell culture and mechanical stretch

RASMCs were isolated from male Sprague-Dawley rats weighing 250–300 g according to previously published methods (20). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). The culture was maintained in a humidified atmosphere containing 5% CO_2 at 37°C. RASMCs from passage three to eight were grown to 70%–80% confluence in collagen I-coated (7.0 $\mu g/cm^2$) silicon chambers (STREX Inc., Osaka, Japan) and then growth-arrested by incubation in serum-free DMEM for 24 h prior to use. The cells were then subjected to mechanical stretch (60 cycles/min, 20% elongation) for a given time period by using the computer-controlled mechanical Strain Unit (STREX Inc, Osaka, Japan) according to previously published methods (21). After cyclic stretch, the medium was replaced with DMEM-containing 0.1% FBS. For western blot analysis, a portion of the RASMCs was lysed immediately after stretch

87 stimulation and lysate proteins were collected in the manner described earlier (15). Immunoreactive
88 bands were visualized using the enhanced chemiluminescence (ECL) plus or ECL prime systems
89 and were quantified using densitometry. In addition, a portion of the RASMCs were further
90 incubated for 24 h to detect cell viability using a 3-[4, 5-dimethylthiazol-2-phenyl]-2,
91 5-diphenyl-tetrazolium bromide (MTT) assay and cell death according to the release of lactate
92 dehydrogenase (LDH) into the medium. In some studies, RASMCs were pre-incubated with
93 olmesartan, a JNK inhibitor (SP600125), and a p38 inhibitor (SB203580) for 10 min, 20 min, and
94 4 h, respectively, before stimulation with cyclic mechanical stretch. Band intensities were quantified
95 using the densitometry of the immunoblot with NIH Image J software.
96
97 ***Materials***
98 Olmesartan (RNH-6270) was kindly provided by Daiichi-Sankyo Co., Ltd. (Tokyo). All other
99 materials were purchased from Wako (Kyoto) or Nakalai Tesque (Kyoto) unless stated otherwise.
100 The antibodies used for western blot analysis, anti-pan- or phospho-SAPK/JNK (Thr183/Tyr185)
101 antibody and anti-pan- or phospho-p38 MAP kinase (Thr180/Tyr182) antibody, were purchased
102 from Cell Signaling Technology. The ECL plus and ECL prime systems were purchased from GE
103 Healthcare. Collagen I was purchased from Nippon Meat Packers, Inc. (Osaka). All chemical
104 compounds were dissolved in dimethyl sulfoxide (DMSO) at a final concentration less than 1%,

except in the case of specific notifications.

Statistical analyses

Data are reported as the mean ± standard deviation (S.D.). We used a Student's *t*-test with Fisher's post-hoc test for intergroup comparison. A *P*-value of <0.05 was considered to indicate statistical significance.

Results

Cyclic mechanical stretch-induced RASMC death evaluated using MTT reduction and LDH release

The effect of cyclic mechanical stretch on RASMC death was examined by measuring the MTT reduction and LDH release from the cells. Figs. 1A and 1B show the viability and death rate of RASMCs subject to cyclic mechanical stretch by 20% elongation for 0–4 h, respectively. It was observed that the cell viability was decreased by stretch in a time-dependent manner and 35% of cells were dead at 4 h, evaluated based on the MTT reduction (Fig. 1A). In accordance with these results, the LDH release from RASMCs was increased by stretch in a time-dependent manner up to 4 h (Fig. 1B). These results suggest that cyclic mechanical stretch induced death in the RASMCs.

123 ***Olmesartan inhibits cyclic mechanical stretch-induced cell death in RASMCs***

124 Next, we examined the effect of olmesartan on cyclic mechanical stretch-induced death in

125 RASMCs. As shown in Fig. 2, it was obvious that cell viability was significantly recovered with

126 olmesartan treatment in a concentration-dependent manner.

