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Mechanical force activates eIF-2α phospho-kinases in fibroblast

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Abstract

Mechanical forces can induce differentiation of fibroblasts into myofibroblasts, a process which requires activation of the MAP kinase p38. Currently, the identification of other phospho-kinases involved in myofibroblast differentiation has not been explored. We applied static tensile forces to rat cardiac fibroblasts via collagen-coated magnetite beads and examined activation of protein phospho-kinases by the Kinexus phospho-antibody screening system. Of 75 candidate protein kinases screened, 39 were detected and, of these, 31 phospho-kinases were analyzed. Following force application, 12 out of 31 phospho-kinases exhibited increases of phosphorylation including PKR (>4-fold), MKK3 (3-fold), MKK6 (~2-fold), and p38 (~2-fold). In several types of mechanically sensitive, contractile fibroblasts including rat cardiac, human gingival, and Rat-2 fibroblasts, tensile forces increased eIF-2α phosphorylation, a downstream effector of PKR. We conclude that phospho-antibody screening is an efficient method for discovery of novel mechanical force-induced phospho-kinases and force can activate eIF-2α phospho-kinases in fibroblasts.

Keywords: Kinase screening; Force application; eIF-2α; Fibroblasts

Gene expression profiles induced by mechanical loading can be divided temporally into immediate early genes (e.g., the transcription factor serum response factor; [1,2]) and late response genes (e.g., collagen; [3,4]). Several signaling pathways that mediate mechanically induced signals have been identified in fibroblasts. Notably, protein kinase phosphorylation is a critical signal transduction mechanism [5,6] and activation of protein kinases has been detected in several cell types exposed to tensile forces including extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), p38 kinase, Src, NF-κB, FAK, PI3K, JAK, and protein kinase C [7–15]. However, a more complete repertoire of force-responsive protein kinases based on screening methods has not been reported.

Fibroblasts respond to increased mechanical tension by elevations of cell number, enhanced gene activation, increased synthesis of matrix proteins, and, depending on the extent of differentiation, a regulation of α-smooth muscle actin (SMA) expression [15,16]. To probe the potential repertoire of force-induced protein kinases that are involved in myofibroblast differentiation, we used the Kinexus phospho-antibody screening system. This is an efficient tool for discovery and definition of kinase signaling pathways [17] that has not been applied previously to the discovery of mechanotransduction systems. We show here that phospho-antibody screening is an effective method for identification of novel, force-induced phospho-kinases and force can activate eIF-2α phospho-kinases in fibroblast.

Materials and methods

Reagents. Collagenase (C-5138) and BSA were purchased from Sigma (Oakville, Ont.). Goat anti-mouse IgG + IgM (H + L) and goat anti-mouse IgG_1 were purchased from Caltag (Burlinghame, CA). Antibodies to eIF-2α as well as the phospho-specific antibodies for each of these kinases were purchased from Cell Signaling Technology. A GAPDH monoclonal antibody (clone# 6C5) was purchased from

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Advanced Immunochemical Inc. Soluble, type I bovine collagen (Vitrogen) was obtained from Celltrix (Palo Alto, CA).

Cell culture. Primary cultures of cardiac fibroblasts were obtained from 1-day-old 10 g Wistar rats. Animal experiments were conducted in accordance with guidelines established by the University of Toronto, Animal Care Committee which provided approval for these experiments. In brief, rats were killed and the hearts were quickly removed under sterile conditions. Ventricular tissue was excised, minced, and digested with 0.3% collagenase containing (w/v) 1.8% sorbitol, 0.05% DNase, 6.25 U/ml elastase, and 0.05% trypsin in Krebs buffer with Zn^{2+}. Non-adherent cells (primarily myocytes, leukocytes, and endothelial cells) were washed away. Cardiac fibroblasts attached to culture dishes within 15 min and proliferated much more rapidly than the other cardiac cell types. These properties enabled us to obtain virtually pure cultures of fibroblasts (>98% by immunophenotyping for vimentin and absence of desmin). Cells were maintained in HG-DME containing 10% fetal bovine serum and a 1:10 dilution of an antibiotic solution (0.17%(w/v) penicillin V, 0.1% gentamicin sulfate, and 0.01 μg/ml amphotericin; Sigma) at 37 °C in a humidified incubator gassed with 95% O_2–5% CO_2. Cells were passaged with 0.01% trypsin (Gibco, Burlington, Ont.). Studies were performed on cells at days 1–3 in HG-DME serum-free medium.

