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Stimulation of multiple MAPK pathways by mechanical overload in the perfused amphibian heart

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Aggeli, Ioanna-Katerina S., Catherine Gaitanaki, Antigone Lazou, and Isidoros Beis. Stimulation of multiple MAPK pathways by mechanical overload in the perfused amphibian heart. *Am J Physiol Regulatory Integrative Comp Physiol* 281: R1689–R1698, 2001.—The mitogen-activated protein kinase (MAPK) signal transduction pathway activated by mechanical stress was investigated in the isolated perfused amphibian (*Rana ridibunda*) heart. High perfusion pressure induced the rapid (30 s) and prolonged (30 min) phosphorylation of a p43-extracellular regulated kinase, a response almost completely inhibited by 25 μM PD-98059. c-Jun NH_2-terminal kinase (JNK) was also phosphorylated with maximal values attained at 15 min and remained elevated over 30 min. In-gel kinase assays verified that phosphorylated JNKs are active, phosphorylating the transcription factor c-Jun. Furthermore, pressure overload rapidly stimulated the p38-MAPK phosphorylation (30 s), a transient process (5 min) abolished by 1 μM SB-203580. In-gel kinase assays revealed that with phosphorylation, active p38-MAPKs phosphorylate their substrate MAP kinase-activated protein kinase 2. Biochemical analysis along with immunohistochemical studies showed that with activation, the three MAPK subfamily members examined are localized not only in the cytoplasm but in the nucleus as well. Present results therefore demonstrate for the first time in an amphibian species the involvement of multiple MAPK pathways in the mechanical overload-induced adaptive responses of the heart as well as their possible physiological roles.

extracellular regulated kinase; c-Jun NH_2-terminal kinase; p38-mitogen-activated protein kinase; mechanical stress

IN LIVING ANIMALS, many cell types are normally exposed to a variety of internal or external stimuli. Among these stimuli, mechanical forces, such as those placed on skeletal and cardiac myocytes with increasing passive and/or active loading, have a variety of effects on the structure and function of the cells by altering their physiology qualitatively and quantitatively (32, 38, 39). In the mammalian heart, mechanical stress induced by pressure overload, increased work or stretching leads to an increase of protein synthesis, atrial natriuretic peptide release, RNA translational activity, and finally to hypertrophy (for review, see Refs. 30, 32, 36). However, little is known as to how mechanical stimuli are converted into intracellular signals of gene regulation and expression.

Among the various signal transduction pathways activated by mechanical stress, the mitogen-activated protein kinases (MAPKs) are included. The MAPK superfamily of protein Ser/Thr kinases is a widely distributed group of enzymes that has been highly conserved through evolution (for review, see Refs. 2, 12, 40). Three subfamilies of the MAPKs have been clearly identified and extensively studied in mammalian experimental models: the extracellular regulated kinases (ERKs); the c-Jun NH_2-terminal kinases (JNKs), which are also known as the stress-activated protein kinases; and the p38-MAPKs. The ERK pathway is primarily responsive to growth factors and mitogens but can also be activated by hypoxia or osmotic stress (3, 4, 22, 33) and appears to be involved predominantly in anabolic processes (cell growth, division, and differentiation). JNKs are only weakly activated by growth factors but show a strong response to cellular stresses such as UV irradiation, heat shock, protein synthesis inhibitors (9, 18), reperfusion following ischemia (15, 27), mechanical stretch (16, 31), and osmotic shock (10, 17). The third MAPK member p38 is activated by various environmental stresses, including hyperosmolarity (13, 40). The MAPKs are final components of three-member MAPK cascades. The nature of these cascades allows considerable potential for signal integration and amplification as well as the possibility for cross talk between the pathways because of the apparently overlapping substrate specificities of some of the components and of the upstream signaling molecules.

During their activation, mammalian MAPKs seem to translocate, to some extent, to the nucleus (25, 40), a well-justified property as many lines of evidence have shown that substrates of the three members of the MAPK superfamily are localized in both the cytoplasm and the nucleus (2, 12, 35). In particular, pressure

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overload-stimulated MAPK activation induces immediate-early genes such as protooncogenes and heat shock protein genes as well as late-responsive genes such as fetal contractile protein genes and the atrial natriuretic peptide gene (30, 32).

In a previous paper, we characterized for the first time the three most studied members of the MAPK superfamily in the isolated perfused amphibian heart (1). The one ERK (p43) detected was activated by 1 μM phorbol 12-myristate 13-acetate (PMA), the most powerful activator of mammalian ERKs yet identified. Two JNKs (p46 and p52) were also identified and found to be phosphorylated by 0.5 M sorbitol as well as reoxygenation following anoxia. p38-MAPK (p38) was strongly activated by 0.5 M sorbitol. The aim of the present study is to elucidate the signal transduction pathways activated by mechanical stress in this experimental model. We examined the activation profile of ERKs, JNKs, and p38-MAPKs, as well as the pattern of their localization after stressing the frog heart mechanically to determine their possible physiological roles under similar conditions in vivo.

MATERIALS AND EXPERIMENTAL PROCEDURES

Most biochemicals used were obtained from Sigma Chemical (St. Louis, MO). Enhanced chemiluminescence (ECL) kit was from Amersham International (Uppsala, Sweden), and alkaline phosphatase kit was from Lipshaw (Pittsburgh, PA). [γ-^{32}ATP] was from NEN Life Sciences (Brussels, Belgium). Bradford protein assay reagent was from Bio-Rad (Hercules, CA). Nitrocellulose (0.45 μm) was obtained from Schleicher & Schuell (Keene, NH). SB-203580 and PD-98059 were obtained from Calbiochem-Novabiochem (La Jolla, CA).

Rabbit polyclonal antibodies to the total ERKs, mitogen-activated protein kinase kinases (MEKs), and p38-MAPK, as well as antibodies specific for the dually phosphorylated ERKs, MEKs, p38-MAPK, and JNKs, were obtained from New England Biolabs (Beverly, MA). Prestained molecular mass markers were from New England Biolabs. Biotinylated anti-rabbit antibody was from DAKO A/S (Glostrup, Denmark). Anti-histone 1 mouse monoclonal antibody was obtained from BioGenex (San Ramon, CA). Peroxidase-labeled anti-mouse antibody was from Amersham International. X-OMAT AR 13 × 18 cm and Elite chrome 100 films were purchased from Eastman Kodak (New York, NY). Glutathione *S*-transferase (GST)-c-Jun (1–135) and GST-MAP kinase-activated protein kinase 2 (MAPKAPK2) (46–400) were kindly provided by Prof. P. H. Sugden (Imperial College, London, UK).

Animals. Frogs (*Rana ridibunda*) weighing 100–120 g were supplied by a local dealer after having been caught in the vicinity of Thessaloniki, Greece. They were kept in containers with fresh water and used a week after arrival. Care of the animals conformed to Good Laboratory Practice.