127

128 ***Cyclic mechanical stretch causes activation of JNK and p38 in RASMCs***

129 The effects of cyclic mechanical stretch on the activation of JNK and p38 were assessed using

130 western blot analysis with phospho-specific antibodies. RASMCs were exposed to cyclic mechanical

131 stretch with a 20% elongation for different periods of time and the phosphorylation of JNK and p38

132 was measured. As shown in Figs. 3A and 3B, both JNK and p38 were activated by cyclic mechanical

133 stretch. For both JNK and p38, the extent of activation increased with the increase in stretch time,

134 reached a peak at 5–30 min, and then decreased to basal level at 60 min.

135

136 ***Olmesartan inhibits cyclic mechanical stretch-induced JNK and p38 activation in RASMCs***

137 To investigate whether stretch-induced JNK and p38 activation are influenced by olmesartan

138 treatment, we examined the effect of olmesartan on cyclic mechanical stretch-induced activation of

139 JNK and p38 in RASMCs. As shown in Figs. 4A and 4B, it was found that stretch-induced JNK and

140 p38 activation were significantly attenuated by olmesartan in a dose-dependent manner.

Olmesartan and JNK and p38 inhibitors inhibit cyclic mechanical stretch-induced RASMC death

To further investigate the role of JNK and p38 activation in stretch-induced RASMC death, we next examined the effects of JNK and p38 inhibitors on stretch-induced RASMC death in comparison with the effect of olmesartan. Fig. 5A compares the relative cell viability of RASMCs after 4 h stretch with or without olmesartan, or JNK and p38 inhibitors. It was found that olmesartan, the JNK inhibitor (SP600125), and the p38 inhibitor (SB203580) all significantly recovered the viability of the RASMCs. Fig. 5B compares the LDH release from the RASMCs after 4 h stretch with or without olmesartan, or JNK and p38 inhibitors. Compared with the positive control, olmesartan, SP600125, and SB203580 significantly reduced the death rate of RASMCs after 4 h stretch. These results indicate that olmesartan, and JNK and p38 inhibitors potentially inhibit RASMC death induced by cyclic mechanical stretch.

Discussion

Hypertension is known as a primary risk factor for AAD, and mechanical stretch is known to be one of the triggers for the onset of cardiovascular diseases (2, 6). However, the mechanism of mechanical stress transmitting signals to induce the onset of AAD is poorly understood. In the present study, we investigated the influence of acute mechanical stretch, which mimics an acute increase in blood pressure, on the viability of aortic SMCs, which are the main constituent cells of

159 the medial layer of the aorta. As shown in Fig. 1A, it was observed that acute cyclic mechanical
160 stretch induced the death of RASMCs in a time-dependent manner, up to 4 h. These results are also
161 supported by the findings that LDH release from RASMCs was increased continually up to 4 h (Fig.
162 1B). Taken together, it can be concluded that acute mechanical stretch causes SMC death, which
163 may be a possible cause of the onset of AAD. Our findings are consistent with other reports that
164 mechanical stretch causes smooth muscle cell death (22, 23). On the other hand, some other
165 researchers have reported that cyclic mechanical stretch results in cell proliferation (22). We also
166 observed such a phenomenon when we exposed RASMCs to 24 h of stretch (data not shown). From
167 these findings, we thought that cell death might occur from the start of acute stretch stimulation up to
168 4 h after which surviving cells entered a proliferation cycle, resulting in a gradual increase in cell
169 numbers that might be higher than that of the initial control cell numbers at the end of 24 h.
170 Therefore, it was suggested that the extent and duration of mechanical stretch may determine the
171 cellular fate, such as death or proliferation. Our experimental findings show that acute mechanical
172 stretch for 4 h causes continuous RASMC death. These findings may imply that an acute rise in
173 blood pressure leads to the death of SMCs, a main component of the aortic medial layer. However,
174 further studies using *in vivo* experimental conditions are required to elucidate whether an acute rise
175 in blood pressure directly causes SMC death.
176 Next, stretch-induced changes in the intracellular signaling of RASMCs were examined. It was