Bead coating. As described earlier [15], 0.4 g of magnetite beads (Sigma–Aldrich) was incubated for one hour with 1 ml of an acidic bovine collagen solution (>95% type I collagen) at 37 °C and neutralized to pH 7.4 with 100 μl of 1 N NaOH. Under these conditions collagen polymerizes and forms fibrils around the beads within 30 min. The beads were sonicated to eliminate clumps and then dispersed. Analysis of bead size was performed by electronic particle counting (Coulter Channelyzer, Coulter Electronics, Hialeah, FL). Particles tended to exhibit a heterogeneous size distribution with a pronounced modal peak at 5 μm although there were many particles with smaller diameters. Beads were rinsed in PBS, washed 3 times, and resuspended in Ca^{2+}–Mg^{2+}-free phosphate-buffered saline.

Force application. A ceramic permanent magnet (Gr. 8, 2.2 cm × 9.6 cm × 11 cm; Jobmaster, Mississauga, Ont.) was used to apply perpendicular forces to beads attached to the dorsal surface of cells. For all experiments, the pole face was parallel with and 2 cm from the cell culture dish surface. At this distance the force on a single fibroblast with ~750 μm^2 area of dorsal bead coverage was 480 pN or 0.65 pN/μm^2. As the surface area of the magnet was larger than those of the culture dishes, and as the bead covering was relatively uniform for all cells, the forces applied to cells across the width of the culture dish were relatively uniform [18]. Constant forces but of varying duration were used for all experiments. Prior to incubation with cells, beads were rinsed in phosphate-buffered saline, washed three times, resuspended in calcium-free buffer, and added to attached cells in complete medium for 10 min. Cells were washed three times to remove unbound beads prior to exposure to force.

Phospho-antibody screening. Cells were rinsed with phosphate-buffered saline, lysed by adding 0.5 ml lysis buffer (20 mM Mops, pH 7.0, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMS, 3 mM benzamidine, 5 μM pepstatin, 10 μM leupeptin, and 0.5% Triton X-100; final pH 7.0) for 5000 rpm for 5 min at 4 °C. Cells were sonicated twice for 15 s and the homogenate was subjected to ultracentrifugation for 30 min at 50,000 rpm. The protein concentration of the supernatant fraction was measured by the Bio-Rad assay and phospho-kinase screening was performed by Kinexus (Vancouver, BC; analyses KPKS-1.0 and KPSS-1.1).

Immunoblotting. For immunoblots, protein from beads or cell lysates prepared from cell cultures (60 mm dishes) that had been subjected to an applied force for specific time intervals was analyzed. Cells were rinsed with phosphate-buffered saline, lysed by adding 200 μl SDS sample buffer (62.5 mM Tris–HCl; pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1%(w/v) bromophenol blue), and transferred to a microfuge tube. The samples were kept on ice and then boiled for 5 min. Protein concentration was assessed by the Bio-Rad assay and equal amounts of protein were loaded in each lane. Isolated proteins were separated by SDS–PAGE (10% acrylamide) and transferred to nitrocellulose. Blots were blocked for 1 h with 5% skim milk in PBS and incubated with the indicated antibody (GAPDH, PKR, and eIF2-α diluted 1:1000 in 0.5% Tween–PBS) for 1 h at room temperature. Blots were washed with 0.5% Tween–PBS for 10 min, incubated with appropriate second antibodies for 1 h, washed 4× in Tween–PBS, and developed by chemiluminescence (ECL; Amersham). X-OMAT Kodak films were exposed to the blots. For all assays, three or more separate experiments were performed.

Results

Mechanical force-induced activation of phospho-kinases

Under conditions of increased mechanical tension, fibroblasts exhibit increased remodeling of the extracellular matrix as shown by elevated levels of synthesis and degradation of collagen [19,20]. This process is thought to be regulated in part by protein kinases [21] but their identity has not been defined. We first examined the possible involvement of protein kinases in mechanotransduction in cardiac fibroblasts by screening for 75 candidate protein kinases (Table 1). Cardiac fibroblasts were subjected to static tensile forces (0.5–2 h) delivered via collagen-coated magnetite beads. Cells were lysed and subjected to screening. We found 39 protein kinases including ERK, JNK, p38, PKR, PKC, FAK, and ZIP, all of which were detectable in rat cardiac fibroblasts using these methods. We next tested tensile force-induced phospho-kinase activation by phospho-antibody screening. Of 31 candidate phospho-protein kinases analyzed, 12 protein kinases were phosphorylated by mechanical force (Fig. 1). Notably, PKR was significantly phosphorylated by tensile force (>4-fold; Table 2). Thus, in addition to the previously described phosphorylation of p38 [15,16], static tensile forces induce phosphorylation of the upstream activators of p38, MKK3, and MKK6, and also PKR.