Heart perfusions. Rana ridibunda (100–120 g of weight) hearts were perfused with the nonrecirculating Langendorff mode at a pressure (P) of 4.5 kPa (31.5 mmHg) with bicarbonate-buffered saline [in mM: 23.8 $NaHCO_3$, 103 NaCl, 1.8 $CaCl_2$, 2.5 KCl, 1.8 $MgCl_2$, and 0.6 NaH_2PO_4 (pH 7.4 at 25°C)] supplemented with 10 mM glucose and equilibrated with 95% O_2-5% CO_2. The temperature of the hearts and perfusates was maintained at 25°C by the use of a water-jacketed apparatus. All hearts were equilibrated for 15 min under these conditions. The perfusion pressure was then changed to 3P/2 (6.75 kPa or 47.25 mmHg) or 2P (9 kPa or 63 mmHg), and hearts were perfused for 30 s up to 30 min. When PMA or sorbitol was added, hearts were perfused at normal pressure (4.5 kPa) with 1 μM PMA or 0.5 M sorbitol (for 10 or 15 min, respectively) after the equilibration period. When the inhibitors PD-98059 or SB-203580 were used, they were added throughout the experiment at a concentration of 1 or 25 μM, respectively. In parallel form, hearts were perfused either with the DMSO solvent or the respective inhibitor alone to examine if they affect any of the variables measured.

At the end of the perfusions, hearts were "freeze-clamped" between aluminum tongs cooled in liquid N_2, and after the removal of the atria, ventricles were pulverized under liquid N_2 and powders were stored at −80°C.

Tissue extractions. Heart powders were homogenized with 3 ml/g of buffer [20 mM Tris·HCl, pH 7.5, 20 mM β-glycerophosphate, 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 5 mM dithiothreitol (DTT), 10 mM benzamidine, 200 μM leupeptin, 120 μM pepstatin A, 10 μM transepoxy succinyl-L-leucylamido-(4-guanidino)butane, 300 μM phenylmethylsulfonyl fluoride (PMSF), 0.5% (vol/vol) Triton X-100] and extracted on ice for 30 min. The samples were centrifuged (10,000 *g*, 5 min, 4°C), and the supernatants were boiled with 0.33 vol of SDS-PAGE sample buffer [0.33 M Tris·HCl, pH 6.8, 10% (wt/vol) SDS, 13% (vol/vol) glycerol, 20% (vol/vol) 2-mercaptoethanol, 0.2% (wt/vol) bromphenol blue]. Protein concentrations were determined using the BioRad Bradford assay (5).

SDS-PAGE and immunoblot analysis. Proteins were separated by SDS-PAGE on 10% (wt/vol) acrylamide, 0.275% (wt/vol) bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm). Membranes were then incubated in Tris-buffered saline T (TBS-T) [20 mM Tris·HCl, pH 7.5, 137 mM NaCl, 0.1% (vol/vol) Tween 20] containing 5% (wt/vol) nonfat milk powder for 30 min at room temperature. Subsequently, the membranes were incubated with the appropriate antibody according to the manufacturer's instructions. After being washed in TBS-T (3 × 5 min), the blots were incubated with horseradish peroxidase-linked anti-rabbit IgG antibodies [1:5,000 dilution in TBS-T containing 1% (wt/vol) nonfat milk powder, 1 h, room temperature]. The blots were washed again in TBS-T (3 × 5 min), and the bands were detected using ECL with exposure to X-OMAT AR film. Blots were quantified by laser-scanning densitometry.

In-gel kinase assays. Proteins (200 μg loaded/lane) were separated on 10% (wt/vol) SDS-polyacrylamide gels with 6% (wt/vol) stacking gel. The 10% (wt/vol) gels were formed in the presence of 0.5 mg/ml GST-MAPKAPK2 (46–400) for the assay of p38-MAPKs (28) or 0.5 mg/ml GST-c-Jun (1–135) for the assay of JNKs (14). After electrophoresis, SDS was removed from the gels by washing in 20% (vol/vol) 2-propanol in 50 mM Tris·HCl, pH 8.0 (3 × 30 min). The 2-propanol was removed by washing in 50 mM Tris·HCl (pH 8.0) and 5 mM 2-mercaptoethanol (3 × 30 min). Proteins were denatured in 6 M guanidine-HCl, 50 mM Tris·HCl (pH 8.0), 5 mM 2-mercaptoethanol (2 × 30 min), and then renatured in 50 mM Tris·HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.04% (vol/vol) Tween 40 (1 × 30 min, 2 × 1 h, 1 × 18 h, 1 × 30 min, 4°C). The gels were equilibrated to room temperature with 40 mM HEPES (pH 8.0), 2 mM DTT, 10 mM $MgCl_2$ (2 × 30 min), and incubated for 3 h with 12.5 μCi/gel [γ-^{32}P]ATP in 5 ml of 40 mM HEPES (pH 8.0), 0.5 mM EGTA, 10 mM $MgCl_2$, 50 μM ATP, and 0.1 μM cAMP-dependent protein kinase inhibitor. The reaction was stopped and gels were washed with 1% (wt/vol) disodium pyrophosphate, 5% (wt/vol) trichloroacetic acid. The gels were dried onto 3MM Whatman chromatogra-

phy paper and autoradiographed. In-gel kinase activities were quantified by laser-scanning densitometry.

Subcellular fractionation. Subcellular fractionation was performed as described by Takemoto et al. (37) with slight modifications. Frozen hearts were homogenized in three volumes of 10 mM HEPES at pH 7.9, which contained 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 10 mM NaF, 1 mM Na_3VO_4, 0.5 mM PMSF, 20 mM β-glycerophosphate, 60 μg/ml aprotinin, and 2 μg/ml leupeptin. After incubation on ice for 15 min, homogenates were centrifuged (5,000 *g*, 10 min, 4°C), and 10% (vol/vol) Nonidet P40 was added. Samples were vigorously mixed and further incubated on ice for 60 min. Homogenates were centrifuged (1,000 *g*, 10 min, 4°C) to obtain pellets. Pellets were washed once and resuspended in a buffer containing 20 mM HEPES at pH 7.9, 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1.5 mM $MgCl_2$, 20% (vol/vol) glycerol, 10 mM NaF, 1 mM Na_3VO_4, 0.5 mM PMSF, 0.2 mM DTT, 20 mM β-glycerophosphate, 60 μg/ml aprotinin, and 2 μg/ml leupeptin. Tubes were incubated on ice for 60 min and then rocked for 15 min at 4°C. After centrifugation (15,000 *g*, 10 min, 4°C), the supernatants containing nuclear protein were boiled with 0.33 vol of SDS-PAGE sample buffer.

Immunohistochemistry. At the end of the perfusions, atria were removed and ventricles were immersed in Uvasol/isopentane precooled in liquid N_2 and stored at −80°C. Tissues were sectioned with a cryostat at a thickness of 5–6 μm, fixed with ice-cold acetone (10 min, room temperature), and specimens were stored at −30°C until use. Tissue sections were washed in TBS-T, and nonspecific binding sites were blocked with 3% (wt/vol) BSA in TBS-T (1 h, room temperature). Specimens were incubated with primary antibody diluted in 3% BSA (wt/vol) in TBS-T (overnight, 4°C) and immunostained by the alkaline phosphatase method using a Kwik kit according to the manufacturer's instructions. The alkaline phosphatase label was visualized by exposing the sections to fast red chromogen, and nuclei were counterstained with hematoxylin. Slides were mounted, examined with a Zeiss Axioplan microscope equipped with Nomarski filter, and photographed with the Kodak Elite chrome 100 film.

