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Mechanical strain-induced c-fos expression in pulmonary epithelial cell line A549 ☆

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Abstract

Pulmonary epithelial cells are exposed to mechanical strain during physiological breathing and mechanical ventilation. It was not clear which style was more related with cell mechanotransduction. c-fos is known to be a component of a transcription factor, activator protein-1, which is induced by oxidative stress. The regulatory pathways involved in the rapid response of the AP-1 transcription factor, c-fos, to mechanical load in the human pulmonary epithelial cell line A549 were investigated using a four-point bending model. In an effort to better understand what processes are involved in mechanotransduction, we have examined whether and how soon c-fos induction occurs in human A549 shortly after the application of the different mechanical strains stimuli.

Keywords: Mechanical strains; c-fos; Pulmonary epithelial cell; A549

The lung is a unique organ in that it is exposed to physical forces derived from breathing, blood flow, and surface tension throughout life. Over the past decade, significant progress has been made at the cellular and molecular levels regarding the mechanisms by which physical forces affect lung morphogenesis, function, and metabolism [1]. Abnormal physical forces exerted on lung tissues contribute to many pathological situations. A better understanding of how physical forces act on lung cells may help us design strategies in the treatment and prevention of physical force-related disorders such as pulmonary hypoplasia, barotrauma, pulmonary hypertension, asthma, and chronic obstructive pulmonary diseases.

A rapid up-regulation in expression of the proto-oncogene c-fos in response to the application of mechanical strains has been documented in a number of cell types, including cardiac [2], muscle [3], and endothelial cells [4] in addition to both osteocytes [5,6] and osteoblasts [7].

The proto-oncogene c-fos is one of a family of transcription factors that includes c-fos, fos-B, fra-1, and fra-2. Many signaling pathways such as tyrosine kinases, p21 ras, MAP kinases, and PKC can induce c-fos mRNA expression [8]. The fos protein is an important component of the transcription factor activating protein-1 (AP-1), which can alter relative gene expression. The up-regulation of c-fos transcription has been shown to be an early member of a larger "cascade" of mechanically stimulated transcriptional responses, which may ultimately result in load-related remodeling of the matrix.

However, the lung is a unique organ in terms of its anatomic structure and physiological role. The lung is subjected to several complex physical forces including breathing, pulmonary blood flow, and surface tension. The effect of mechanical stress on pulmonary epithelial cells at the cellular level is relatively unknown. In particular, when pulmonary epithelial cells are subjected to

☆ *Abbreviations:* AP-1, activating protein-1; PKC, protein kinase C; NF-κB, nuclear factor κB.

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different mechanical stress. In an effort to better understand what processes are involved in mechanotransduction, we have examined whether and how soon c-fos induction occurs in human pulmonary epithelial cell line A549 shortly after the application of the different mechanical stimuli.

Materials and methods

Cell culture. A549 cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes buffer (Invitrogen Life Technologies), pH 7.3 (CRPMI), and containing 10% defined FBS (HyClone) at 37 °C in 5% CO_2-enriched air. After reaching 80% confluence the cells were harvested and replaced on six-well, flexible-bottomed plates (No. 353135, BD Falcon) at a density of 2×10^5 cells/cm^2. We used early-passaged cells in the following experiments.

Application of mechanical stress. The plates were subjected to cyclic uniaxial compressive or tensile strain by the self-made four-point bending system at 0.5 Hz for 5 min, 15 min, 30 min, 1 h, 3 h, and 6 h, respectively. In each time-phase, cells were loaded with tensile or compressive stress at 1000 μstrain. Control cells were cultured on similar plates and kept in the same incubator without mechanical stress loading. The treatments were repeated for three times.