177 reported that a high level of phosphorylated JNK was observed in AAD tissues, and that
178 degeneration and tear of the aortic media had occurred in the AAD lesion. (2, 13). In addition, it was
179 reported that inhibition of the phosphorylation of JNK lead to regression of AAD (24). In the present
180 study, we found that acute mechanical stretch causes rapid phosphorylation of JNK and p38 (Figs.
181 3A and 3B), which may lead to SMC death. In fact, we also observed that SP600125, a JNK
182 inhibitor, and SB203580, a p38 inhibitor, both recovered stretch-induced RASMC death evaluated
183 based on the MTT reduction and LDH release from the cells (Figs. 5A and 5B). Altough we also
184 found that ERK1/2 is phosphorylated by mechanical stretch, ERK inhibitors failed to inhibit
185 stretch-induced RASMC death (data not shown). Taking these observations together, mechanical
186 stretch causes phosphorylation of JNK and p38, which may result in SMC death that may ultimately
187 lead to the onset of AAD. On the other hand, a previous study showed that angiotensin II acted as an
188 agonist for a potent inducer of AAD (1). In contrast to these findings, mechanical stretch itself,
189 which is independent of angiotensin II stimulation, phosphorylated JNK and p38, and induced SMC
190 death in our experiments. Although we did not measured the amount of angiotensisn II in the
191 medium, angiotensin II itself will not be involved in JNK and p38 phosphorylation because
192 stretch-induced AT1 receptor activation was also observed in the mesenteric and renal arteries from
193 angiotensinogen knockout mouse (25). Therefore, it is conceivable that not only agonist stimulation,
194 but also mechanical stretch could have an important role in triggering the occurrence of AAD.

ARBs are used all over the world for the treatment of patients with hypertension (26). Olmesartan, one of the ARBs, is known as an inverse agonist, which inhibits basic and stretch-induced activation of the AT1 receptor (17, 27). In our present study, we found that olmesartan inhibited phosphorylation of JNK and p38 (Figs. 4A and 4B), and SMC cell death (Fig. 2) induced by acute mechanical stretch. These results suggest that olmesartan inhibits stretch-induced SMC death by suppression of phosphorylation of JNK and p38. Therefore, it is assumed that inhibition of phosphorylation of JNK and p38 by each inhibitor causes a reduction of stretch-induced SMC death. This notion is supported by the findings that SP600125 and SB203580, as well as olmesartan, all recovered stretch-induced RASMC death (Figs. 5A and 5B). We previously reported that azelnidipine, a calcium channel blocker, also inhibits stretch-induced RASMC death (21). Since azelnidipine also inhibited stretch-induced JNK and p38 phosphorylation and SMC cell death, suppression of phosphorylation of JNK and p38 would be important to inhibit SMC death induced by acute mechanical stretch (21). Consistent with our results, it was reported that stretch-induced-cardiac hypertrophy was inhibited by candesartan, another known inverse agonist of the AT1 receptor (17). Therefore, further studies should be performed in the future using ARBs other than olmesartan with an aim of comparing their effects on stretch-induced death of RASMCs.

In the present study, we found that olmesartan inhibited acute mechanical stretch-induced RASMC death through the inhibition of JNK and p38 phosphorylation. Although future studies

using *in vivo* animal models are required to confirm whether olmesartan also inhibits the onset of AAD without affecting the blood pressure, our present study may shed light on the development of a new pharmacotherapy for the prevention of AAD.

Conclusion

In this study, we found that acute mechanical stretch causes JNK and p38 phosphorylation, resulting in the death of cultured RASMCs. It was suggested that olmesartan inhibited stretch-induced RASMC death through the inhibition of JNK and p38-mediated intracellular signaling pathways. Olmesartan is a potential candidate for the prevention of AAD, independent of its blood pressure lowering effect. Our findings may provide new insights into alternative pharmacotherapy for patients with acute AAD.