Statistical evaluations. All data are presented as means ± SE. Comparisons between control and treatments were performed using Student's paired *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Mechanical overload stimulates ERKs. To simulate mechanical overload, hearts were perfused under increased perfusion pressure (3/2- and 2-fold of normal P = 4.5 kPa). It is known that increased pressure leads to direct mechanical stress due to the fact that heart work output is also increased. The different values of pressure examined could represent two conditions of mechanical overload, an intermediate and a more extreme, in terms of physiology, compared with the normal arterial frog pressure corresponding to 4.5 kPa (19, 34). Perfusion under high pressure activated the 43-kDa ERK as evidenced by immunoblotting with an antibody that detects phosphorylated Thr^{202} and Tyr^{204} of ERK1/2. Increased phosphorylation of ERK was detected from as early as 30-s perfusion at 3P/2 compared with the control (Fig. 1*A*, *top*). Maximal activation was observed at 1 min (~3.6 ± 0.1-fold relative to controls) and remained elevated over 30 min, with a progressive decline after 5 min (Fig. 1*A*, *top*, and *C*). This activation profile was similar to that obtained after perfusion at an even higher pressure (2P) (Fig. 1, *B* and *C*). Frog hearts perfused with 1 μM PMA for 10 min, which is known to activate ERK in this experimental model (1), were used as positive controls (Fig. 1, *A* and *B*). The response of ERK to mechanical overload was comparable with that in-

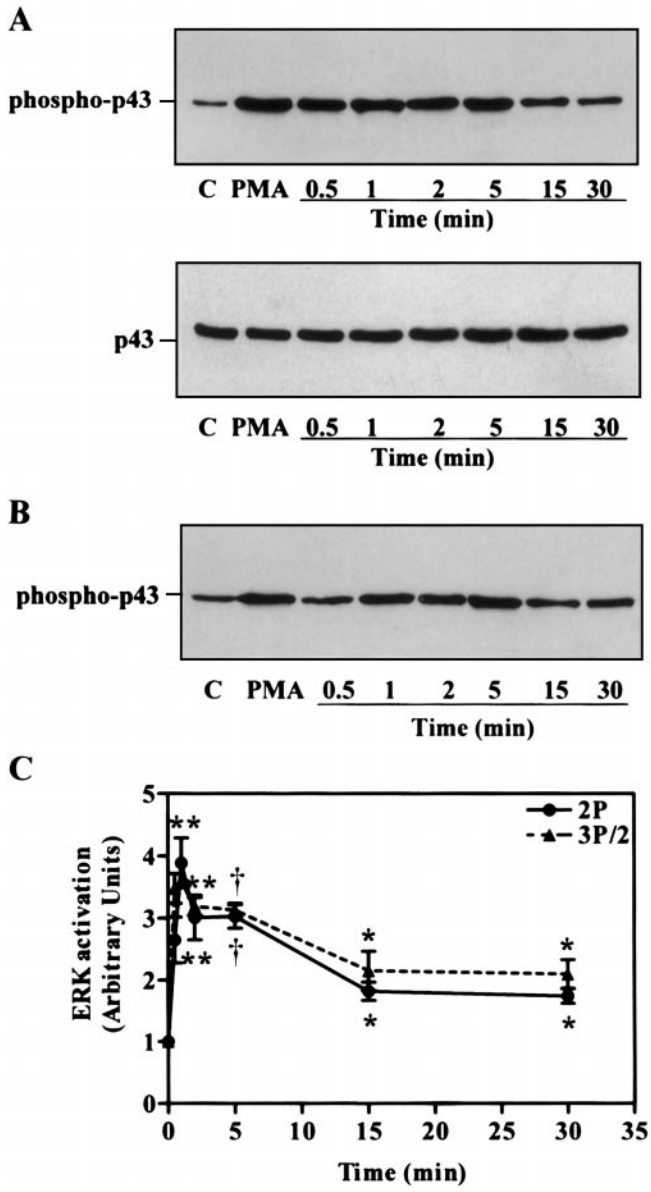

Fig. 1. Phosphorylation of extracellular regulated kinases (ERKs) in hearts perfused under high pressure (P) (3P/2 and 2P relative to control condition, P = 4.5 kPa). ERK phosphorylation was assayed by Western immunoblotting in samples (50 μg protein) from *Rana ridibunda* hearts perfused at 3P/2 (*A*, *top*) and 2P (*B*) for the times indicated, as described in MATERIALS AND EXPERIMENTAL PROCEDURES. Samples from hearts perfused at 3P/2 for the times indicated were also assayed for total ERK immunoreactivity (*A*, *bottom*). Bands were quantified by laser-scanning densitometry and plotted (*C*). Each point represents the mean ± SE for the relative time points (4 separate heart perfusions at each time point). *$P < 0.05$ vs. control (C) value; **$P < 0.01$ vs. control value; and †$P < 0.001$ vs. control value. PMA, phorbol 12-myristate 13-acetate.

duced by 1 μM PMA (~3.3 ± 0.2-fold relative to controls). MEK1/2, the upstream activator of ERKs, was also rapidly phosphorylated by an increased load, with the phosphorylation remaining elevated over 30 min (Fig. 2, *A*, *B*, *top*, and *C*). Equal protein loading was verified with Western blotting for total ERK and MEK1/2 (Figs. 1*A*, *bottom*, and 2*B*, *bottom*, respectively).

PD-98059 (25 μM in DMSO/18.5 mM final concentration), a selective inhibitor of the phosphorylation and activation of MEK, was tested. To ensure that any effects were independent of DMSO, hearts were also perfused with 18.5 mM DMSO included in the perfusion media. The inclusion of this concentration of DMSO slightly activated p43-ERK. This effect was taken into consideration during the evaluation of the effect of PD-98059 on the activation of ERK. PD-98059 abolished the phosphorylation of this kinase (Fig. 3*A*, *top*, and *B*) by high pressure (2P), demonstrating that p43-ERK was indeed the kinase activated by pressure overload in the assays mentioned above. To verify that equal amounts of protein were loaded, Western blots for total ERK (phosphorylation state independent) were performed (Fig. 3*A*, *bottom*).

