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Mitogen-activated Protein Kinases and Mitogen-activated Protein Kinase Phosphatases Mediate the Inhibitory Effects of All-*trans* Retinoic Acid on the Hypertrophic Growth of Cardiomyocytes*

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All-*trans* retinoic acid (RA) has been implicated in mediation of cardiac growth inhibition in neonatal cardiomyocytes. However, the associated signaling mechanisms remain unclear. Utilizing neonatal cardiomyocytes, we demonstrated that RA suppressed the hypertrophic features induced by cyclic stretch or angiotensin II (Ang II). Cyclic stretch- or Ang II-induced activation of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAP kinase) was dose- and time-dependently inhibited by RA. Significant inhibition was observed by 5 μM RA, from 8 to 24 h of pretreatment. This inhibitory effect was not mediated at the level of mitogen-activated protein kinase kinases (MKKs), because RA had no effect on stretch- or Ang II-induced phosphorylation of MEK1/2, MKK4, and MKK3/6. However, the phosphatase inhibitor vanadate reversed the inhibitory effect of RA on MAP kinases and protein synthesis. RA up-regulated the expression level of MAP kinase phosphatase-1 (MKP-1) and MKP-2, and the time course was correlated with the inhibitory effect of RA on activation of MAP kinases. Overexpression of wild-type MKP-1 inhibited the phosphorylation of JNK and p38 in cardiomyocytes. These data indicated that MKPs were involved in the inhibitory effect of RA on MAP kinases. Using specific RAR and RXR antagonists, we demonstrated that both RARs and RXRs were involved in regulating stretch- or Ang II-induced activation of MAP kinases. Our findings provide the first evidence that the anti-hypertrophic effect of RA is mediated by up-regulation of MKPs and inhibition of MAP kinase signaling pathways.

Retinoid is a generic term for synthetic and natural vitamin A-related compounds, of which all-*trans* retinoic acid (RA)[1] is a biologically active prototype. Retinoids participate in a wide range of biological processes, including vision, neoplasia, embryonic development, normal reproductive function, regulation of epithelial and hematopoietic cellular differentiation, and cardiovascular development (2, 3). Retinoids function by binding to nuclear receptor proteins. Two families of retinoid receptors exist as follows: the retinoic acid receptor (RAR) family (RARα, -β, and -γ), bind both RA and *9-cis* RA, and the retinoid X receptor (RXR) family (RXRα, -β, and -γ), preferentially bind 9-*cis* RA (4, 5). These ligand-activated retinoid receptors act as transcription factors, which bind to RA response elements in the promoters/enhancers of numerous target genes, leading to transcriptional stimulation or repression. Deficiency of vitamin A has been linked to a variety of defects in cardiovascular development, such as tetralogy of Fallot and supracristal ventricular septal defect (3). Embryonic deletions of individual retinoid receptors induce a number of developmental abnormalities in the cardiovascular system (6, 7), and a predilection for many of the same malformations seen with vitamin A deficiency (8). On the other hand, overexpression of a constitutively active RAR in developing atria and/or in postnatal ventricles is relatively benign, while ventricular expression during gestation can lead to dilated cardiomyopathy and significant cardiac dysfunction (9, 10). It has been demonstrated that RA-mediated signaling suppresses myocardial cell hypertrophy in response to angiotensin II (Ang II), endothelin-1 (ET-1), and the α-adrenergic receptor agonist phenylephrine (PE) (11–13). These studies demonstrate that retinoid-dependent pathways are involved in promoting the ventricular phenotype in embryonic heart, and indicate that retinoids may have an important role in maintaining the normal ventricular phenotype in the postnatal state. Although the anti-hypertrophic effect of RA in neonatal cardiomyocytes has been demonstrated, the signaling mechanisms of RA action are not well understood.

Cardiac hypertrophy is associated with the activation of numerous signal transduction factors, including G protein-coupled receptors, receptor-tyrosine kinases, protein kinase C, and members of the mitogen-activated protein kinase (MAP kinase) signaling cascade (14–18). MAP kinases are serine/threonine kinases that target substrates in a multilayered signaling network composed of the MAP kinases, the MAP kinase kinases (MKKs), and the MEK kinases (MEKKs). MAP kinases can be divided into three major subfamilies: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAP kinase. A large number of *in vitro* and *in vivo* studies have identified important roles for the three branches of the MAP kinase signaling pathway in cardiomyocyte hypertrophy (19–24). It has been shown that MAP kinase pathways are activated and involved in various hypertrophic stimuli (growth

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[1] The abbreviations used are: RA, retinoic acid; MOI, multiplicity of infection; RAR, retinoic acid receptor; RXR, retinoic X receptor; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; JNK, Jun N-terminal kinase; Ang II, angiotensin II; MKP, MAP kinase phosphatase; Act D, actinomycin D; MKK, MAP kinase kinase; MEKK, MEK kinases; CHX, cycloheximide.

factors, cytokines, and mechanical stretch) induced cardiac hypertrophy, *in vitro* and *in vivo* (25–30). In response to MAP kinase activation, a family of dual-specificity phosphatases, MAP kinase phosphatases (MKPs), become transcriptionally induced, leading to the specific dephosphorylation and inactivation of MAP kinases (31). MKP-1 is an important member of the dual-specificity phosphatase family that is expressed in the heart, where it regulates inactivation of p38, JNK1/2, and ERK1/2 (32–34). Studies have shown that MKP-1 limits the cardiac hypertrophic response *in vitro* and *in vivo*, via dephosphorylation and inactivation of MAP kinases (34–36). Previous studies have indicated a role of RA in regulating MAP kinase pathways. RA inhibits serum-induced phosphorylation of JNK, which is associated with a post-translational increase in the level of MKP-1 protein in tumor cells (37). Up-regulation of MKP-1 and inhibition of JNK phosphorylation is involved in the antiapoptotic effect of RA on H_2O_2-exposed mesangial cells (38). Although these observations clearly suggest a potential link between retinoids and MAP kinase signaling, the mechanisms involved, particularly in hypertrophic cardiomyocytes, have not been elucidated.

In the present study, we determined the effects of RA on cyclic stretch- or Ang II-induced cardiac hypertrophy, and the associated signaling mechanisms. Our results show that RA had inhibitory effects on the activation of MAP kinases induced by cyclic stretch or Ang II, and that the up-regulation of MKPs by RA was involved. These results identify a mechanism whereby RA regulates expression of MKPs and inhibition of MAP kinases, which play an important role in the anti-hypertrophic effects of RA.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents (Dulbecco's modified Eagle's medium/F12 Medium, horse serum, fetal bovine serum, and antibiotics) were from Invitrogen (Baltimore, MD). Polyclonal antibodies against ERK1/2, JNK1, p38, MKP-1, MKP-2, MKP-3, actin, RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ were from Santa Cruz Biotechnology (San Diego, CA). Phospho-MEK1, -MKK3, -MKK4, -ERK1/2, -JNK, and -p38 antibodies were from Cell Signaling Technology (Beverly, MA). PD98059, SB202190, and JNK inhibitor II (SP600125) were from Calbiochem (San Diego, CA). [γ-^{32}P]ATP and [^{3}H]leucine were from PerkinElmer Life Science (Boston, MA). AGN193109 was a gift from Allergan (Irvine, CA). HX531, a gift from Dr. Kagechika (The University of Tokyo, Japan) (39).