Mechanical force-induced eIF-2α phosphorylation in cardiac fibroblasts

The discovery of force-induced phosphorylation of PKR was novel and we sought to confirm this response using conventional immunoblotting of cell lysates from cardiac fibroblasts that had been pre-incubated with collagen-coated magnetite beads and treated with force (Fig. 2A; 0.65 pN/μm^2; [16]). As anticipated, within 30 min of tensile force application, there was strongly enhanced phosphorylation of PKR at Thr 451. We next determined if potential downstream targets of PKR were phosphorylated in response to force. We immunoblotted cell lysates for eIF-2α phosphorylation, a PKR downstream effector [22] that has not previously been associated with mechanotransduction processes.

Table 1
Protein kinases in cardiac fibroblasts

Full name of protein	Abbreviation	Type	Average of trace quantity	SEM
3-Phosphoinositide-dependent protein kinase 1 (PKB kinase) (56)	PDK1(56)	PSTK	0	
Bone marrow X kinase (69)	BMX (69)	PSTK	6750	1400.3
Bruton agammaglobulinemia tyrosine kinase (65)	BTK (65)	PYK	0	
Calmodulin-dependent kinase 1 (43)	CaMK1 (43)	PSTK	<1000	
Calmodulin-dependent kinase 4 (60)	CaMK4 (60)	PSTK	0	
Calmodulin-dependent kinase kinase (52)	CaMKK (52)	PSTK	0	
Cancer Osaka thyroid oncogene (Tpl2) (53)	COT (53)	PSTK	4577	310.2
Casein kinase 1 δ (39)	CK1d (39)	PSTK	0	
Casein kinase 1 ϵ (35)	CK1e (35)	PSTK	0	
Casein kinase 2 (35)	CK2 (35)	PSTK	10,323	1889.6
Casein kinase 2 (37)	CK2 (37)	PSTK	6421	419.4
Casein kinase 2 (39)	CK2 (39)	PSTK	9359	833.3
c-SRC tyrosine kinase (43)	CSK (43)	PYK	<1000	
Cyclin-dependent kinase 1 (cdc2) (30)	CDK1 (30)	PSTK	<1000	
Cyclin-dependent kinase 2 (31)	CDK2 (31)	PSTK	<1000	
Cyclin-dependent kinase 4 (35)	CDK4 (35)	PSTK	1961	242.3
Cyclin-dependent kinase 5 (28)	CDK5 (28)	PSTK	6587	81
Cyclin-dependent kinase 6 (34)	CDK6 (34)	PSTK	1889	423.4
Cyclin-dependent kinase 7 (35)	CDK7 (35)	PSTK	14,002	3192.5
Cyclin-dependent kinase 9 (35)	CDK9 (35)	PSTK	1040	451.5
Death associated protein kinase 1 (156)	DAPK (156)	PSTK	0	
DNA-activated protein kinase (460)	DNAPK (460)	PSTK	0	
dsRNA-dependent kinase (75)	PKR (75)	PSTK	0	
Extracellular-regulated kinase 1 (40)	ERK1 (40)	PSTK	17,497	902.4
Extracellular-regulated kinase 1 (41)	ERK1 (41)	PSTK	2990	618.6
Extracellular-regulated kinase 2 (37) (Gel A, lane 3)	ERK2 (37)	PSTK	7984	714.3
Extracellular-regulated kinase 2 (37) (Gel A, lane 4)	ERK2 (37)	PSTK	17,190	649
Extracellular-regulated kinase 2 (39) (Gel A, lane 3)	ERK2 (39)	PSTK	1871	139.8
Extracellular-regulated kinase 2 (39) (Gel A, lane 4)	ERK2 (39)	PSTK	0	
Extracellular-regulated kinase 3 (52)	ERK3 (52)	PSTK	0	
Extracellular-regulated kinase 3 (56)	ERK3 (56)	PSTK	2292	372.5
Extracellular-regulated MAP kinase 6 (45)	ERK6 (45)	PSTK	0	
Focal adhesion kinase (123)	FAK (123)	PYK	<1000	
Fyn oncogene related to SRC (46)	FYN (46)	PYK	0	
Fyn oncogene related to SRC (48)	FYN (48)	PYK	0	
G protein-coupled receptor kinase 2 (BARK2) (74)	GRK2 (74)	PSTK	7059	1669.5
Germinal centre kinase (86)	GCK (86)	PSTK	<1000	
Glycogen synthase kinase 3α (44)	GSK3a(44)	PSTK	4274	861.3
Glycogen synthase kinase 3β (40)	GSK3b(40)	PSTK	3170	1692.5
Hematopoietic progenitor kinase 1 (87)	HPK1 (87)	PSTK	0	
Inhibitor NF-κB kinase α (72)	IKKa (72)	PSTK	3297	967.6
Janus kinase 1 (125)	JAK1 (125)	PTK	5322	562.9
Janus kinase 2 (116)	JAK2 (116)	PTK	0	
Kinase suppressor of Ras 1 (89)	KSR1 (89)	PSTK	0	
Lymphocyte-specific protein tyrosine kinase (46)	LCK (46)	PYK	0	
Mammalian sterile 20-like 1 (57)	MST1 (57)	PYK	0	
MAP kinase interacting kinase 2 (55)	MNK2 (55)	PSTK	0	
MAP kinase kinase 1 (MKK1) (40)	MEK1 (40)	PTYK	4341	719.5
MAP kinase kinase 2 (MKK2) (41)	MEK2 (41)	PTYK	3011	623.9
MAP kinase kinase 4 (MEK4) (36)	MEK4 (36)	PTYK	2509	314.8
MAP kinase kinase 6 (MEK6) (33)	MEK6 (33)	PTYK	<1000	
MAP kinase kinase 7 (MEK7) (37)	MEK7 (37)	PTYK	<1000	
NIMA (never in mitosis)-related kinase 2 (52)	NEK2 (52)	PSTK	0	
NIMA (never in mitosis)-related kinase 2 (54)	NEK2 (54)	PSTK	0	
Oncogene Lyn (45)	LYN (45)	PYK	0	
Oncogene Raf 1 (69)	RAF1 (69)	PSTK	1150	109.9
Oncogene Raf 1 (73)	RAF1 (73)	PSTK	11,583	1271.8
Oncogene SRC (48)	SRC (48)	PYK	0	
p21-activated kinase 1 (PAKα) (62)	PAK1 (62)	PSTK	0	
p21-activated kinase 3 (PAKβ) (62)	PAK3 (62)	PSTK	0	
p38 Hog MAP kinase (37)	38 MAPK (37)	PSTK	29,301	4310.4
Pim1 (35)	Pim1 (35)	PSTK	2294	295.5