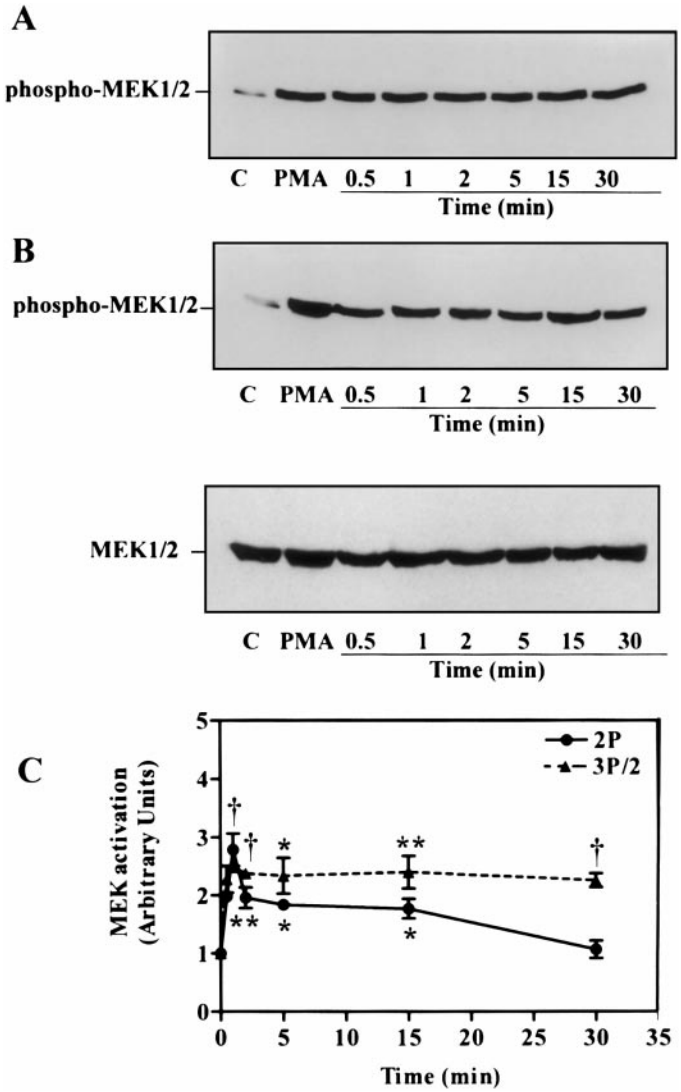

Fig. 2. High perfusion pressure rapidly activates mitogen-activated protein kinase kinase (MEK) 1/2. MEK1/2 phosphorylation was assayed by Western immunoblotting in samples (100 μg protein) from hearts perfused at 3P/2 (*A*) and 2P (*B, top*) for the times indicated, as described in MATERIALS AND EXPERIMENTAL PROCEDURES. Samples from hearts perfused at 2P for the times indicated were also assayed for total MEK1/2 immunoreactivity (*B, bottom*). Bands were quantified by laser-scanning densitometry and plotted (*C*). Each point represents the mean ± SE for the relative time points (3 separate heart perfusions at each time point). *$P < 0.05$ vs. control value; **$P < 0.01$ vs. control value; and †$P < 0.001$ vs. control value.

Activation of JNKs by mechanical stress. Two JNKs have been detected in the frog heart by Western blot analysis and in-gel kinase assays, with molecular masses of ~46 kDa (JNK1) and 52 kDa (JNK2) (1). As phosphorylation of JNKs at Thr^{183} and Tyr^{185} is essential for kinase activity, JNK activation was examined by Western blot analysis using an antibody that detects their dual phosphorylated isoforms. Hearts perfused at 3P/2 showed immediate activation of JNK1 from as early as 30 s (Fig. 4*A*). Maximal activation was observed at 15 min (~10.2 ± 0.4-fold relative to controls) and remained elevated over 30 min (Fig. 4*A*). The second protein kinase detected, JNK2, was less intense than JNK1, with an activation that followed a similar time course. Maximal stimulation (~3.6 ± 0.4-fold relative to controls) was reached at 15 min (Fig. 4*A*). A similar profile of activation was observed with perfusion at 2P (~13 ± 0.1-fold for JNK1 and 3.8 ± 0.3-fold for JNK2 relative to controls) (Fig. 4*B*). Hearts perfused with 0.5 M sorbitol (15 min), which strongly activates JNKs in the amphibian heart (1), were used as positive controls.

The next set of experiments was designed to evaluate whether JNK phosphorylation contributes to c-Jun activities. Two protein kinases (~46 and 52 kDa) that phosphorylate GST-c-Jun (1–135) were detected by in-gel kinase assays. No activity was detected in control hearts or when GST-c-Jun was omitted from the gels (data not shown). c-Jun phosphorylation by both JNKs followed a similar time course with maximal values being attained at 15 min, 4.7 ± 0.2-fold (JNK 1) and 3.7 ± 0.3-fold (JNK 2), respectively (Fig. 4*C*).

Effects of high perfusion pressure on the activation of p38-MAPK. Immunoblot analysis of total extracts of hearts perfused under high pressure (3P/2) revealed phosphorylation of p38-MAPKs. The band detected at 39 kDa may represent another form of p38-MAPK, recognized by the same antibody. The response was immediate, and maximal intensities were attained after a period of 30 s for p38 (~12.1 ± 1.0-fold relative to controls) and 2 min for p39 (~4.7 ± 0.5-fold relative to controls) (Fig. 5*A*). Perfusion at an even higher pressure (2P) also caused the direct dual phosphorylation of p38 and p39 with maximal values being attained after a period of 1 min (11.3 ± 0.4-fold) and 3 min (4.4 ± 0.1-fold), respectively. Activation levels declined drastically after 5 min and reached basal levels at 15 min (Fig. 5*B*). Hearts that had been perfused with 0.5 M sorbitol for 15 min, a potent activator of amphibian p38-MAPKs (1), were used as positive controls. The response of p38-MAPK to mechanical stress was comparable with that induced by sorbitol, which stimu-

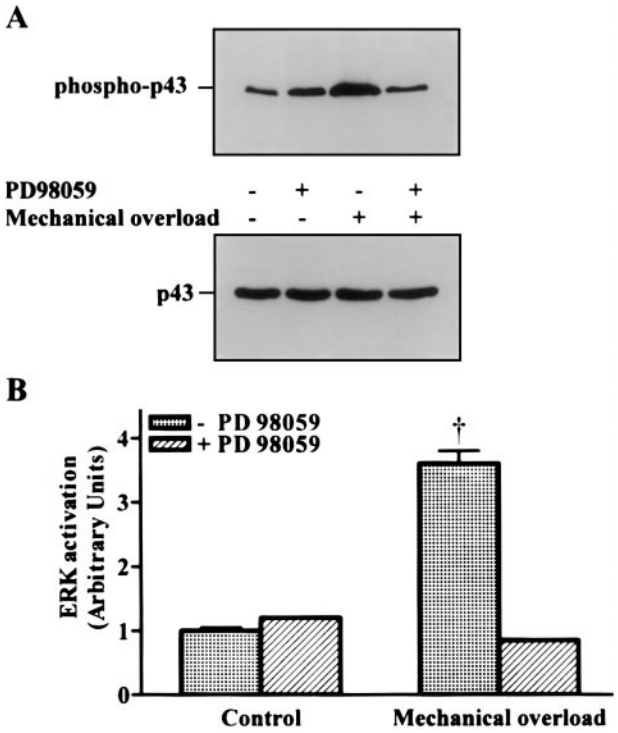

Fig. 3. PD-98059 inhibits high perfusion pressure-mediated ERK activation. PD-98059 was added at a concentration of 25 μM throughout the experiment and the perfusion under high pressure (2P). Phosphorylated ERK (*A*, *top*) and total ERK immunoreactivity (*A*, *bottom*) were assayed as described in MATERIALS AND EXPERIMENTAL PROCEDURES. The experiment was repeated on 2 further occasions with similar results. Bands were quantified by laser-scanning densitometry and plotted (*B*). †$P < 0.001$ vs. control value.

lated p38 and p39 by 16.5 ± 1.7- and 11 ± 0.9-fold, respectively, relative to controls (Fig. 5, *A* and *B*).