Cell Cultures—Monolayer cultures of neonatal cardiomyocytes were prepared from the ventricles of 1–3-day-old Sprague-Dawley rat pups, as previously described (40) and were cultured in 5% horse serum, 10% fetal bovine serum, antibiotics, and 0.1 mM bromodeoxyuridine (prevent proliferation of nonmyocytes) for 48 h at a density of 1.5×10^5 cells/cm^2 in 100-mm culture dishes (for immunostaining in response to Ang II, in 2-well chamber slide) or in laminin-coated six-well BioFlex culture plates (Flex Cell International Corporation). Cells were serum-starved for 24 h prior to intervention. For the cyclic stretch model, serum-starved cells were subjected to cyclic stretch using the Flexcell 3000 Strain Unit. The vacuum produced a 15% elongation on the flexible bottom membranes at a frequency of 60 cycles/min. Culture plates not subjected to cyclic stretch were used as controls.

Adenovirus Generation—The replication-defective adenovirus-encoding wild-type MKP-1 (AdMKP-1, a gift from Dr. Molkentin, Children's Hospital Medical Center, Cincinnati, OH) was plaque purified, and amplified using HEK293 cells. The multiplicity of viral infection (MOI) for each virus was determined by dilution assay in HEK293 cells. Cardiomyocytes were infected with adenovirus at a MOI of 25–50 plaque-forming units for 8 h at 37 °C. Subsequently, the cells were cultured in serum-free Dulbecco's modified Eagle's medium/F12 media for an additional 24 h before treatment or analysis.

Leucine Incorporation—Serum-starved cardiomyocytes were subjected to cyclic stretch or exposed to Ang II for 24 h. [^{3}H]Leucine (1μCi/ml) was added 4 h before harvest. The total radioactivity of incorporated [^{3}H]leucine into proteins was measured by liquid scintillation counting, as described previously (41).

Quantification of Total Cell Protein Content—The total protein content per cell number was determined as previously described (13). Briefly, the same amount of cardiomyocytes were cultured in laminin-coated 6-well BioFlex culture plates. After 24 h of serum starvation, cardiomyocytes were pretreated, with or without RA (5 μM) for 24 h, and subjected to cyclic stretch (24 h) or exposed to Ang II (1 μM, 36 h). Cells were washed three times with 1× phosphate-buffered saline, and dissolved in 2% SDS. Protein concentrations were measured using a DC protein assay (Bio-Rad).

Immunocytochemistry—After 24 h of cyclic stretch or Ang II stimulation, cardiomyocytes were fixed in 3.7% paraformaldehyde, and permeabilized with 0.3% Triton X-100. Cells were incubated with Texas Red-X phalloidin (1:40). Immunostained cardiomyocytes were viewed by fluorescence microscopy. Quantitation of cell surface area was performed on actin-stained cardiomyocytes.

Western Blot Analysis—Cardiomyocytes were lysed in lysis buffer as previously described (40, 42). Equal amounts of extracted proteins (50 μg) were separated on 10% SDS-PAGE, transferred to nitrocellulose membrane, and the Western blots probed with anti-MKP-1, MKP-2, or MKP-3 antibody or antibodies specific for the phosphorylated forms of MEK1, MKK3, MKK4, ERK1/2, JNK, or p38. Membranes were reprobed with unphosphorylated forms of corresponding antibodies to confirm equal loading. Primary antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody and visualized by chemiluminescence (PerkinElmer Life Sciences).

Extraction of Nuclear Proteins and the Expression of RARs and RXRs—Cardiomyocytes were rinsed with phosphate-buffered saline at 4 °C and scraped into the same buffer. Nuclear extracts were prepared according to standard methods described previously (40, 43). Equal amounts of extracted nuclear proteins (20 μg) were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The expression level of RARs and RXRs was determined using anti-RARα, -β, -γ, RXRα, -β, and -γ antibodies. Another set of samples was probed with anti-histone antibody, as a control for equal protein loading.

Gel Mobility Shift Assay—To determine the transcriptional activation of RAR and RXR, gel shift assay was performed as described previously (40). Five micrograms of nuclear extract were incubated with 1 μg of poly(dI-dC)-poly(dI-dC) and radiolabeled probes (~10,000 cpm) in 20 μl of 10 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM EDTA, and 10% glycerol for 30 min at 25 °C. The probes were purchased from Santa Cruz Biotechnology, and their sequences have been described (RAR: 5′-AGGGTAGGGTTCACCGAAAGTTCACTC-3′, RAR mutant: 5′-AGGG TAGGGAACACCGAAAGTTCACTC-3′; RXR: 5′-AGCTTCAGGTCAGAGGTCAGAGAG CT-3′, and RXR mutant: AGCTTCAGCACAGAGCACAGAGAGCT-3′). The oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Binding reactions were resolved on a 4% native polyacrylamide gel containing 1× TAE buffer (40 mM Tris, pH 7.5, 40 mM sodium acetate, and 1 mM EDTA) for 3 h at 150 V in 4 °C. The gel was dried, and exposed to x-ray film.

Statistical Analysis—Data were expressed as the mean ± S.E. Statistical differences were determined using a Student's t test ($p < 0.05$).

RESULTS

RA Inhibits Cyclic Stretch- and Ang II-induced Cardiomyocyte Hypertrophy—To determine the effects of RA on cardiac hypertrophy, cardiomyocytes were pretreated with or without RA for 24 h, and subjected to cyclic stretch or exposed to Ang II for an additional 24 h. Cell morphological changes were observed. Cyclic stretch and Ang II stimulation induced a marked increase in cell size, which was significantly inhibited by RA (Fig. 1, *A* and *B*). RA treatment also reduced the accumulation of sarcomeric fibers and the myofibrillar reorganization in response to Ang II and cyclic stretch (Fig. 1*A*). RA alone had no effect on myocardial cell phenotype, indicating that the effect of RA was not secondary to a toxic cellular effect. In addition to the specific effects on cell morphology, cardiac hypertrophy is accompanied by an increase in protein synthesis. In an effort to determine the effects of RA on protein synthesis, we performed [^{3}H]leucine incorporation, which is an index of protein synthesis. As shown in Fig. 1*C*, cyclic stretch and Ang II stimulation resulted in a significant increase in [^{3}H]leucine incorporation, which was inhibited by RA. RA alone had little inhibitory effect on the basal level, but no significant difference compared with control. We also determined the effect of RA on total protein