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Competing Interests: The authors have declared no competing interests exist.

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312 **Figure Legends**

313 Fig. 1. Time course for the effects of cyclic mechanical stretch (20% elongation) on cell viability (A)
314 (evaluated by 3-[4, 5-dimethylthiazol-2-phenyl]-2, 5-diphenyl-tetrazolium bromide (MTT) assay)
315 and cell death (B) (evaluated by lactate dehydrogenase (LDH) release) in rat aortic smooth muscle
316 cells (RASMCs) up to 4 h. Colorimetric analysis of each value was normalized by arbitrarily setting
317 the colorimetric value of the non-stimulated control cells to 1. Each value represents the mean ±
318 standard deviation (S.D.; n = 3) (*$P < 0.05$, compared with control. **$P < 0.01$, compared with
319 control).

320

321 Fig. 2. Inhibitory effect of olmesartan at different concentrations on stretch-induced cell death in rat
322 aortic smooth muscle cells (RASMCs). Olmesartan is abbreviated as Olm. Colorimetric analysis of
323 each value was normalized by arbitrarily setting the colorimetric value of the control cells without
324 stretch to 1. (*$P < 0.05$)

325

326 Fig. 3. Time courses for the effects of cyclic mechanical stretch (20% elongation) on the activation
327 of c-Jun N-terminal kinase (JNK) (A) and p38 (B) in rat aortic smooth muscle cells (RASMCs).
328 Olmesartan is abbreviated as Olm. Densitometric analysis of each value was normalized by
329 arbitrarily setting the densitometric value of the control cells without stretch to 1. Each value

represent the mean ± S.D. (n = 3). (*$P < 0.05$ compared with control without stretch)

Fig. 4. Effects of different concentrations of olmesartan on the activation of c-Jun N-terminal kinase (JNK) (A) and p38 (B) induced by cyclic mechanical stretch in rat aortic smooth muscle cells (RASMCs). Olmesartan is abbreviated as Olm. Densitometric analysis of each value was normalized by arbitrarily setting the densitometric value of the control cells without stretch to 1. Each value represents the mean ± standard deviation (S.D.; n = 6). (*$P < 0.05$ compared with control without stretch, #$P < 0.05$ compared with 20 min stretch without olmesartan, ## $P < 0.01$ compared with stretch 20 min. without olmesartan.).

Fig. 5. Comparison of the cell viability (A) and lactate dehydrogenase (LDH) release (B) induced by cyclic mechanical stretch in rat aortic smooth muscle cells (RASMCs) with or without olmesartan or mitogen-activated protein (MAP) kinase inhibitors. Olmesartan, SP600125, and SB203580 are abbreviated as Olm, SP, and SB, respectively. Colorimetric analysis of each value was normalized by arbitrarily setting of the colorimetric value of the control (Ctrl.) cells without stretch to 1. Each value represents the mean ± standard deviation (S.D.; n = 11). (*$P < 0.05$ compared with control without stretch, #$P < 0.05$ compared with stretch only).

(A)

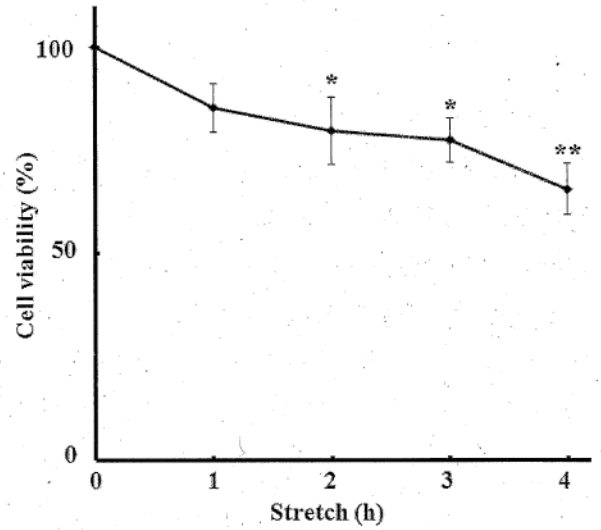


Figure 1

(B)