SB-203580 (1 μM in DMSO/1.27 mM final concentration), a bicyclic imidazole widely used as a specific inhibitor of p38-MAPK, abolished the phosphorylation of p38 and p39 by all these interventions (Fig. 5*C*, *top*). To ensure that equal amounts of protein were loaded, Western blots for total p38 were also performed (Fig. 5*C*, *bottom*).

The identities of the kinases detected by Western blot were confirmed as p38-MAPK activities by in-gel kinase assays using GST-MAPKAPK2 (46–400) as a substrate. The results presented in Fig. 5*D* show that high perfusion pressure (2P) stimulated a considerable increase in the p38-MAPK activity, with maximal value being attained at 5 min (~3.6 ± 0.4-fold relative to controls), reaching basal levels at 30 min.

Subcellular localization of phosphorylated MAPKs under mechanical overload. All three members of the MAPK superfamily are involved in the phosphorylation of substrates localized not only in the cytoplasm but in the nucleus as well. Thus it was of interest to examine the subcellular localization of activated MAPKs modulated by mechanical stress. For this purpose, extracts of hearts perfused under high pressure (2P) were fractionated. Purity and protein homogeneity of the nuclear fractions were confirmed by immunodetection of the nuclear marker protein histone 1 (Fig. 6*D*).

Results in Fig. 6 (*A*, *B*, *C*, *top* panels) demonstrate that the three MAPKs examined (phosphorylation

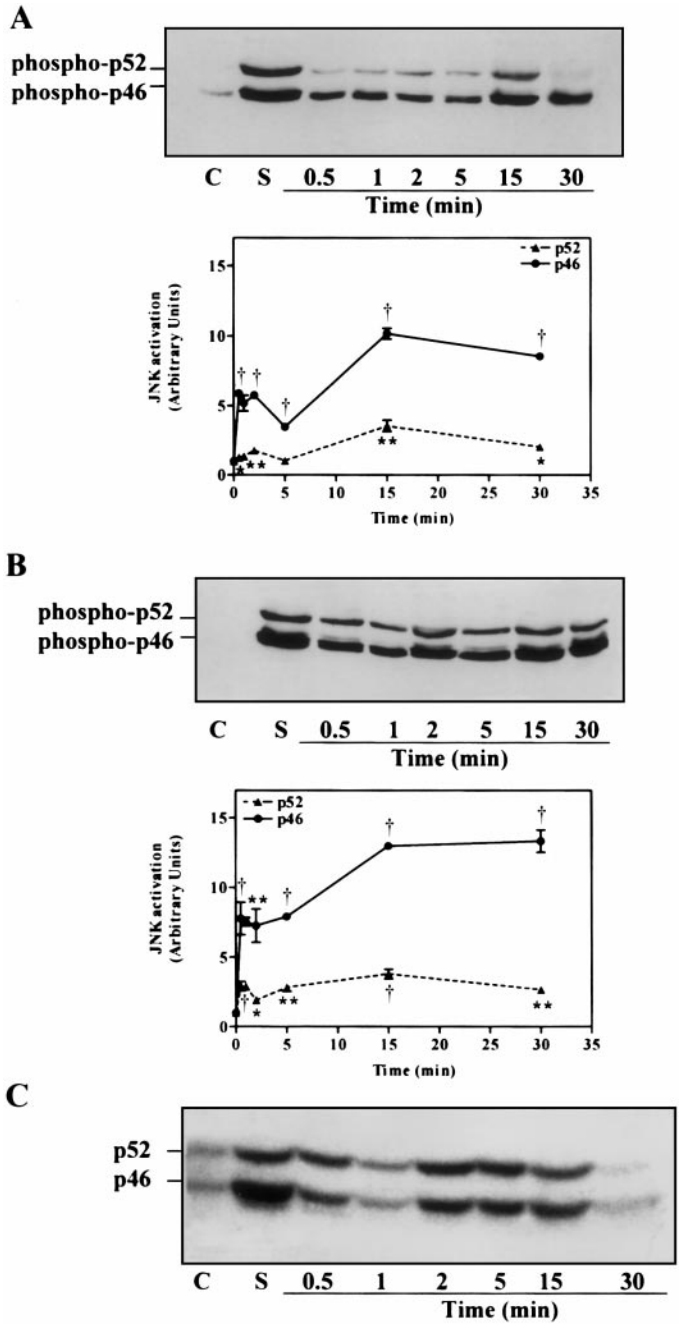

Fig. 4. High perfusion pressure activates c-Jun NH_2-terminal kinases (JNKs). JNK phosphorylation was assayed by Western immunoblotting in samples (100 μg protein) from control *Rana ridibunda* hearts, hearts perfused with 0.5 M sorbitol (S) for 15 min, or hearts perfused at 3P/2 (*A*, *top*) and 2P (*B*, *top*) for the times indicated. Bands were quantified by laser-scanning densitometry and plotted (*A* and *B*, *bottom* panels). *C*: JNK activities in whole extracts (150 μg protein) from hearts perfused at 3P/2 for the times indicated were determined by in-gel kinase assays with glutathione *S*-transferase (GST)-c-Jun (1–135) as substrate. The experiment was repeated on 2 further occasions with similar results. *$P < 0.05$ vs. control value; **$P < 0.01$ vs. control value; and †$P < 0.001$ vs. control value.