(*continued on next page*)

Table 1 (*continued*)

Full name of protein	Abbreviation	Type	Average of trace quantity	SEM
Protein kinase A (cAMP-dependent protein kinase) (38)	PKA (38)	PSTK	<1000	
Protein kinase Bα (57)	PKBa (57)	PSTK	3902	595.7
Protein kinase Bα (60)	PKBa (60)	PSTK	0	
Protein kinase Cα (77)	PKCa (77)	PSTK	3895	559.6
Protein kinase Cβ1 (80)	PKCb (80)	PSTK	1409	513.9
Protein kinase Cδ (71)	PKCd (71)	PSTK	23,694	2464.6
Protein kinase Cϵ (94)	PKCe (94)	PSTK	2277	181.7
Protein kinase Cϵ (98)	PKCe (98)	PSTK	1443	143.4
Protein kinase Cγ (76)	PKCg (76)	PSTK	0	
Protein kinase Cλ (62)	PKCl (62)	PSTK	0	
Protein kinase Cμ (110)	PKCm (110)	PSTK	0	
Protein kinase Cμ (120)	PKCm (120)	PSTK	1945	38.3
Protein kinase Cθ (74)	PKCt (74)	PSTK	0	
Protein kinase Cζ (78)	PKCz (78)	PSTK	19,278	1050.3
Protein kinase Cζ (83)	PKCz (83)	PSTK	0	
Protein kinase G1 (cGMP-dependent protein kinase) (70)	PKG1 (70)	PSTK	7081	638
Protein tyrosine kinase 2 (104)	PYK2 (104)	PYK	0	
Protein tyrosine kinase 2 (110)	PYK2 (110)	PYK	0	
RhoA kinase (154)	ROKa (154)	PYK	1294	232
Ribosomal S6 kinase 1 (77)	RSK1 (77)	PSTK	1359	488.2
Ribosomal S6 kinase 2 (86)	RSK2 (86)	PSTK	1587	504.7
S6 kinase p70 (59)	S6K p70 (59)	PSTK	<1000	
S6 kinase p70 (61)	S6Kp70 (61)	PSTK	0	
S6 kinase p70 (63)	S6K p70 (63)	PSTK	0	
Spleen tyrosine kinase (70)	SYK (70)	PYK	0	
Stress-activated protein kinase (JNK) (38)	SAPKb (38)	PSTK	1277	332.6
Stress-activated protein kinase (JNK) (45)	SAPKb (45)	PSTK	0	
v-mos Moloney murine sarcoma viral oncogene homolog 1 (34)	MOS1 (34)	PSTK	4429	922.1
v-raf murine sarcoma viral oncogene homolog B1 (92)	RAFB (92)	PSTK	<1000	
Yamaguchi sarcoma viral oncogene homolog 1 (54)	YES1 (54)	PYK	<1000	
ζ-chain (TCR) associated protein kinase (76)	ZAP70 (76)	PYK	<1000	
ZIP kinase (death associated protein kinase 3) (45)	ZIP (45)	PSTK	3303	403.5
ZIP kinase (death associated protein kinase 3) (48)	ZIP (48)	PSTK	3016	180.1