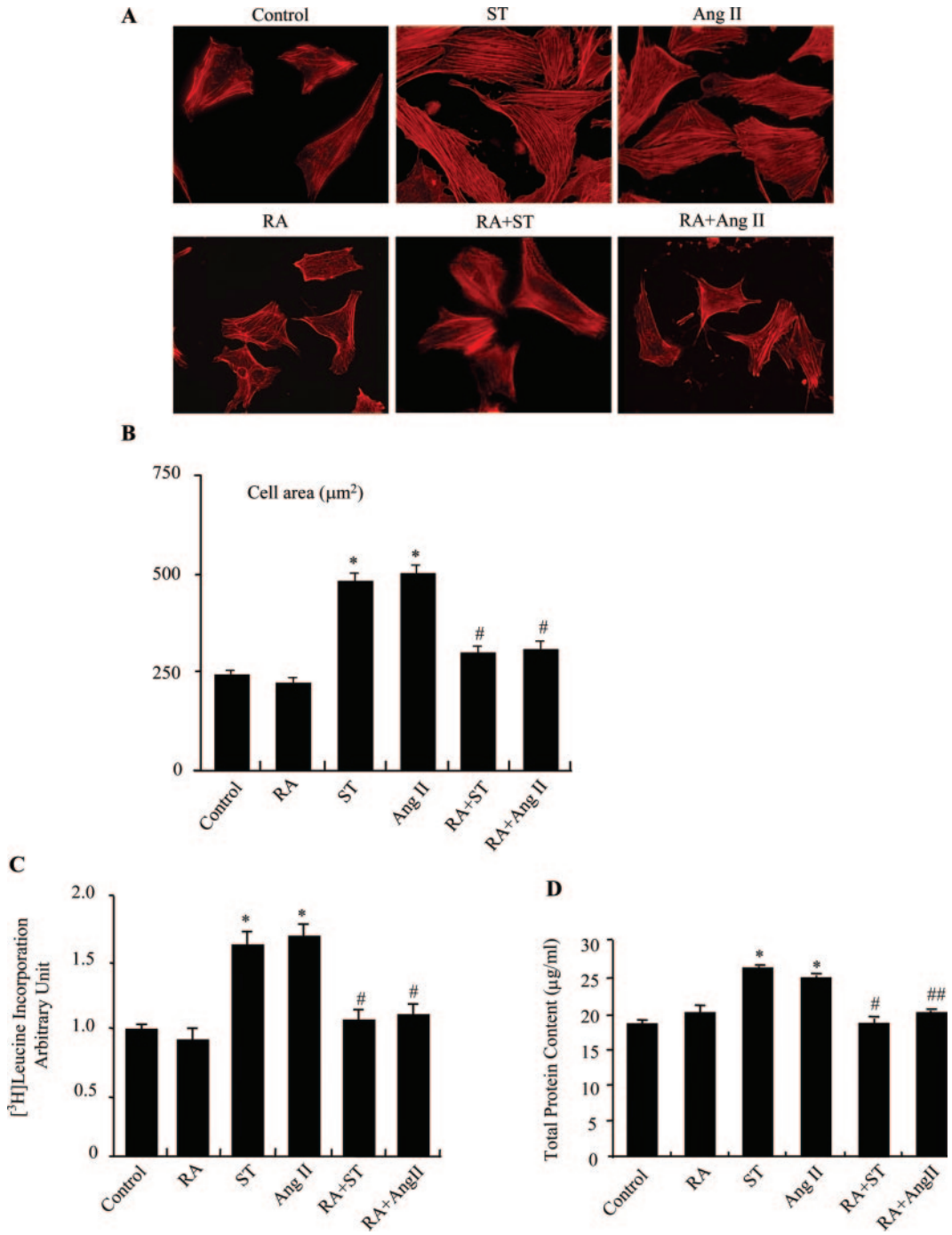

FIG. 1. **RA inhibits cyclic stretch- and Ang II-induced cardiac hypertrophy.** *A*, cardiomyocytes were pretreated with or without RA (5 μM) for 24 h, and subjected to cyclic stretch (*ST*) or exposed to Ang II for another 24 h. Control cells were pretreated with Me_2SO. Immunofluorescence was performed with Texas Red pholloidin antibody. *B*, pholloidin-stained cells were imaged and cell surface areas calculated with the Image J Program ($n = 100$ cells each). *, $p < 0.001$ *versus* control; #, $p < 0.001$ *versus* stretch or Ang II without RA. *C*, cardiomyocytes were subjected to stretch or exposed to Ang II for 24 h, with or without RA pretreatment for 24 h. [^{3}H]Leucine (1 μCi/ml) was added 4 h before cell harvest. The total radioactivity of incorporated [^{3}H]leucine into proteins, was measured by liquid scintillation counting. Each *bar* represents the mean ± S.E. of six separate experiments. *, $p < 0.05$ *versus* control and #, $p < 0.05$ *versus* stretch or Ang II. *D*, effects of RA on total protein content. Cardiomyocytes were pretreated with or without RA (5 μM) for 24 h, and subjected to stretch (24 h) or exposed to Ang II (1 μM, 36 h). Total protein content was determined using the DC protein assay. Values are means ± S.E. ($n = 12$ in each group). *, $p < 0.05$ *versus* control; #, $p < 0.001$ *versus* stretch; ##, $p < 0.05$ *versus* Ang II.

content. As shown in Fig. 1*D*, 24 h of stretching or 36 h of Ang II stimulation induced approximately a 40 and 35% increase in total protein content, respectively (compared with control), which was inhibited by RA pretreatment. Total protein content increased about 8% in the myocytes incubated with 5 μM RA alone, but there was no significant difference compared with control. These results suggest that RA suppresses cyclic stretch- or Ang II-induced cardiac hypertrophy.

RA Inhibits Cyclic Stretch-induced Activation of MAP Kinases—Accumulating data have shown that MAP kinases are important mediators in cardiac hypertrophy. We determined the involvement of MAP kinases in the anti-hypertrophic effect of RA. Cardiomyocytes were pretreated with or without RA for different times and subjected to cyclic stretch for 8 min. ERK, JNK, and p38 MAP kinase activity was determined by Western blot analysis, using specific antibodies against phospho-

ERK1/2, -JNK1/2, and p38. RA time-dependently inhibited stretch-induced phosphorylation of ERK, JNK, and p38 (Fig. 2*A*). Although the inhibitory effect was observed beginning at 2 h of RA pretreatment ($p > 0.05$, compared with stretch, *bottom panel*), the most significant inhibition was observed from 8 to 24 h ($p < 0.05$, compared with stretch). Stretch-induced phosphorylation of MAP kinases was also dose-dependently inhibited by 24 h of RA pretreatment (Fig. 2*B*). The phosphorylation of MAP kinases was significantly inhibited by 5–10 μM of RA ($p < 0.05$, compared with stretch, *bottom panel*), whereas a low dose of RA (0.1–1 μM) had no effect. The involvement of MAP kinases in stretch-induced cardiac hypertrophy was further determined using specific inhibitors for ERK (PD98059, PD), JNK (SP600125, SP), and p38 (SB202190, SB). Cardiomyocytes were subjected to stretch for 24 h, in the presence or absence of inhibitors. [^{3}H]leucine incorporation was performed. As shown in Fig. 2*C*, the stretch-induced increase in leucine incorporation was significantly inhibited by 70, 50, and 40%, respectively, in PD98059-, SP600125-, or SB202190-treated cardiomyocytes, indicating that all three MAP kinases were involved in the stretch-mediated hypertrophic process. Taken together, our results suggested that MAP kinase pathways were involved in the inhibitory effects of RA on cyclic stretch-induced cardiac hypertrophy.