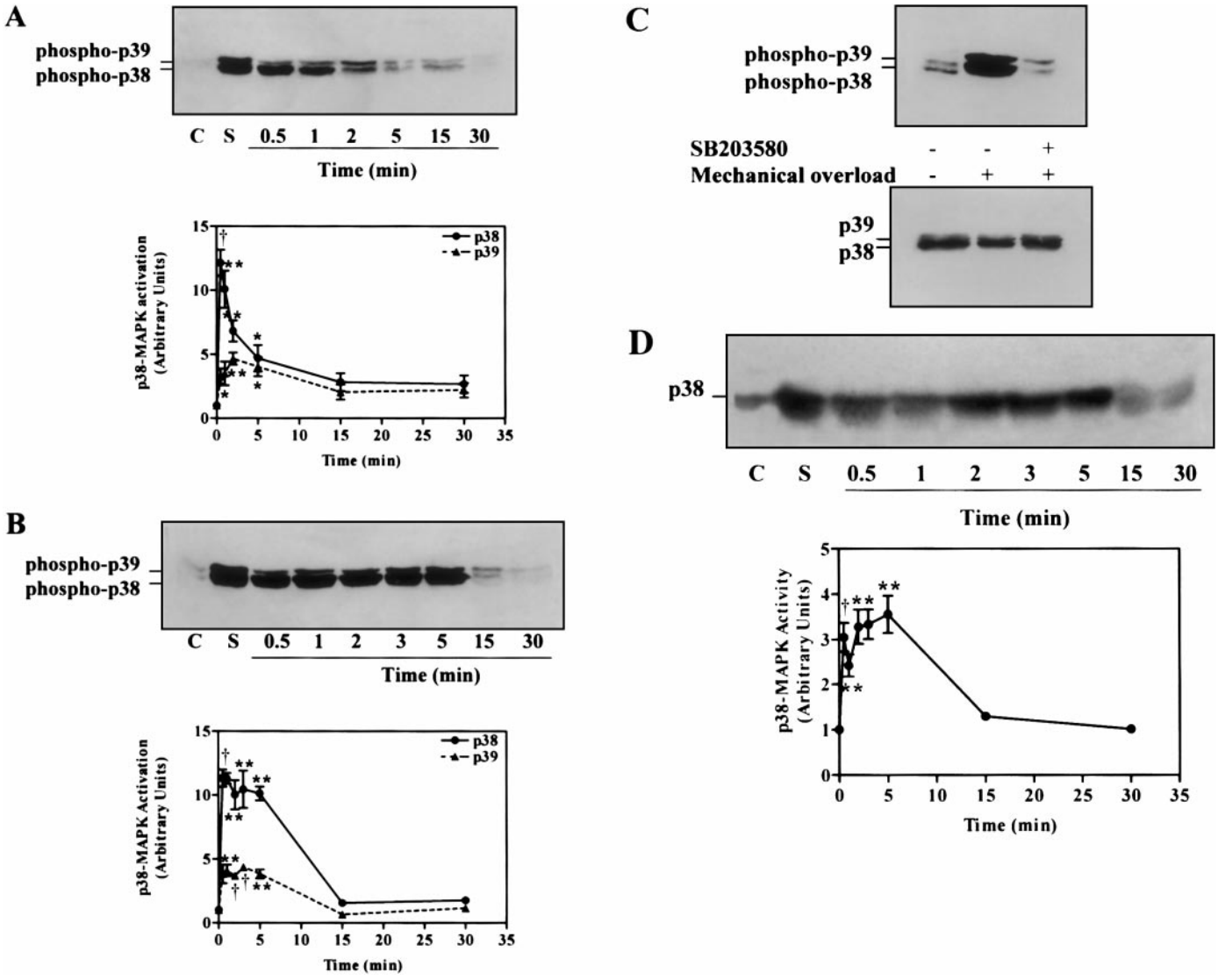

Fig. 5. High perfusion pressure rapidly activates p38-mitogen-activated protein kinase (MAPK). p38-MAPK phosphorylation was assayed by Western immunoblotting in samples (100 μg protein) from control *Rana ridibunda* hearts, hearts perfused with 0.5 M S for 15 min, or hearts perfused at 3P/2 (*A*, *top*) and 2P (*B*, *top*) for the times indicated. *C*: SB-203580 was added at a concentration of 1 μM throughout the experiment. Phosphorylated p38-MAPK (*top*) as well as total p38-MAPK (*bottom*) immunoreactivity were assayed, and uniform protein loading was verified. *D*: p38-MAPK activity in whole extracts (150 μg) from hearts perfused at 2P (*top*) for the times indicated was determined by in-gel kinase assays with GST-MAP kinase-activated protein kinase 2 (46–400) as substrate. Bands were quantified by laser-scanning densitometry and plotted (*A*, *B*, *D*, *bottom* panels). Each point represents the mean ± SE of at least 3 separate samples for the relative time points and the conditions indicated. *$P < 0.05$ vs. control value; **$P < 0.01$ vs. control value; and †$P < 0.001$ vs. control value.

state-independent levels) are present in both cytoplasmic and nuclear fractions. High perfusion pressure induces the phosphorylation of cytoplasmic as well as nuclear MAPKs (Fig. 6*A*, *B*, *C*, *bottom* panels). In particular, in the cytoplasmic fraction, mechanical overload resulted in a considerable phosphorylation of p43-ERK (3.6 ± 0.2-fold relative to control, $P < 0.01$), JNK1 (p46) and JNK2 (p52) (9.8 ± 0.4- and 4.6 ± 0.1-fold relative to control, $P < 0.01$, respectively), and p38-MAPK (7.3 ± 0.2-fold relative to control, $P < 0.01$). In parallel form, mechanical overload also induced a significant activation of the respective kinases in the nuclear fraction by 3.5 ± 0.2-fold for p43-ERK, 2.3 ± 0.2-fold for JNK1, 2.7 ± 0.1-fold for JNK2, and 3.9 ± 0.3-fold for p38-MAPK, relative to the respective controls ($P < 0.01$).

Immunohistochemical localization of phosphorylated ERK and p38-MAPK. On the basis of the results of the biochemical analysis performed, we further investigated the immunohistochemical localization pattern of phospho-ERK and phospho-p38-MAPK, using the respective antibodies. Frog hearts perfused under normal or high pressure (2P) for 2 min in the absence or presence of the respective inhibitor (PD-98059 for ERK or SB-203580 for p38-MAPK) were sectioned and processed using an alkaline phosphatase kit as described

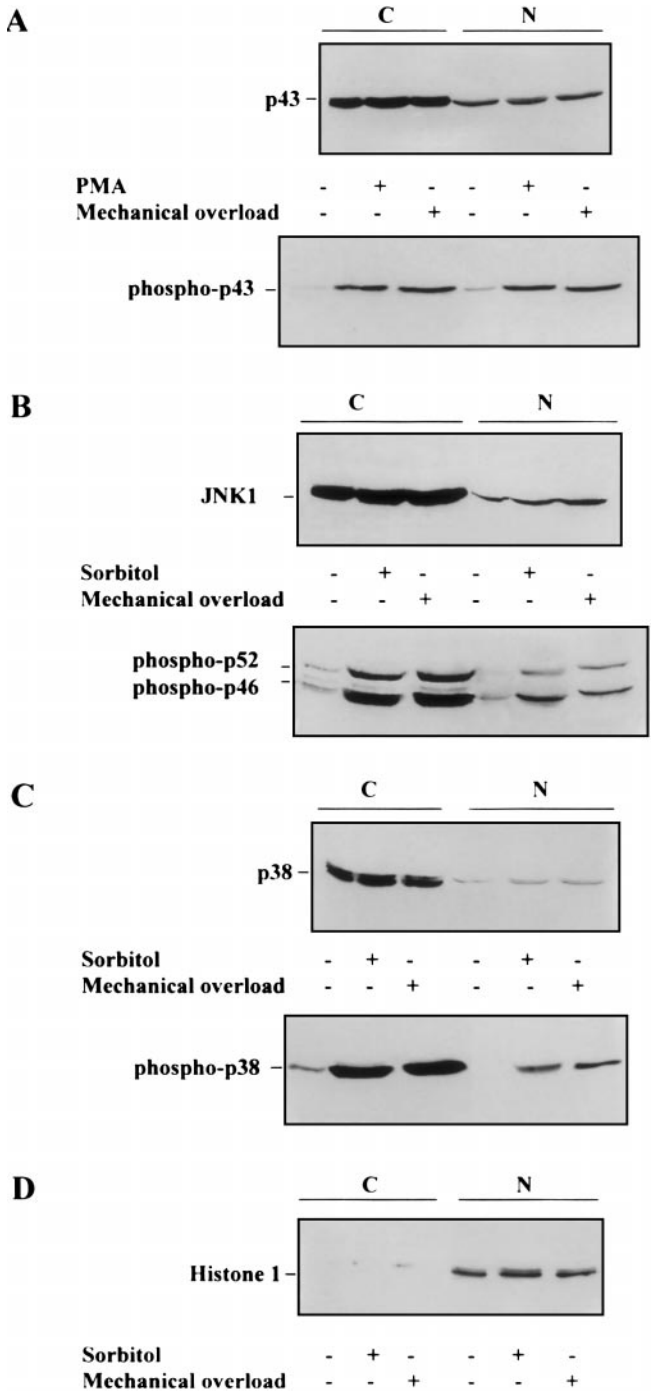

Fig. 6. Subcellular localization of ERK, JNKs, and p38-MAPKs during mechanical overload. Subcellular fractions were prepared from hearts perfused under the conditions indicated, and these fractions were subjected to immunoblotting with their respective antibodies. *A-C*, *top* panels: total ERK, JNK1, and p38-MAPK, respectively. *A-C*, *bottom* panelzs: phospho-ERK, phospho-JNKs, and phospho-p38-MAPK, respectively. *D*: densities of the nuclear (N) marker protein histone 1 are also shown. In this case, proteins were separated by SDS-PAGE on 15% (wt/vol) acrylamide, 0.332% (wt/vol) bisacrylamide slab gels. C, cytosol.