Cardiac fibroblasts were lysed and a total of 75 protein kinases were analyzed by KPKS-1.0.
The data are means of three different assays and are an estimate of blot density. Proteins that were undetected are given a value of 0 and those with blot densities <1000 are indicated.

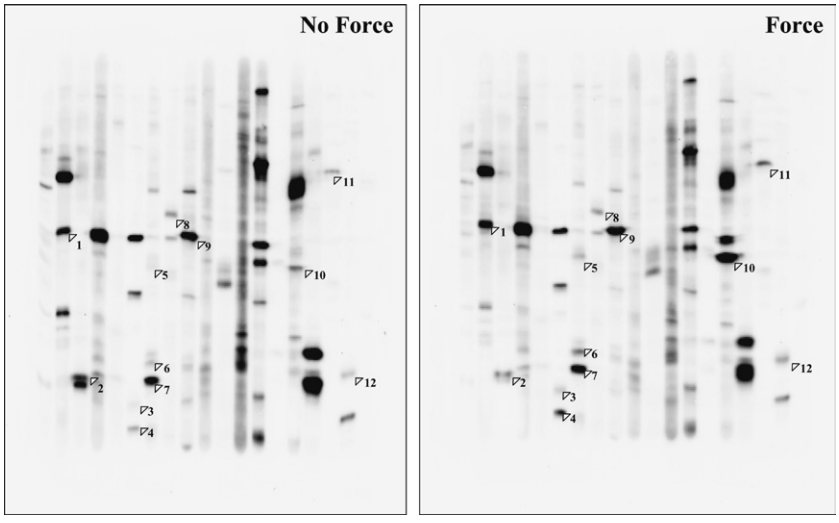

Fig. 1. Discovery of force-responsive protein kinases using phospho-site screening in cardiac fibroblasts. Rat cardiac fibroblasts were incubated with collagen-coated beads, subjected to force for 2 h or not, and analyzed by the KPSS-1.1 screening system. The identities of protein targets are indicated by arrows and markers. 1, adducin gamma; 2, p38; 3, MKK3; 4, MKK6; 5, p70S6k 6, ERK1; 7, ERK2; 8, PKC; 9, STAT3; 10, PKR; 11, RB; and 12, MKK1/2. Note that phospho-p38 is reduced at 2 h but is increased at 30 min after force, as is shown in Table 2 which is consistent with previous data [16].