QRT RT-PCR assay. Total RNA was extracted using a modification of the guanidium thiocyanate method [9] as described by Ghu et al. [10]. Total RNA was quantified by spectrophotometric analysis of the absorbance at 260 nm. mRNA level was quantified by quantitative real time reverse transcription polymerase chain reaction (QRT RT-PCR) [11]. Briefly, this technique is based on the detection of a fluorescent signal produced by an OPN-specific oligonucleotide probe during PCR primer extension (Prism 7700 sequence detection system; Applied Biosystems, Frost City, CA). The RNeasy mini kit (Qiagen Inc., Valencia, CA) was used to extract total RNA after lysis and homogenization with the QIAshredder mini column system (Qiagen Inc., Valencia, CA). Human c-fos cDNA primers and probes were designed using sequence data from GenBank and the QRT RT-PCR probe/primer design software Primer Express (version 1.0; Applied Biosystems, Frost City, CA). The fluorogenic oligonucleotide probe for human c-fos was 5′- cactccaa gcggagacagacc-3′ (Synthetic Genetics, San Diego, CA). The forward and reverse PCR primers were 5′-gcgcagagcattggcagga-3′, and 5′-cct tcagcttggcaatct-3′, respectively. The fluorogenic oligonucleotide probe for human GAPDH was 5′-ctgcaccaccaactgcttagc-3′ (Synthetic Genetics, San Diego, CA). The forward and reverse PCR primers were 5′-gggtg tgaaccatgagaagt-3′, and 5′-ccaaagttgtcatggatgacct-3′, respectively. These sequences were synthesized, and PCR conditions were optimized with respect to concentrations of Mg^{2+}, probe, and both primers.

Immunoblotting. Total cell proteins were extracted as described previously [12]. Protein concentrations were determined using a protein determination kit (Sigma). Proteins were separated on SDS–polyacrylamide gels using a Mini Protean II (Bio-Rad) gel kit according to the manufacturer's instructions. Proteins were transferred to Hybond ECL membrane (Amersham Pharmacia Biotech) using a Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Blots were blocked with 10% defatted milk protein overnight at 4 C. Blots were then washed with TBS-T20 (TBS plus 0.1% Tween 20) three times for 15 min and two times for 5 min. Blots were probed with primary antibody (rabbit anti-human c-fos polyclonal antibody, 1:1000 (Santa Cruz US)) in TBS-T20 containing 1% defatted milk protein for 1 h. Filters were washed as before and then incubated with secondary antibody (anti-rabbit (Santa Cruz US), 1:2000) in TBS-T20 for 30 min. Filters were rinsed and washed three times in TBS-20 and then probed with streptavidin 3 antibody (1:2000) in TBS for 20 min before rinsing and washing three times for 5 min in TBS-T20. Detection was then performed by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Results

To determine whether A549 cells showed express difference of c-fos stimulation by different mechanical strains, we loaded two type stress on the A549 in different time, and the mRNA level changes of c-fos were showed in Figs. 1–3. After a 5 min loading, c-fos mRNA levels are

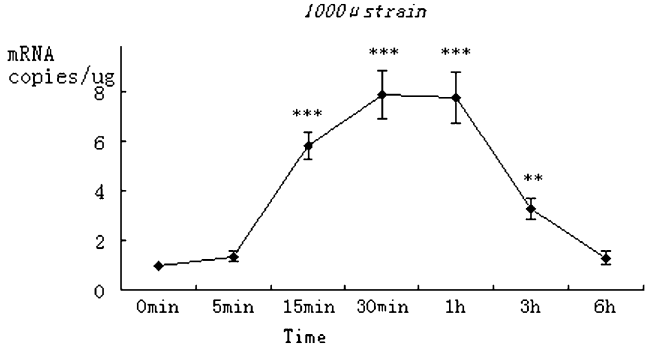

Fig. 1. Time–effect relationship of c-fos mRNA level inducted by tensile stress of 1000 μstrain (contrast to control (0 min): $^{**}p < 0.01$, $^{***}p < 0.001$).