in MATERIALS AND EXPERIMENTAL PROCEDURES. In control hearts, myofibrillar organization with the characteristic of cardiac myocyte Z-disks was observed, but no immunoreactivity was detected (Figs. 7*A* and 8*A*). In specimens incubated either with the secondary antibody or the chromogen alone, no immunoreactivity was detected (results not shown). In hearts perfused under high pressure, phospho-ERK immunocomplexes were detected between myocyte bundles and in the perinuclear region (Fig. 7, *B* and *C*). No immunoreactivity was seen in hearts perfused under high pressure in the presence of 25 μM PD-98059 (Fig. 7*D*).

In specimens from hearts perfused under double pressure and incubated with phospho-p38-MAPK-specific antibody, the immunoreactivity complexes were localized between myocyte bundles and within the cytoplasm as well as in the perinuclear region (Fig. 8*B*). Furthermore, certain immunocomplexes seem to correspond rather to granular structures (Fig. 8*C*). No immunoreactivity was observed in hearts perfused under double pressure in the presence of 1 μM SB-203580 (Fig. 8*D*).

DISCUSSION

Eukaryotic cells use a variety of evolutionarily conserved intracellular signaling pathways to respond to changes in their external environment. Among these pathways, those that activate kinases belonging to the MAPK family have received particular attention because many of these enzymes play a vital multidimensional role regulating various cell functions. With respect to heart physiology, several recent reports have focused attention on the presence of MAPKs in mammalian ventricular myocytes or neonatal cells and have investigated the role of this signaling pathway in the development of hypertrophy and the adaptive response to ischemia. However, very little has been reported regarding the presence of MAPKs in the hearts of lower vertebrates or the role of this kinase cascade in physiological processes other than hypertrophy.

In the present study, we investigated the response of ERK, JNKs, and p38-MAPK to mechanical overload in the perfused amphibian (*Rana ridibunda*) heart. We further examined the subcellular localization of the activated MAPKs under this particular form of stress. The model system we studied allows the elucidation of another diverse role of MAPKs in organisms with a different physiology compared with that of mammals.

Phosphorylation and activation of MAPKs. Mechanical stress stimulated the activation of all three MAPKs examined in a time-dependent manner. The time course of ERK activation was markedly different from that of JNKs and p38-MAPK. In particular, ERK was rapidly activated, reaching maximal values at 1 min (3.6 ± 0.1- and 3.9 ± 0.2-fold relative to control for 3P/2 and 2P, respectively) (Fig. 1). Activation of both JNKs was rather slow, requiring 15 min to reach the maximum (Fig. 4). ERK and JNK activation levels remained elevated over a 30-min period. On the other hand, although p38-MAPK activation was considerable, reaching a maximum at 5 min (12.1 ± 1- and 11.3 ± 0.4-fold relative to control for 3P/2 and 2P, respectively), it was transient, declining to control lev-

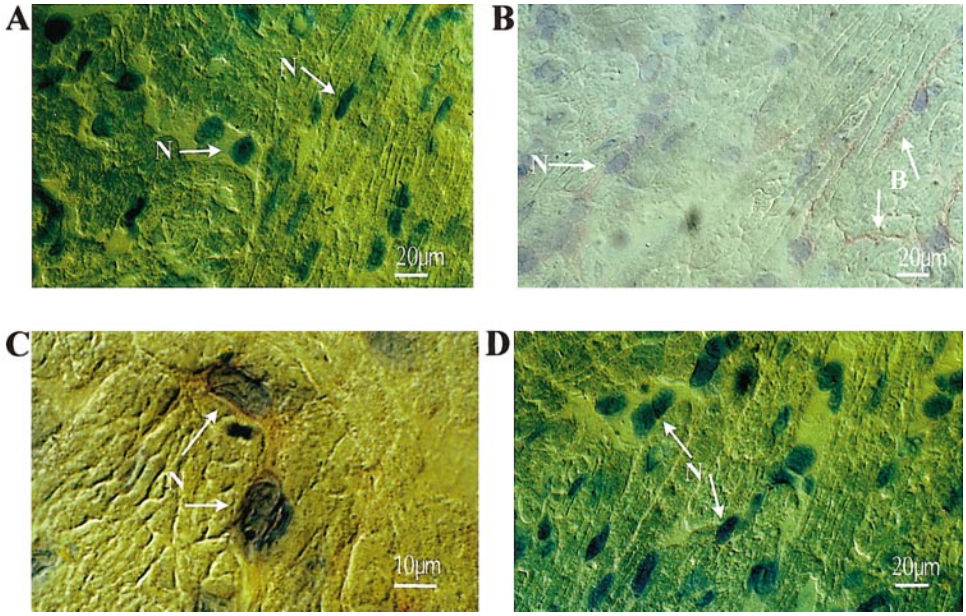

Fig. 7. Immunohistochemical localization of phospho-p43-ERK during pressure overload. Hearts were perfused under normal pressure (*A*) or subjected to mechanical overload for 2 min in the absence (*B*, *C*) or presence of 25 μM PD-98059 (*D*). After the removal of atria, the ventricles were cryosectioned and fixed with ice-cold acetone. Specimens were incubated with phospho-ERK antibody and counterstained with hematoxylin. The figure shows representative photographs from 3 independent experiments. N, nucleus; B, myocyte bundle. Bars: 10 or 20 μm.

els after 15 min of perfusion under high pressure (Fig. 5). There are limited data available concerning the response of MAPKs under mechanical overload in the mammalian whole heart (8, 20). Most of the existing data refer to the stretch-induced activation of MAPKs in neonatal cardiac myocytes (32, 42) where mechanical stretch has been shown to induce a slow ERK activation maximized at 10 min, with the phosphorylation levels returning to control values at 30 min, whereas JNK response is maximized at 15 min.