Table 2
Force-responsive protein kinases identified by phospho-site screening in cardiac fibroblasts

Full name of protein	Abbreviation	Lane	Epitope	Trace quantity		
			Force:	0 (h)	0.5 (h)	2 (h)
Adducin-α (121)	Adducin a (121)	3	S724	19,645	19,474	24,090
Adducin-γ (80)	Adducin g (80)	3	S662	5622	10,000	10,798
CAMP response element binding protein (44)	CREB (44)	13	S133	3306	5417	2373
dsRNA-dependent protein kinase (71)	**PKR (71)**	**16**	**T451**	**2284**	**11,813**	**19,674**
Extracellular-regulated kinase 1 (42)	ERK1 (42)	8	T202/Y204	1574	1838	4098
Extracellular-regulated kinase 2 (40)	ERK2 (40)	8	T185/Y187	6544	8429	11,163
Glycogen synthase kinase 3α (45)	GSK3a (45)	17	Y279	14,009	17,138	13,452
Glycogen synthase kinase 3α (45)	GSK3a (45)	15	S21	518	1633	1060
Glycogen synthase kinase 3β (41)	GSK3b (41)	17	Y216	15,422	22,566	12,698
Glycogen synthase kinase 3β (41)	GSK3b (41)	15	S9	155	440	526
MAP kinase kinase 1/2 (41)	MKK1/2 (41)	19	S221/S225	1596	4719	3646
MAP kinase kinase 3 (33)	**MKK3 (33)**	**7**	**S189/T193**	**1674**	**2142**	**5186**
MAP kinase kinase 6 (37)	**MKK6 (37)**	**7**	**S207/T211**	**678**	**1258**	**1734**
Mitogen-and stress-activated protein kinase 1/2 (79)	MSK1/2 (79)	15	S376	774	1158	848
N-methyl-D-aspartate glutamate receptor subunit 1(112)	NR1 (112)	2	S896	1674	3079	1921
Oncogene JUN (40)	JUN (40)	11	S73	2373	1722	2543
Oncogene Raf 1 (61)	RAF1 (61)	12	S259	3656	4631	4704
Oncogene Raf 1 (66)	RAF1 (66)	12	S259	1800	2249	3072
Oncogene SRC (49)	SRC (49)	3	Y529	3888	3427	3248
Oncogene SRC (49)	SRC (49)	5	Y418	987	1025	1111
p38-α MAP kinase (39)	**p38 MAPK (39)**	**4**	**T180/Y182**	**3185**	**7329**	**3382**
Protein kinase Cα (S657)	PKCa (80)	5	S657	25,737	36,679	34,599
Protein kinase Cα/β (T 368)	PKCa/b (80)	7	T 638/641	7198	6825	7706
Protein kinase Cδ	PKCd (73)	13	T505	2276	2226	1996
Protein kinase Cϵ	PKCe (94)	9	S719	1997	2939	2483
Retinoblastoma 1 (126)	RB (126)	18	S780	2042	4258	4204
Retinoblastoma 1 (126)	RB (126)	20	S807/S811	444	308	119
Ribosomal S6 kinase 1 (86)	RSK1 (86)	6	T 360/S364	80	739	219
S6 kinase p70 (72)	p70 S6K (72)	8	T 389	728	779	2041
S6 kinase p70 (79)	p70 S6K (79)	8	T 389	517	915	622
Signal transducer and activator of transcription 3 (83)	STAT3 (83)	10	S727	10,045	24,361	16,262
SMA-and MAD-related protein 1	SMAD1 (61)	9	S463/465	543	545	579
Stress-activated protein kinase (JNK) (41)	SAPK (41)	6	T 183/Y185	643	943	540
Stress-activated protein kinase (JNK) (47)	SAPK (47)	6	T 183/Y185	419	451	494

Rat cardiac fibroblasts were incubated with collagen-coated magnetite beads, subjected to tensile force for 0, 0.5 or 2 h, lysed, and analyzed for phospho-kinase expression.
A total of 31 phospho-protein kinases were analyzed by KPSS-1.1.

Cardiac fibroblasts from one-day-old rats were subjected to static tensile forces delivered via collagen-coated magnetite beads. As shown by immunoblotting (Fig. 2B), force application induced higher levels of eIF-2α phosphorylation within 30 min (>1.7-fold) of force application which increased over 4 h. Application of force caused no significant change of the protein content of eIF-2α and the relative abundance of GAPDH (as a loading control) also did not change. Thus, eIF-2α is not only phosphorylated by amino acid starvation, viral infection, iron deficiency, and ER stress [23–26], but is a novel, mechanical force-induced phospho-kinase.

eIF-2α phosphorylation in other fibroblast types

To determine if mechanical force induces eIF-2α phosphorylation in other types of fibroblasts, we examined force-induced eIF-2α phosphorylation in human gingival fibroblasts and Rat-2 fibroblasts, both of which express abundant levels of SMA [27]. Rat-2 cells and human gingival fibroblasts were incubated with collagen-coated beads, subjected to tensile forces (0.5–4 h), and immunoblotted for eIF-2α and phospho-eIF-2α. In human gingival fibroblasts, eIF-2α phosphorylation was detected at 30 min after force and reached maximum levels at 4 h (2.5- to 3.5-fold) (Fig. 3A). In Rat-2 cells, eIF-2α phosphorylation was maximal at 30 min (2.3-fold) after force and decreased thereafter (Fig. 3B). These data indicated that mechanical force-induced eIF-2α phosphorylation is a common response in different types of fibroblasts but the kinetics of phosphorylation are not identical.