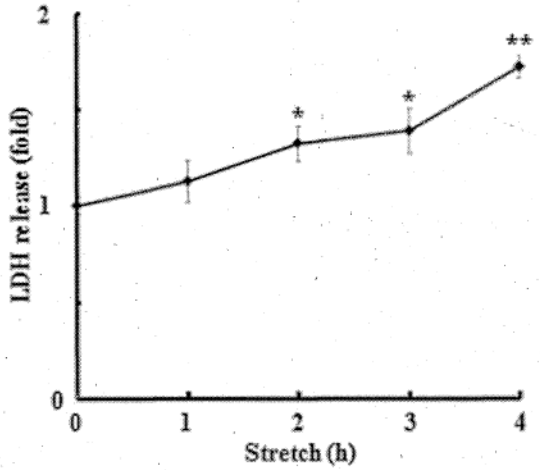


Figure 1

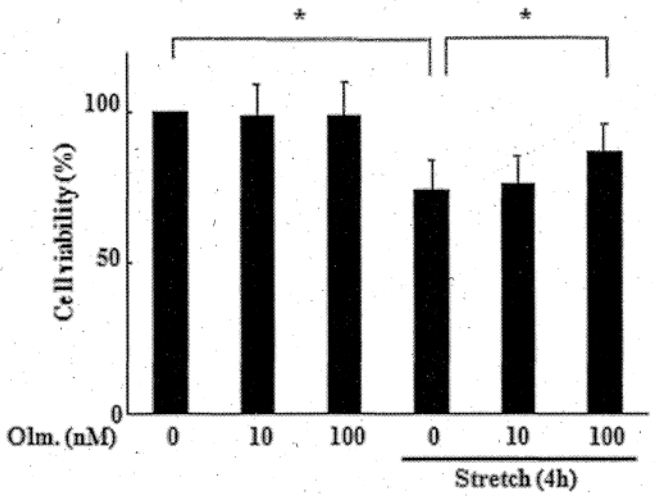


Figure 2

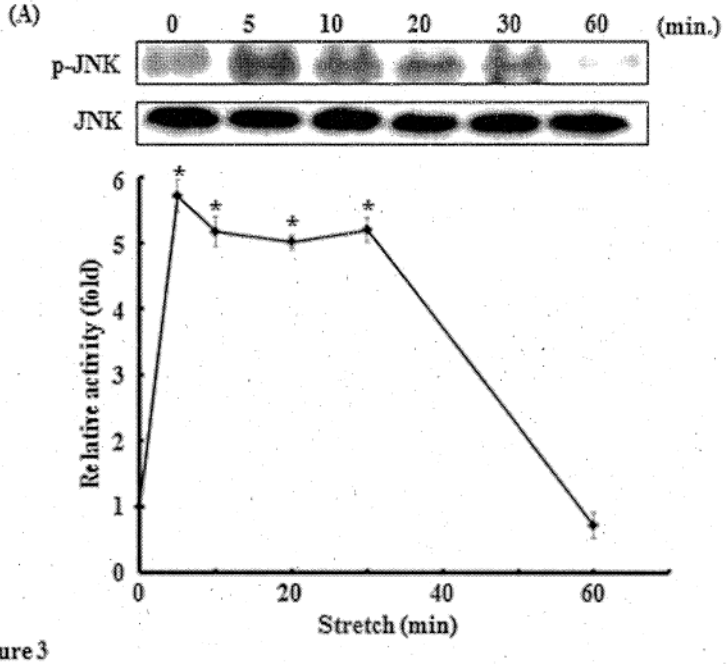
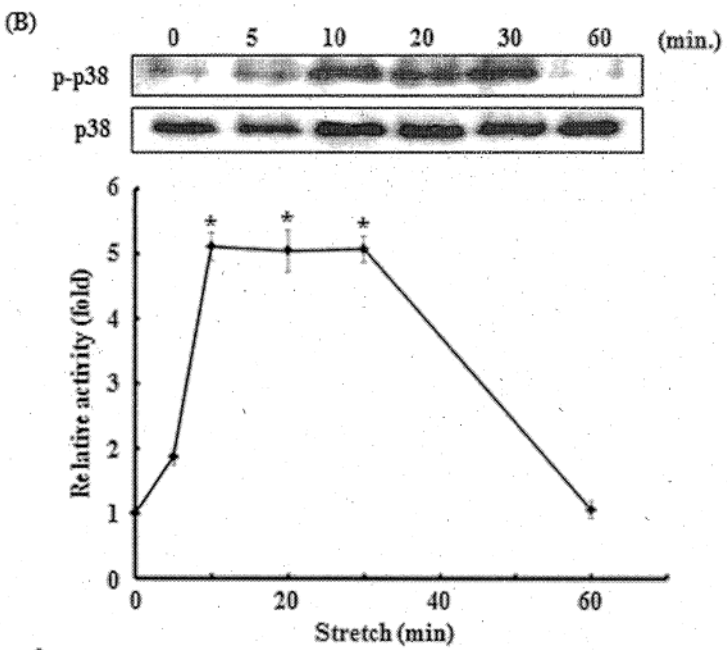