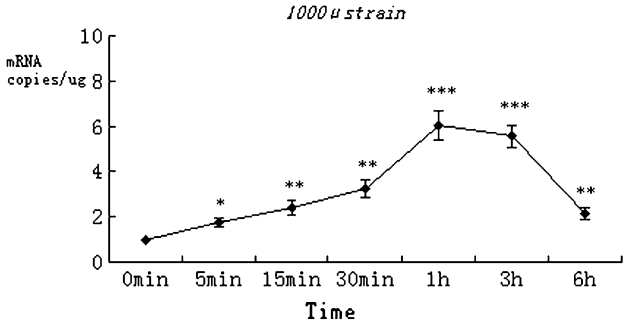

Fig. 2. Time–effect relationship of c-fos mRNA level inducted by compressive stress of 1000 μstrain (contrast to control (0 min): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

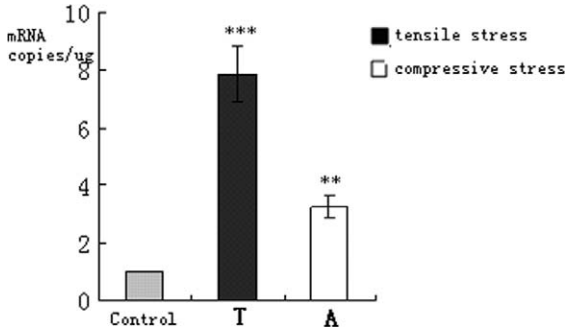

Fig. 3. Effect of c-fos mRNA stimulation by different type stress in 30 min (contrast to control: $^{**}p < 0.01$, $^{***}p < 0.001$) (T, 1000 μstrain tensile stress; C, 1000 μstrain compressive stress). Induction after 30 min by stress, results showed the tensile stress had a more significant effect on c-fos mRNA than compressive stress.

increased under induction by two type strains, but their change tendencies were not same. Under induction of tensile stress, the c-fos mRNA level reached peak in 30 min, while it is 1 h under the effect of compressive stress (Figs. 1–3), and the tensile stress will induce higher level mRNA of c-fos than compressive stress (Fig. 3).

To observe the change tendency of c-fos protein level under induction by two stresses, we next performed Western analysis on proteins isolated from total cellular lysates of A549 cells. The results show the same tendency as mRNA of c-fos (Figs. 4–8).

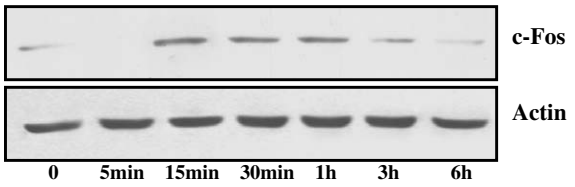

Fig. 4. Time–effect relationship of c-fos protein level inducted by tensile stress of 1000 μstrain.

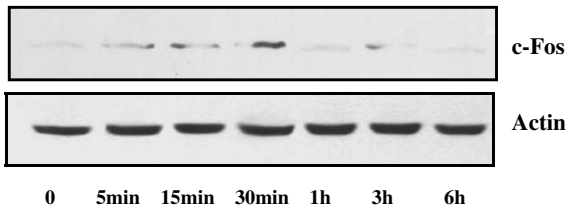

Fig. 5. Time–effect relationship of c-fos protein level inducted by compressive stress of 1000 μstrain.

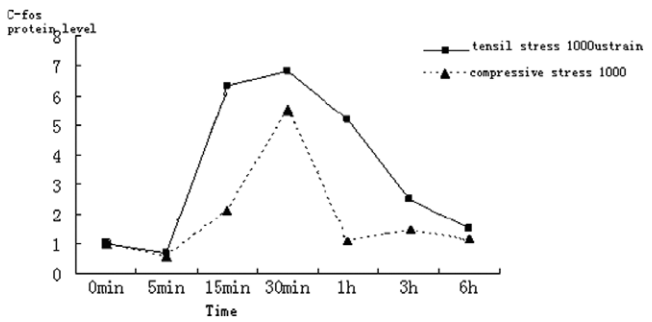

Fig. 6. Time–effect relationship of c-fos protein level inducted by different stress. Figs. 4–6 show the results of c-fos protein change induction by different stress indicated that c-fos protein would express fast in a short time and reach a peak in 30 min. Subsequently, it would decrease to normal level. These results were same to change of c-fos mRNA.