RA Inhibits Ang II-induced Activation of MAP Kinases—Studies have shown that RA suppressed Ang II-induced cardiac hypertrophy (13). Thus, we determined whether MAP kinases were also involved in RA action. As shown in Fig. 3*A*, Ang II induced rapid phosphorylation of ERK1/2 and p38 at 2 min, which was sustained to 60 min, and returned to a basal level at 120 min. On the other hand, the phosphorylation of JNK1/2 in response to Ang II, was observed from 2 min, with peak phosphorylation observed at 40–60 min. The involvement of MAP kinases in Ang II-induced protein synthesis was determined. Cardiomyocytes were exposed to Ang II for 24 h, in the presence or absence of PD98059, SP600125, or SB202190, and [^{3}H] leucine incorporation was determined. The Ang II-induced increase in leucine incorporation was inhibited by PD98059, SP600125, or SB202190 (inhibited by 60, 45, and 30%, respectively, compared with Ang II). We further determined the effect of RA on Ang II-induced activation of MAP kinases. Western blot analysis demonstrated that RA pretreatment inhibited Ang II-induced activation of MAP kinases, in a time- and dose-dependent manner. Significant inhibition by RA was observed from 8 to 24 h, at a dose of 5–10 μM ($p < 0.05$, compared with Ang II, Fig. 3, *C* and *D*). RA pretreatment for 2–4 h at 0.1–1 μM did not significantly inhibit Ang II-induced activation of MAP kinases ($p > 0.05$, Fig. 3, *C* and *D*, *bottom panel*).

RA Has No Effect on the Activation of MKKs—All MAP kinase pathways share a three-component cascade mode of activation, in which MKKK phosphorylates a dual-specificity kinase MKK, which specifically recognizes and activates different MAP kinases (44). Therefore, we determined whether the inhibitory effect of RA on the activation of MAP kinases, was caused by the inhibition of MKK activation. The phosphorylation of MKKs was examined by immunoblotting with specific antibodies against phospho-MEK1, -MKK3/6, and -MKK4. Cardiomyocytes were pretreated with or without 5 μM RA, up to 24 h, and subjected to cyclic stretch for 1 and 3 min (Fig. 4*A*), or exposed to Ang II for 3 min (Fig. 4*B*). Cyclic stretch or Ang II stimulation induced rapid phosphorylation of MEK1, MKK3/6, and MKK4. Pretreatment of cardiomyocytes with RA did not affect the phosphorylation of MEK1, MKK4, and MKK3/6. These results indicated that the inhibitory effect of RA on the activation of MAP kinases was not mediated at the level of MKKs.

Vanadate Reverses the Inhibitory Effect of RA on the Activation of MAP Kinases and Leucine Incorporation—Once activated, MAP kinases are rapidly inactivated by the family of dual-specificity protein phosphatases. To determine the role of phosphatases in regulating RA action on MAP kinases, we examined whether the inhibition of MAP kinases by RA could be prevented by the phosphatase inhibitor, vanadate. Cardiomyocytes were pretreated with RA for 24 h in the absence or presence of vanadate, and subjected to cyclic stretch for 8 min, or exposed to Ang II for 3 min (for ERK and p38) or 40 min (for JNK). Western blot analysis demonstrated that the inhibitory effect of RA on stretch- or Ang II-induced activation of MAP kinases was significantly reversed by vanadate (Fig. 5, *A* and *B*). Treatment with vanadate alone minimally increased the basal level of MAP kinases, but there was no significant difference compared with control. Under similar conditions, the role of phosphatases in the anti-hypertrophic effect of RA was determined. After 24 h of stimulation with cyclic stretch or Ang II, [^{3}H]leucine incorporation was performed. RA inhibited the increased leucine incorporation in response to cyclic stretch or Ang II, and vanadate treatment increased leucine incorporation in the presence of RA, indicating the inhibitory effect of RA on leucine incorporation was reversed by vanadate (Fig. 5*C*). Vanadate alone had no effect. These data suggested that protein phosphatases were involved in regulating the inhibitory effects of RA on MAP kinases and protein synthesis.

Induction of MKPs by RA in Cardiomyocytes—MKPs are a family of inducible dual-specificity phosphatases (45), which dephosphorylate and inactivate MAP kinases (46, 47). To determine whether the suppression of MAP kinases by RA is mediated by MKPs, we tested the protein expression level of MKPs in cardiomyocytes. Cells were treated with RA up to 48 h, and Western blot analysis was performed. RA increased the protein level of MKP-1 and MKP-2 in a dose- and time-dependent manner (Fig. 6, *A–C*). The expression level of MKP-1 and MKP-2 was up-regulated by RA from 2 h of treatment, and peaked at 24–48 h. The expression level of MKP-3 was not significantly affected by RA. RA-induced up-regulation of MKP-1 and MKP-2 was consistent with the inhibitory pattern of RA on activation of MAP kinases, suggesting that up-regulated MKP-1 and MKP-2, may contribute to the inhibitory effect of RA on the activation of MAP kinases.

RA Increases MKPs Expression at the Transcriptional and Translational Levels—Previous studies have shown that the induction of MKPs by RA is variable (37, 38), suggesting that RA may differentially regulate the expression of MKPs depending on the cell type. To determine the regulatory mechanisms of MKP expression by RA in neonatal cardiomyocytes, a RNA synthesis inhibitor actinomycin D (ActD), and a protein synthesis inhibitor cycloheximide (CHX) was used. Cardiomyocytes were treated with RA for 3, 6, and 24 h, in the presence or absence of ActD or CHX. Western blot analysis showed that RA-induced expression of MKP-1 and MKP-2 was blocked by ActD or CHX (Fig. 7, *A* and *B*). These data suggested that RA regulated the expression of MKPs at both transcriptional and translational levels in neonatal cardiomyocytes.

MKP-1 Overexpression Inhibits the Activation of MAP Kinases and Leucine Incorporation—To determine the role of MKPs in regulating the activation of MAP kinases in cardiomyocytes, an MKP-1-expressing adenovirus (AdMKP-1) was used. Cardiomyocytes were infected with different doses of AdMKP-1, which resulted in a dose-dependent overexpression of MKP-1 (Fig. 8*A*). The role of MKP-1 in cyclic stretch- or Ang II-mediated cardiac hypertrophy was also determined. After infection with AdMKP-1, cells were subjected to stretch or exposed to Ang II for 24 h. [^{3}H]leucine incorporation assay