(A)
0
5
10
20
30
60
(min.)
p-JNK
JNK
Relative activity (fold)
0
1
2
3
4
5
6
*
*
*
*
0
20
40
60
Stretch (min)

Figure 3


(B)
0
5
10
20
30
60
(min.)
p-p38
p38
Relative activity (fold)
0
1
2
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4
5
6
*
*
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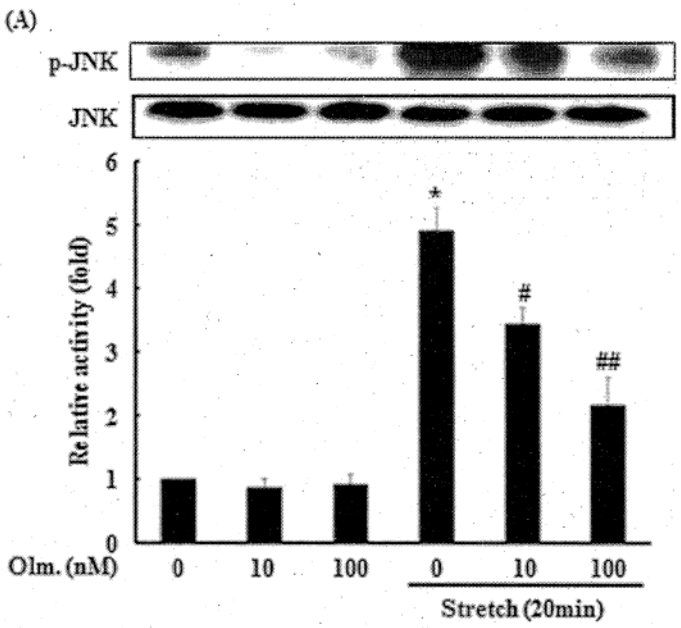
0
20
40
60
Stretch (min)

Figure 3


(A)
p-JNK
JNK
Relative activity (fold)
0
1
2
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6
*
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##
Olm. (nM)
0
10
100
0
10
100
Stretch (20min)