Discussion

The major finding of this study is that phospho-kinase screening for mechanically induced signaling

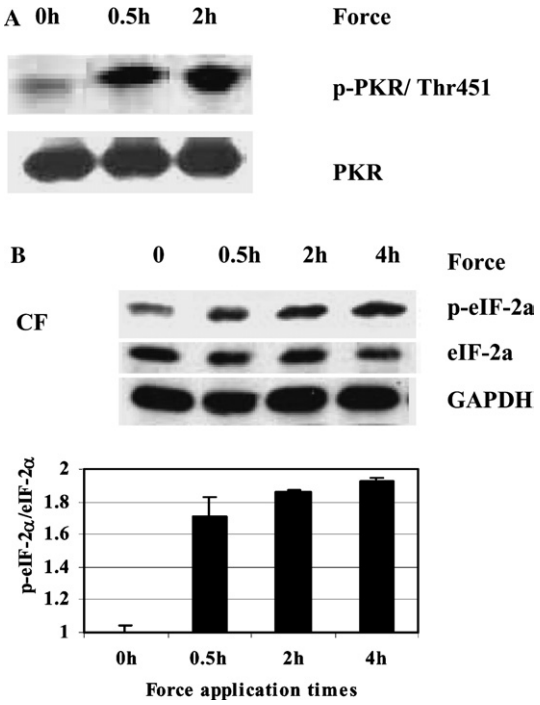

Fig. 2. Force induces a time-dependent increase of phosphorylated PKR and eIF-2α. Immunoblots of (A) PKR and phospho-PKR (Thr 451) and of (B) phospho-eIF-2α, eIF-2α, and GAPDH. Force promotes increased phospho-PKR, phospho-eIF-2α but has no effect on PKR, eIF-2α, and GAPDH content. Data are means ± SEM of ratios of phospho-eIF-2α/eIF-. ($p < 0.05$; $n = 3$ replicates).

pathways can facilitate the discovery of novel mechanotransduction elements, as shown here for PKR. This enzyme has been previously associated with cell stress responses leading to apoptosis [28,29]. As we have previously found small but significant increases of the proportion of dead cells after 24 h of tensile force application to fibroblasts [30], PKR, and its downstream effector, eIF-2α, may be relevant elements in the mechanotransduction pathways that control force-induced apoptosis.

Screening for mechanical force-induced phospho-kinases

Mechanical forces strongly influence a large number of cellular processes including motility, proliferation, regulation of cell shape, and tissue homeostasis [31,32]. Notably, mechanical forces are important regulators of the cell and tissue differentiation programs that occur in development and mechanical forces also impact on cell differentiation and tissue remodeling in mature tissues [33]. Mechanical force-induced activation of MAP kinase and NF-κB signaling pathways has been previously described in fibroblasts [34]. The Kinexus screening method provides an efficient, systematic approach to explore mechanical force-induced activation of novel signaling pathways. We used a well-characterized, collagen-coated magnetite bead system [14–16,21,35–37] to deliver perpendicular forces to cell surface integrins of neonatal cardiac fibroblasts (0.65 pN/μm^2 cell surface area). These forces are of comparable levels to those generated in functional myocardium in vivo [38]. We found that the MAPKKs MKK3 and MKK6 were significantly phosphorylated by force. The finding of increased phosphorylation of upstream activators of p38 after application of tensile force is consistent with previous immunoblotting studies of cardiac fibroblasts and human gingival fibroblasts [14,15]. Recent reports have indicated that p38 can regulate myofibroblast differentiation and cellular responses to applied force [39,40] and may be a crucial mediator of mechanical force-induced SMA expression [41].