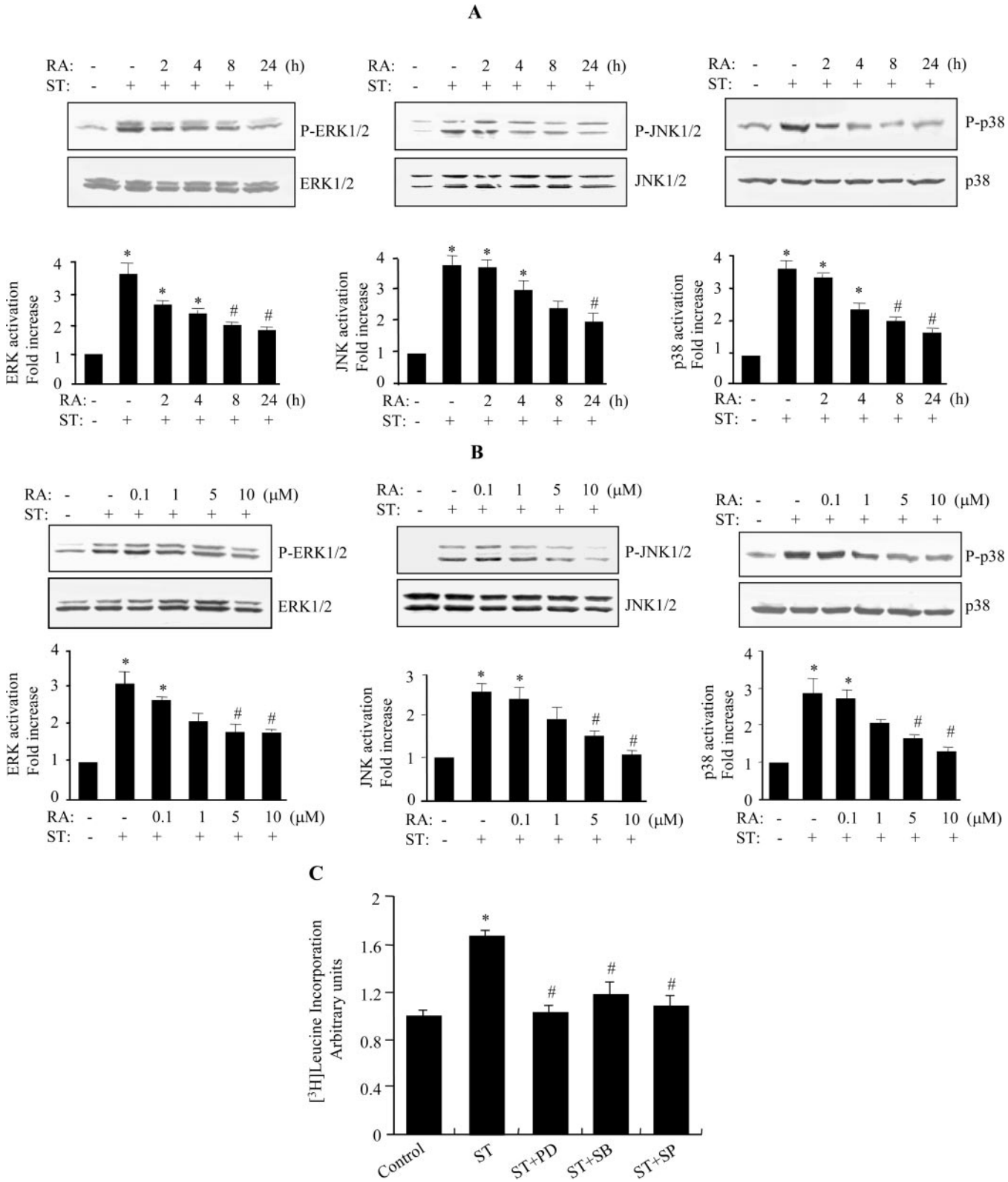

FIG. 2. **RA inhibits cyclic stretch-induced phosphorylation of MAP kinases in a time- and dose-dependent manner.** *A*, cardiomyocytes were pretreated with or without RA (5 μM) for different times, then subjected to cyclic stretch for 8 min. Cell extracts (50 μg) were subjected to 10% SDS-PAGE and immunoblotted with an antibody specific for phospho-ERK1/2, -JNK, or -p38 MAP kinase. Membranes were stripped and reprobed for total ERK1/2, JNK1, or p38, as a control for equal protein loading (*upper panel*). *B*, cardiomyocytes were pretreated with different doses of RA for 24 h as indicated, and subjected to stretch for 8 min. The phosphorylation of ERK1/2, JNK1/2, and p38 was determined (*upper panel*). Phosphorylated levels of ERK1/2, JNK1/2, or p38 were quantified by densitometric scanning, and normalized to the level of total ERK, JNK, or p38 (*A* and *B*, *bottom panel*). Data represent average fold increase of controls from three independent experiments (mean ± S.E.). *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* stretch. *C*, role of MAP kinases in the stretch-induced increase in protein synthesis. Cardiomyocytes were subjected to stretch for 24 h, in the presence or absence of PD98059 (PD, 50 μM), SB202190 (SB, 10 μM), or SP600125 (SP, 20 μM). The incorporated [^{3}H]leucine was measured by liquid scintillation counting. Each *bar* represents the mean ± S.E. of six separate experiments. *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* stretch.