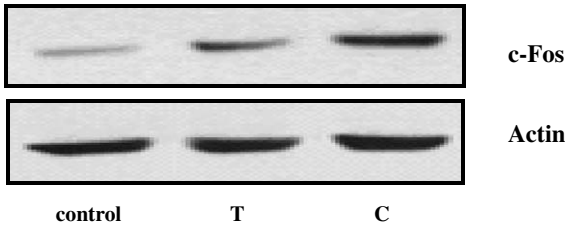

Fig. 7. Express change of c-fos protein after different stress 30 min.

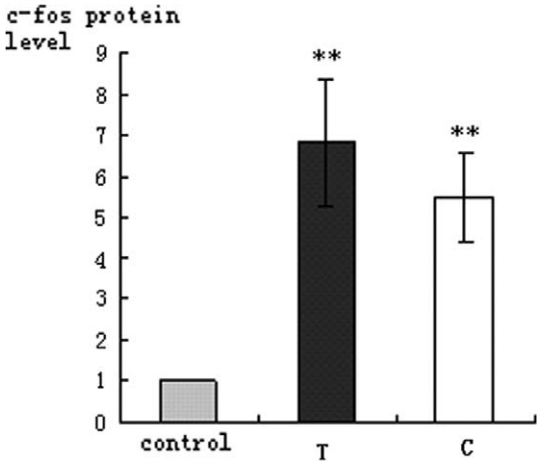

Fig. 8. Express change of c-fos protein after different stress 30 min (contrasted to control: $^{**}p < 0.01$) (T, 1000 μstrain tensile stress; C, 1000 μstrain compressive stress). Induction after 30 min by stress, results showed the tensile stress had a more significant effect on c-fos mRNA than compressive stress.

Discussion

Lung expansion and relaxation during breathing movements apply deformation to lung cells. In addition to shear stress, bulk air and blood flow also generate pressures that stretch the walls of the airways and blood vessels. Therefore, several devices have been designed to simulate this type of mechanical stimulation on lung cells. These can be divided into different groupings according to their major physical features, for example, static versus cyclic stretch, uniaxial versus biaxial stretch, and two-dimensional versus three-dimensional stretch [13–15].

During evolution, mechanical stimulation, as well as other physical factors such as temperature, pH, and light, has been a critical environmental signal sensed by cells. It is plausible that the intracellular signal transduction pathways originally developed by cells to respond to those basic physical stimuli have been preserved during evolution and further developed to respond to other external stimuli. Thus the biochemical reactions involved in mechanotransduction may be shared by other stimuli. The signaling events initiated by mechanical stimulation include generation of second messengers, change of phosphorylation status of proteins, amplification through enzymatic cascades, and transmission via a complicated network of signaling molecules.

c-fos is a transcription factor that belongs to the AP-1 family, which includes the c-fos (c-fos, fos B, fra-1, and fra-2) and c-jun (c-jun, jun B, and jun D) families. These transcription factors are activated after the activation of specific intracellular signal-transduction cascades, and activated members of the families bind to form heterodimers. The binding of jun–fos dimers to AP-1 sites within the promoter/regulatory region of genes modulates the transcriptional machinery and leads to alterations in their expression pattern [16].

Response of cell inducted by different character stress may be different. In our research, we induced the A549 cells using two type stress (compressive stress and tensile stress). Regardless of different magnitude strains, tensile stress would induce higher expression of c-fos than compressive stress. These results cued us response process of cell was complex. Different character stress could activate different signal transduction passageway in cell or passageway activated degree was different. Tensile stress had more significant influence than compressive stress on c-fos expression. This may be explained using tensegrity [17,18] of mechanics signal transduction. This theory considered that each member of cystoskeleton in cell existed prestress, microfilament, microbule and intermediate fiber had different effect on cell. During stress loading on the cell, accordingly internal strain in cystoskeleton would effect redistribution and respond. If this change were based on the internal strain, so response would be different facing different stress.

In conclusion, it is clear that rapid mechanotransducers, capable of responding within milliseconds, promote the induction of c-fos within 1 h of applied mechanical load. This activation of mechanotransducers may involve stretch or volume activation, which is followed by initiation of a series of downstream signaling pathways. Although clearly the AP-1 transcription factor is one of the steps in the load cascade, further work should identify whether expression of the gene is a critical step for downstream activation of bone matrix protein genes. One of the potential functions of load-related activation of multiple cellular pathways may be to initiate independent activation of a series of genes in response to mechanical load.