The differences we observed in the ERK activation by mechanical overload may be attributed to the fact that the amphibian heart is fundamentally different in terms of structure and function from the mammalian heart. Furthermore, amphibians, being ectotherms, represent a species more tolerant against external stimuli. However, the mechanical stress-induced activation of ERK in both models similarly precedes that of JNKs. This delay in JNK activation may result from the absence of an immediate upstream mechanosensitive signaling molecule that could directly transduce the stimulus to JNKs. It is also possible that JNKs are activated as a "side effect" due to cross talk with the other two MAPK subfamilies (ERK and p38-MAPK), which respond immediately to this specific form of stress in the amphibian heart. Overall, it is likely that the different time-dependent responses observed indicate that these delicately coordinated responses of ERK, p38-MAPK, and JNKs may lead to differential expression of multiple mechanical stress-sensitive genes.

Moreover, there may be alternate pathways for the activation of different members of one subfamily by a

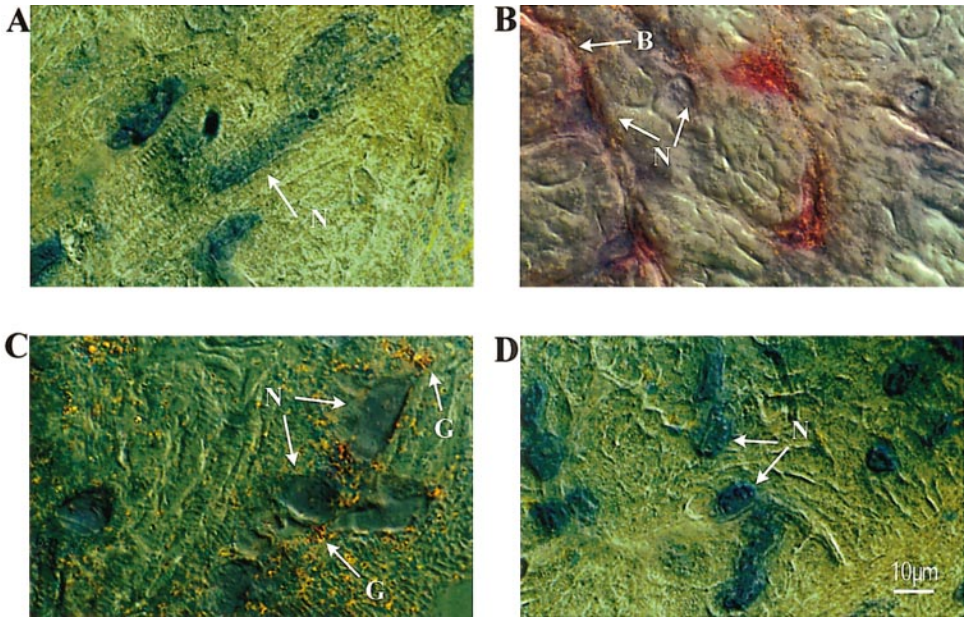

Fig. 8. Immunohistochemical localization of phospho-p38-MAPK in the amphibian heart during pressure overload. Hearts were perfused under normal pressure (*A*) or subjected to mechanical overload for 2 min in the absence (*B*, *C*) or presence (*D*) of 1 μM SB-203580. After the removal of atria, the ventricles were cryosectioned and fixed with ice-cold acetone. Specimens were incubated with phospho-p38-MAPK antibody and counterstained with hematoxylin. The figure shows representative photographs from 3 independent experiments. G, granules. Bar: 10 μm.

specific form of stress. Thus, unlike JNK1 (p46) activation, which was intense (10.2 ± 0.4- and 13 ± 0.1-fold relative to control for 3P/2 and 2P, respectively), JNK2 (p52) did not respond in the same way, with its phosphorylation levels reaching relatively moderate values (3.6 ± 0.4- and 3.8 ± 0.3-fold relative to control for 3P/2 and 2P, respectively) (Fig. 4). Studying the rat heart, Bogoyevitch et al. (4) have found similar results. The mechanisms leading to these diverse rates of phosphorylation (activation) remain to be identified.

MAPKs subcellular localization in the mechanical overload-subjected amphibian heart. One of the characteristic features of all phosphorylated MAPKs in mammalian tissues is their localization in both the cytoplasm and nucleus, where they interact with their substrates, phosphorylating and activating other upstream or downstream protein kinases, cytoskeletal proteins, transcription factors, etc. (26). The biochemical analysis we performed demonstrates that mechanical stress elicits a rapid increase in the cytosolic and nuclear ERK, JNK, and p38-MAPK phosphorylation that is associated with the presence of these proteins in both compartments (Fig. 6). In mammals, ERK is known to be first activated in the cytoplasm by MEK and then translocated to the nucleus (11, 21). Although the nuclear translocation of MAPKs is generally thought to be associated with their activation (6), Mizukami et al. (25) demonstrated that in rat hearts subjected to ischemia/reperfusion, JNK1 translocates while inactive, from the cytosol to the nucleus where it is then activated.

The immunohistochemical studies we performed showed that in specimens from hearts perfused under mechanical overload and incubated with an antibody detecting phosphorylated ERK, strong positive signals were observed between myocyte bundles and in the perinuclear region (Fig. 7). In respective specimens incubated with phospho p38-MAPK-specific antibody, immunoreactive complexes were localized between myocyte bundles, within the cytoplasm, and around the elongated myocyte nuclei (Fig. 8). The different immunolocalization patterns of phosphorylated ERK and p38-MAPK possibly represent these kinases' interaction with certain generally suggested substrates of theirs.

In conclusion, the present study demonstrates the mechanical overload-induced activation profiles of ERK, p38-MAPK, and JNKs in the amphibian heart, presenting detailed information on the magnitude and timing of these responses, as well as their immunolocalization pattern, a promising approach reported in this field for the first time. The roles of the different MAPKs in this physiological setting remain to be established, as the ultimate biological effects of their activation may depend on the duration and extent of the latter. The conservation of the MAPK pathway from vertebrates to yeast and its involvement in a variety of functions suggest universality and significance of this ancient mechanism of bioinformatics, making its study in a variety of model systems quite intriguing.

Perspectives

As mentioned above, activated MAPKs are present in both the cytosolic and nuclear fractions. Our studies correlate with this determination (Fig. 6). However, as there must be several pathways for MAPK activation in the cell, further investigation is needed to clarify and define the exact cascade of events that leads to MAPKs' presence in the nucleus in the mechanically overloaded amphibian heart. It is likely that either cytosolic MAPKs translocate to the nucleus on activation or that they are constantly present in both cytoplasm and nucleus where they are, respectively, activated, thereby ensuring the direct and effective response of the kinases in both cellular compartments.

In addition, the results from the immunohistochemical approach we performed extend this body of research, demonstrating that the distribution pattern of the granular-like immunoreactive structures detected may be linked to the localization pattern of a number of granules observed in the amphibian heart (Figs. 7 and 8). One limitation is the hematoxylin staining that obscures key data regarding intranuclear MAPK localization. The evident next step of this study is to clarify the localization pattern of MAPKs by electron transmission microscopy. In an amphibian heart, most of the natriuretic peptide granules (e.g., atrial natriuretic peptide) are found at the poles of the elongated nuclei and are also widely dispersed in the cytoplasm (7, 24). One cannot also exclude the possibility these may also correspond to ANG II secretory granules. ANG II is known to act as a mediator of stretch-induced adaptive responses in vertebrate cardiac myocytes and fibroblasts (29), and the importance of the renin-angiotensin regulatory system in frogs has previously been reported (23, 41).

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