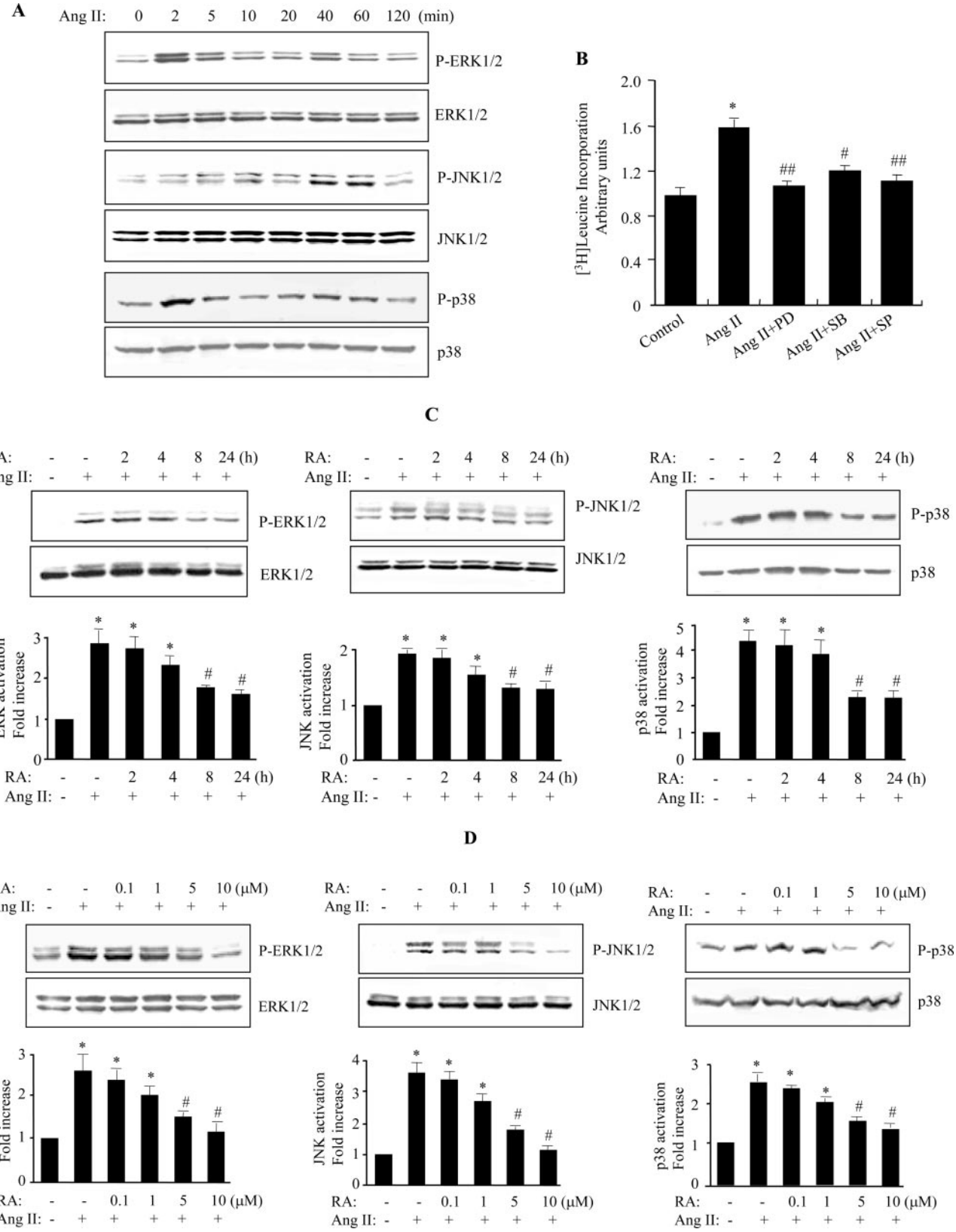

FIG. 3. **RA inhibits Ang II-induced phosphorylation of MAP kinases in a time- and dose-dependent manner.** *A*, Ang II induces phosphorylation of MAP kinases. Cardiomyocytes were exposed to Ang II (0.1 μM) for the times indicated. The phosphorylation of ERK1/2, JNK1/2, and p38 was determined, as described in Fig. 2*A*. *B*, cardiomyocytes were exposed to Ang II for 24 h, in the presence or absence of PD98059 (PD, 50 μM), SB202190 (SB, 10 μM), or SP600125 (SP, 20 μM), and [^{3}H]leucine incorporation was measured. Each *bar* represents the mean ± S.E. of six separate experiments. *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* Ang II; ##, $p < 0.001$ *versus* Ang II. *C*, cardiomyocytes were pretreated with RA (5 μM) for the times indicated, then exposed to Ang II for 3 min (for ERK1/2 and p38) or 40 min (for JNK), and the phosphorylation of ERK1/2, JNK1/2, and p38 determined (*upper panel*). *D*, cardiomyocytes were pretreated with different doses of RA for 24 h, exposed to Ang II for 3 min (ERK and p38), or 40 min (JNK), and the phosphorylation of ERK1/2, JNK1/2, and p38 determined by Western blotting (*upper panel*). Phosphorylated levels of ERK1/2, JNK1/2, or p38 were quantified by densitometric scanning, and normalized to the level of total ERK1, JNK, or p38 (*C* and *D*, *bottom panel*). Data represent the average fold increase of controls from three independent experiments (mean ± S.E.). *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* Ang II.

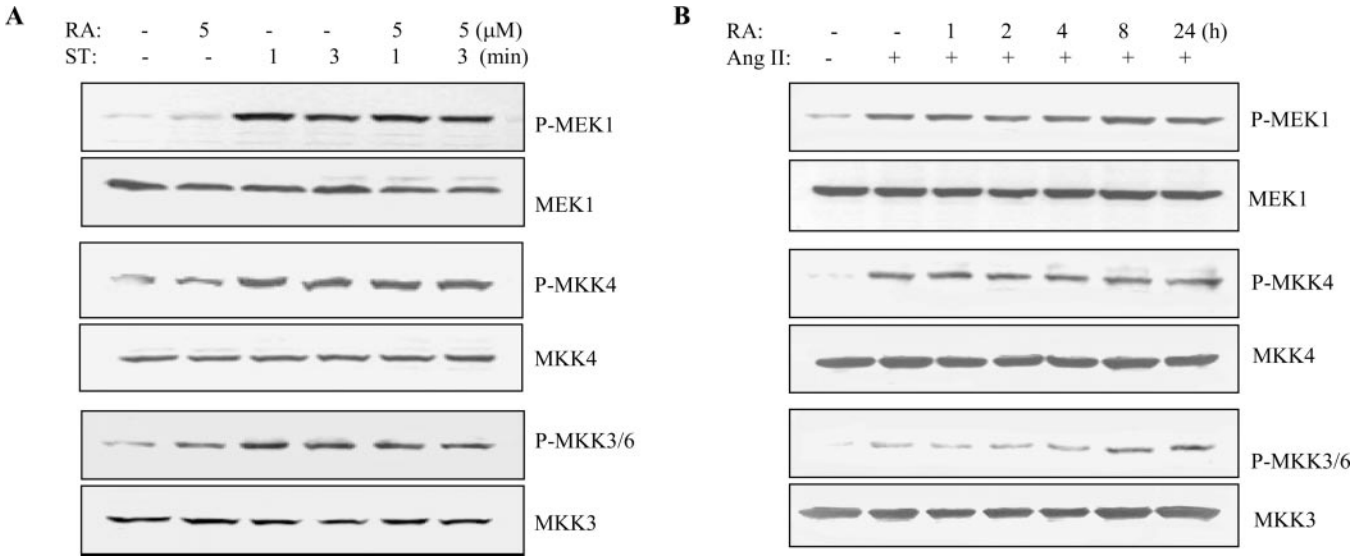

FIG. 4. **Effect of RA on stretch- or Ang II-induced phosphorylation of MKKs.** *A*, cardiomyocytes were pretreated with or without RA (5 μM) for 24h, and subjected to cyclic stretch for 1 and 3 min. An equal amount of proteins (50 μg) was subjected to 10% SDS-PAGE, and the phosphorylation of MKKs detected by immunoblotting with antibody specific to phospho-MEK1, -MKK4, or MKK3/6. Membranes were reprobed for total MEK1, MKK4, and MKK3, as a control for equal protein loading. *B*, cardiomyocytes were pretreated with RA (5 μM) for the times indicated, then exposed to Ang II (0.1 μM) for 3 min. The phosphorylation of MKKs was determined, as described above.

demonstrated that the increased leucine incorporation induced by cyclic stretch or Ang II was significantly inhibited by overexpression of MKP-1. AdLacZ (control virus) or AdMKP-1 alone had no effect on the basal level of [^{3}H]leucine incorporation (Fig. 8*B*). Additionally, the effect of AdMKP-1 on activation of MAP kinases was determined. After 24 h of infection, cardiomyocytes were subjected to cyclic stretch, or exposed to Ang II, and the phosphorylation of ERK1/2, JNK1/2, and p38 was examined. Cyclic stretch or Ang II-induced phosphorylation of JNK1/2 and p38 was significantly inhibited by overexpression of MKP-1 ($p < 0.05$, compared with stretch or Ang II stimulation group, Fig. 8, *C* and *D*), and that of ERK1/2 was partially inhibited ($p > 0.05$, compared with stretch or Ang II). AdLacz or AdMKP-1 alone had no effect on the phosphorylation of MAP kinases, whereas total protein levels of each MAP kinase were unchanged.

RA Induces Expression and Activation of RARs and RXRs in Cardiomyocytes—It has been demonstrated that both RAR and RXR, especially RAR/RXR heterodimers, mediate suppression of PE and ET-1-induced cardiac hypertrophy (11). To further establish the role of RAR and RXR in RA-induced anti-hypertrophic effects, we determined whether RA influences the expression and activation of RARs and RXRs in cardiomyocytes. Cells were exposed to RA up to 24 h, and nuclear extracts prepared following standard methods (40, 48). The protein level of RARs and RXRs was determined by Western blot, using specific anti-RARα, -RARβ, -RARγ, -RXRα, -RXRβ, and -RXRγ antibodies. As shown in Fig. 9*A*, all of the RARs and RXRs were expressed in neonatal cardiomyocytes. The expression of RARα, RXRα, and RXRγ was found to be the most abundant, whereas RARγ was expressed weakly in neonatal cardiomyocytes. RA increased the expression level of RARs and RXRs after 30 min of treatment, which was sustained to 24 h. DNA binding activity of RAR and RXR was determined by electrophoretic mobility shift assays (EMSA), using oligonucleotides corresponding to the RAR or RXR binding site, as described previously (49). The DNA-protein complex with RAR was observed after 30 min of RA treatment, and peaked at 1–2 h. The DNA-protein complex with RXR was observed after 30 min, peaked at 2–4 h, and decreased thereafter (Fig. 9*B*). These results indicated that both RAR and RXR were induced and activated by RA.