Acknowledgments

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References

[1] R.H. Notter, D.L. Shapiro, Lung surfactants for replacement therapy: biochemical, biophysical, and clinical aspects, Clin. Perinatol. 14 (1987) 433–479.
[2] J. Sadoshima, S. Izumo, Mechanotransduction in stretch-induced hypertrophy of cardiac myocytes, J. Recept. Res. 13 (1993) 777–794.
[3] N.J. Dawes, V.M. Cox, K.S. Park, H. Nga, D.F. Goldspink, The induction of c-fos and c-jun in the stretched latissimus dorsi muscle of the rabbit: responses to duration, degree and re-application of the stretch stimulus, Exp. Physiol. 81 (1996) 329–339.
[4] B.J. Ballermann, A. Dardik, E. Eng, A. Liu, Shear stress and the endothelium, Kidney Int. Suppl. 67 (1998) S100–S108.
[5] A. Kawata, Y. Mikuni-Takagaki, Mechanotransduction in stretched osteocytes—temporal expression of immediate early and other genes, Biochem. Biophys. Res. Commun. 246 (1998) 404–408.
[6] J. Klein-Nulend, A. van der Plas, C.M. Semeins, N.E. Ajubi, J.A. Frangos, P.J. Nijweide, E.H. Burger, Sensitivity of osteocytes to biomechanical stress in vitro, FASEB J. 9 (1995) 441–445.
[7] J. Roelofsen, J. Klein-Nulend, E.H. Burger, Mechanical stimulation by intermittent hydrostatic compression promotes bone-specific gene expression in vitro, J. Biomech. 28 (1995) 1493–1503.
[8] J. Sadoshima, S. Izumo, Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism, EMBO J. 12 (1993) 1681–1692.
[9] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[10] Y. Gu, M.R. Preston, A.J. el Haj, J. Hamid, G.W. Zamponi, J. Howl, S.J. Publicover, Osteoblasts derived from load-bearing bones of the rat express both L- and T-like voltage-operated calcium channels and mRNA for alpha 1C, alpha 1D and alpha 1G subunits, Pflugers Arch 438 (1999) 553–560.
[11] J. You, C.E. Yellowley, H.J. Donahue, Y. Zhang, Q. Chen, C.R. Jacobs, Substrate deformation levels associated with routine physical activity are less stimulatory to bone cells relative to loading-induced oscillatory fluid flow, J. Biomech. Eng. 122 (2000) 387–393.
[12] R.A. Hess, D.H. Gist, D. Bunick, D.B. Lubahn, A. Farrell, J. Bahr, P.S. Cooke, G.L. Greene, Estrogen receptor (alpha and beta) expression in the excurrent ducts of the adult male rat reproductive tract, J. Androl. 18 (1997) 602–611.
[13] A.J. Banes, J. Gilbert, D. Taylor, O. Monbureau, A new vacuum-operated stress-providing instrument that applies static or variable duration cyclic tension or compression to cells in vitro, J. Cell Sci. 75 (1985) 35–42.
[14] J.A. Gilbert, P.S. Weinhold, A.J. Banes, G.W. Link, G.L. Jones, Strain profiles for circular cell culture plates containing flexible surfaces employed to mechanically deform cells in vitro, J. Biomech. 27 (1994) 1169–1177.
[15] T.J. Kulik, S.P. Alvarado, Effect of stretch on growth and collagen synthesis in cultured rat and lamb pulmonary arterial smooth muscle cells, J. Cell Physiol. 157 (1993) 615–624.
[16] C.T. Rubin, L.E. Lanyon, Regulation of bone mass by mechanical strain magnitude, Calcif. Tissue Int. 37 (1985) 411–417.
[17] D.E. Ingber, Tensegrity I. Cell structure and hierarchical systems biology, J. Cell Sci. 116 (2003) 1157–1173.
[18] D.E. Ingber, Tensegrity II. How structural networks influence cellular information processing networks, J. Cell Sci. 116 (2003) 1397–1408.