Figure 4

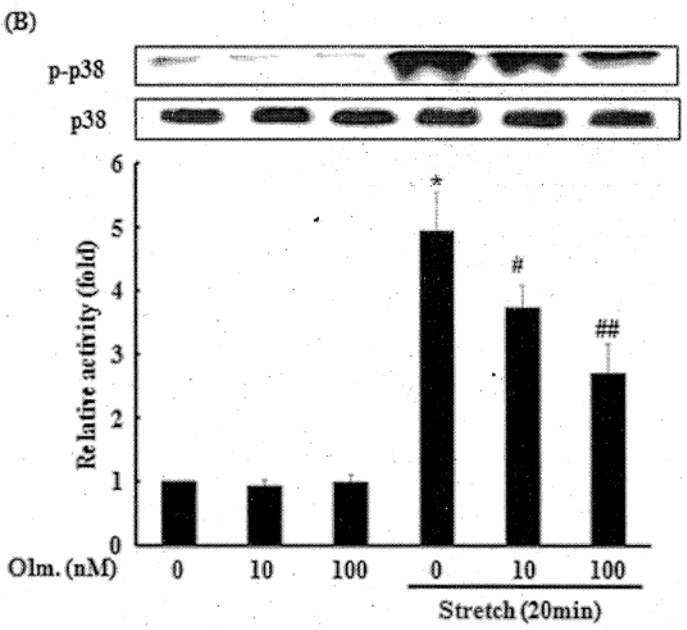
(B)
p-p38
p38
Relative activity (fold)
0
1
2
3
4
5
6
*
#
##
Olm. (nM)
0
10
100
0
10
100
Stretch (20min)

Figure 4

(A)

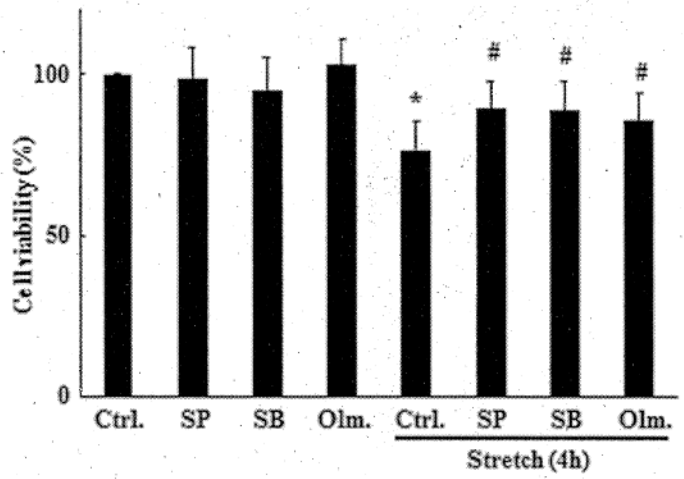
Cell viability (%)
100
50
0
Ctrl.
SP
SB
Olm.
Ctrl.
SP
SB
Olm.
Stretch (4h)
*
#
#
#

Figure 5

(B)

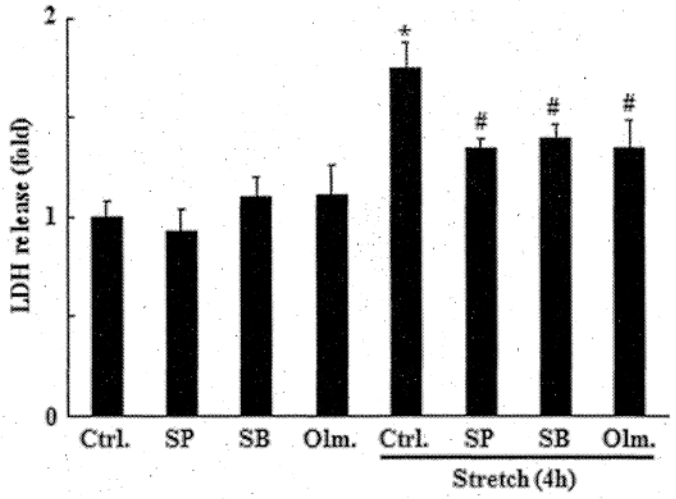

Figure 5