Role of RAR and RXR in Regulating the Phosphorylation of MAP Kinases—To determine the role of RAR and RXR in the regulation of the activation of MAP kinases, a specific RAR antagonist (AGN193109) and RXR antagonist (HX531) were used. Cells were pretreated with or without AGN193109 or HX 531 for 1 h, and then exposed to RA for 24 h, and subjected to stretch for 8 min (Fig. 10*A*) or exposed to Ang II for 3 min (ERK and p38) or 40 min (JNK) (Fig. 10*B*). Cyclic stretch- or Ang II-induced phosphorylation of ERK1/2, JNK1/2, and p38 was inhibited by RA, as demonstrated (Figs. 2 and 3). Although AGN193109 partially reversed the inhibitory effect of RA on stretch- or Ang II-induced phosphorylation of ERK1/2, JNK1/2, and p38, there was no significant difference compared with the RA treatment group ($p > 0.05$, Fig. 10, *A* and *B*, *bottom panel*). HX531 almost completely reversed the inhibitory effect of RA on both stretch and Ang II-induced activation of MAP kinases ($p < 0.05$, compared with RA treatment group). Based on these observations, activation of RXR or the heterodimerization of RAR and RXR may have an important role in the regulation of the activation of MAP kinases induced by cyclic stretch or Ang II.

DISCUSSION

RA suppresses ET-1, PE, and Ang II-induced hypertrophic responses in neonatal cardiomyocytes (11–13). However, the signaling mechanisms of RA action were not known. We provide the first evidence that RA inhibits the activation of MAP kinases induced by cyclic stretch or Ang II, by regulating the expression of MKP-1 and MKP-2 in cardiomyocytes. This represents a novel mechanism by which RA regulates cardiomyocyte growth inhibition.

In the present study, we demonstrated that cyclic stretch- or Ang II-induced cardiac hypertrophic features, including increased total protein content, protein synthesis, cell size, and myofibrillar reorganization were significantly inhibited by RA pretreatment, suggesting that retinoid-mediated signaling pathways have an important role in regulating cyclic stretch- or Ang II-induced cardiomyocyte growth. Previous studies have suggested that RA has a role in regulating the activation of

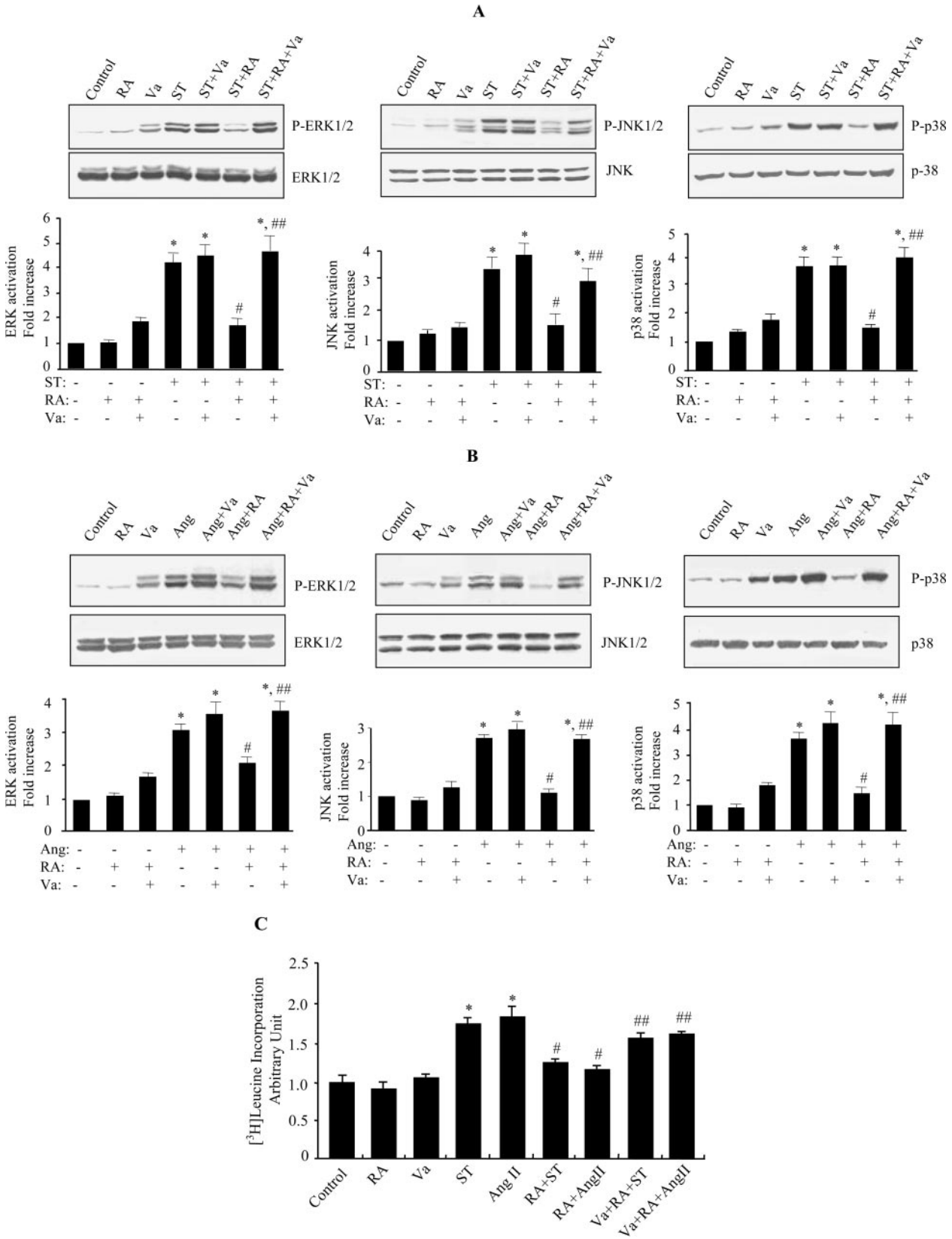

FIG. 5. **Vanadate reverses the inhibitory effect of RA on MAP kinases and protein synthesis.** To determine whether RA-mediated suppression of MAP kinases is regulated by protein phosphatases, cardiomyocytes were pretreated, with or without vanadate (Va, 10 μM) for 1 h, treated with or without RA (5 μM) for 24 h, then subjected to cyclic stretch for 8 min (*A*, *upper panel*), or exposed to Ang II for 3 (ERK and p38) or 40 min (JNK) (*B*, *upper panel*), the phosphorylation of ERK1/2, JNK1/2, and p38 was detected by Western blotting, as described in the legend to Fig. 2*A*. Phosphorylated levels of ERK1/2, JNK1/2, or p38 were quantified by densitometric scanning, and normalized to the level of total ERK, JNK, or p38 (*A* and *B*, *bottom panel*). Data represent average fold increase of controls from three independent experiments (mean ± S.E.). *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* stretch or Ang II. ##, $p < 0.05$ *versus* RA plus stretch or Ang II. *C*, under similar conditions, cardiomyocytes were stimulated by cyclic stretch or Ang II for 24 h, after pretreatment with vanadate and RA. [^{3}H]Leucine incorporation was performed, as described in the legend to Fig. 1*C*. Each *bar* represents the mean ± S.E. of six separate experiments. *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* stretch or Ang II; ##, $p < 0.05$ *versus* RA plus stretch or Ang II.