With the Kinexus screening system we found phosphorylation of PKR after application of mechanical tension. PKR is a double-stranded, RNA-dependent protein kinase. It is ubiquitously expressed by all cells at low levels but is transcriptionally induced by α and β interferons, and in response to anti-viral and anti-proliferative factors secreted by viral-infected cells [42]. Phosphorylation of PKR is associated with viral

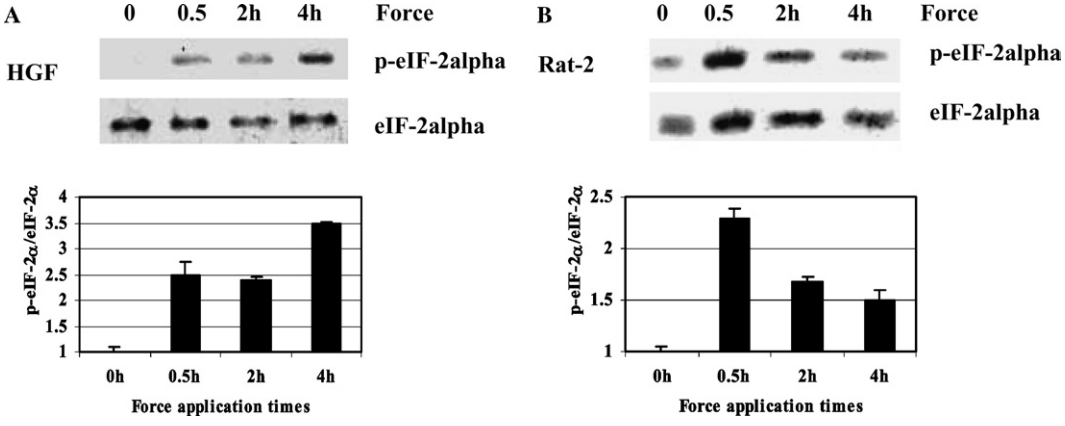

Fig. 3. Force induces p-eIF-2α in human gingival fibroblasts and Rat-2 cells. Immunoblots of phosphorylated eIF-2α and eIF-2α in human gingival fibroblasts (A) and Rat-2 cells (B) after force application. There is increased phosphorylation of eIF-2α but no effect on total eIF-2α content. Note that the kinetics of phosphorylation in response to force are different between the two cell types. Data are means ± SEM of ratios of phospho-eIF-2α/eIF-. ($p < 0.05$; $n = 3$ replicates).

infection, cell stress, and events leading up to cell death, including a global block of protein translation, activation of p53, participation in the JNK and p38 MAPK pathways, and activation of NF-κB. Until now there have been no published reports on mechanical force-induced PKR activation. Although we found no alteration of the levels of total (non-phosphorylated) PKR following application of tensile forces (Fig. 2), the marked increases of phosphorylated PKR induced by force suggested that we examine potential downstream targets of PKR, including eIF-2α.

Mechanical force-induced eIF-2α phosphorylation

eIF-2α is a highly conserved protein kinase in eukaryotes that facilitates adaptation to a wide variety of stressful conditions including amino acid starvation [23], viral infection [26], iron deficiency [25], and endoplasmic reticulum stress [24]. eIF-2α is a heterotrimer composed of three subunits (α, β, and γ) that functions by associating with GTP [43]. We found that mechanical force induces eIF-2α phosphorylation in cardiac fibroblasts. Similar results from Rat-2 cells (from kidney) and human gingival fibroblasts indicated that mechanical forces can indeed induce eIF-2α phosphorylation in multiple cell types. Unlike previous studies of eIF-2α activation, force stimulation does not promote amino acid starvation, viral infection, iron deficiency or high levels of interferon expression starvation [23,24,26].

Phosphorylation of eIF-2α generally leads to inhibition of protein synthesis [44]. In myofibroblasts with high SMA content, tensile forces reduce SMA content [15,16], an effect which may be mediated by an eIF-2α inhibitory pathway. However, in the same model system, other studies have shown increased mRNA and protein expression of filamin A [41], an actin binding protein that protects cells against apoptosis. As one of the consequences of eIF-2α phosphorylation is PKR-mediated apoptosis [45], we suggest that multiple apoptosis regulating factors may be invoked by eIF-2α, including filamin A. Further, as eIF-2α has been implicated in cell stress responses that lead to apoptosis, we speculate that it is at a nodal decision point determining if cells survive or succumb to force-induced apoptosis. The discovery of this protein and PKR was dependent on the Kinexus phospho-kinase screening system, a simple and effective search system that allowed us to identify novel phospho-kinases implicated in mechanotransduction processes.

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