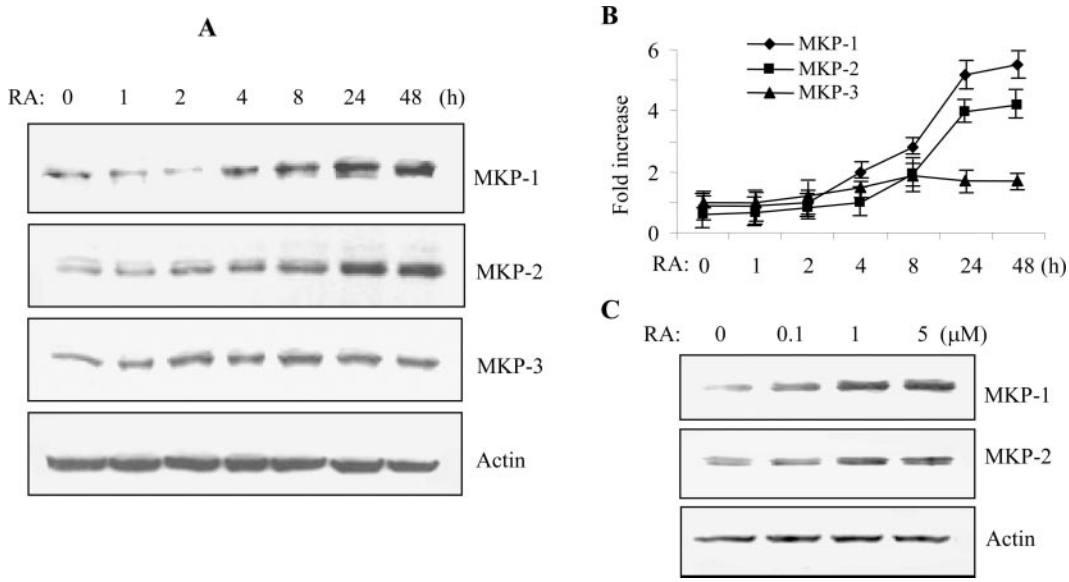

FIG. 6. **RA induces expression of MKPs.** *A*, cardiomyocytes were treated with RA (5 μM) for the times indicated, and the expression level of MKPs determined by Western blot using anti-MKP-1, -MKP-2, and -MKP-3 antibodies. Another set of samples was Western-blotted using anti-actin antibody, to serve as a loading control. Densitometric analysis of data is shown in *B*. The intensity of each message was normalized to the level of actin. *C*, cells were treated with different doses of RA for 24 h, and the expression of MKP-1 and MKP-2 was determined. The data are representative of three separate experiments.

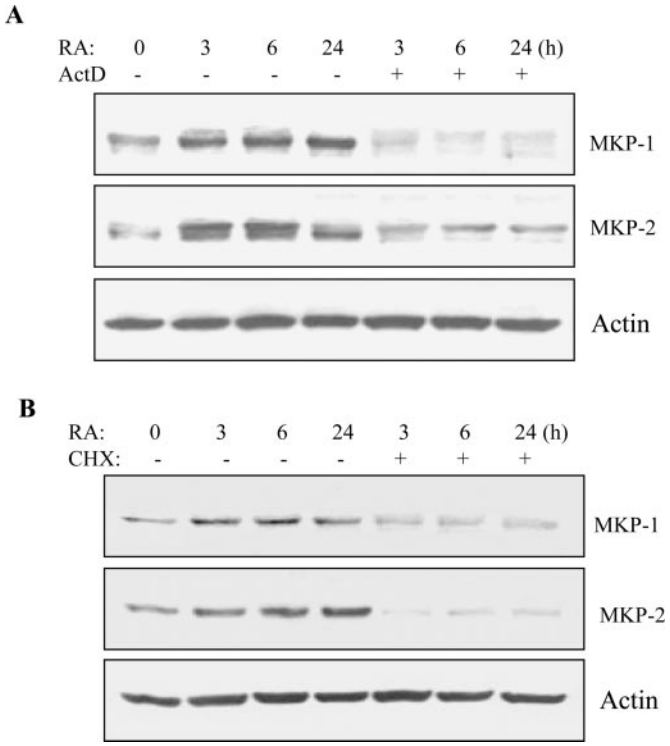

FIG. 7. **Effect of actinomycin D and cycloheximide on RA-induced expression of MKPs.** Cardiomyocytes were treated with RA (5 μM) for 3, 6, and 24 h, in the presence or absence of actinomycin D (*ActD*, 5 μg/ml, *A*) or cycloheximide (*CHX*, 50 μM, *B*), and subjected to Western blot analysis. The expression level of MKP-1 and MKP-2 was normalized to actin.

MAP kinase signaling pathways in other cell lines (37, 38). Here we determined whether there was a link between RA and MAP kinase signaling in hypertrophic cardiomyocytes. Our results showed that RA time- and dose-dependently inhibited cyclic stretch- and Ang II-induced activation of ERK1/2, JNK1/2, and p38. Significant inhibition was observed following 5–10 μM RA pretreatment. Although the activation of MAP kinases was inhibited by 5 μM RA from 2 h of pretreatment, significant inhibition was observed from 8–24 h of RA treatment, suggesting that retinoid receptor transcriptional activation was involved in regulating the late, sustained suppression of MAP kinases by RA. All three MAP kinases have been implicated as important transducers of the hypertrophic growth response, both in cell culture-based studies and in the intact heart (50–52). Using specific inhibitors for MEK1/2, JNK, or p38 MAP kinase, we demonstrated that all three MAP kinases were differentially involved in regulating the cyclic stretch- and Ang II-induced increase in protein synthesis. One potential mechanism whereby MAP kinases promote hypertrophy is by regulating the activation of GATA4, a cardiac-expressed transcription factor, which has been demonstrated to be involved in cardiac hypertrophy (53–55). GATA4 is directly regulated by ERK1/2 and p38 MAP kinase signaling effectors, resulting in augmented hypertrophic gene expression (56, 57). It has recently been shown that RA inhibits the hypertrophic gene expression through regulation of the transcriptional activity of GATA4 (58). In the present study, we provided additional evidence for understanding the signaling mechanism of RA action in neonatal cardiomyocytes. RA inhibits the activation of MAP kinases, which may lead to inactivation of GATA4, or other transcription factors such as AP-1, resulting in inhibition of the increase in cell size and protein synthesis induced by cyclic stretch and Ang II.

The precise mechanism by which RA mediates suppression of MAP kinases is not well defined. The activation of MAP kinases by stretch or Ang II is regulated by their upstream kinases (MKKs) (50, 51, 59). We demonstrated that both cyclic stretch and Ang II induced rapid phosphorylation of MEK1/2, MKK3, and MKK6. It is possible that the inhibitory effects of RA on MAP kinases may be regulated through inhibition of MKKs. Surprisingly, RA had no effect on the cyclic stretch- or Ang II-induced phosphorylation of MEK1/2, MKK4, and MKK3/6, suggesting that the inhibitory effects of RA on MAP kinases were mediated through MKK-independent pathways.

It has previously been shown that inactivation of MAP kinases is primarily achieved by a group of MKPs (60, 61). To date, 10 MKP family members have been cloned, some of which are encoded by immediate-early genes, such as MKP-1 and MKP-3 (62–65). MKPs differ by properties such as tissue-

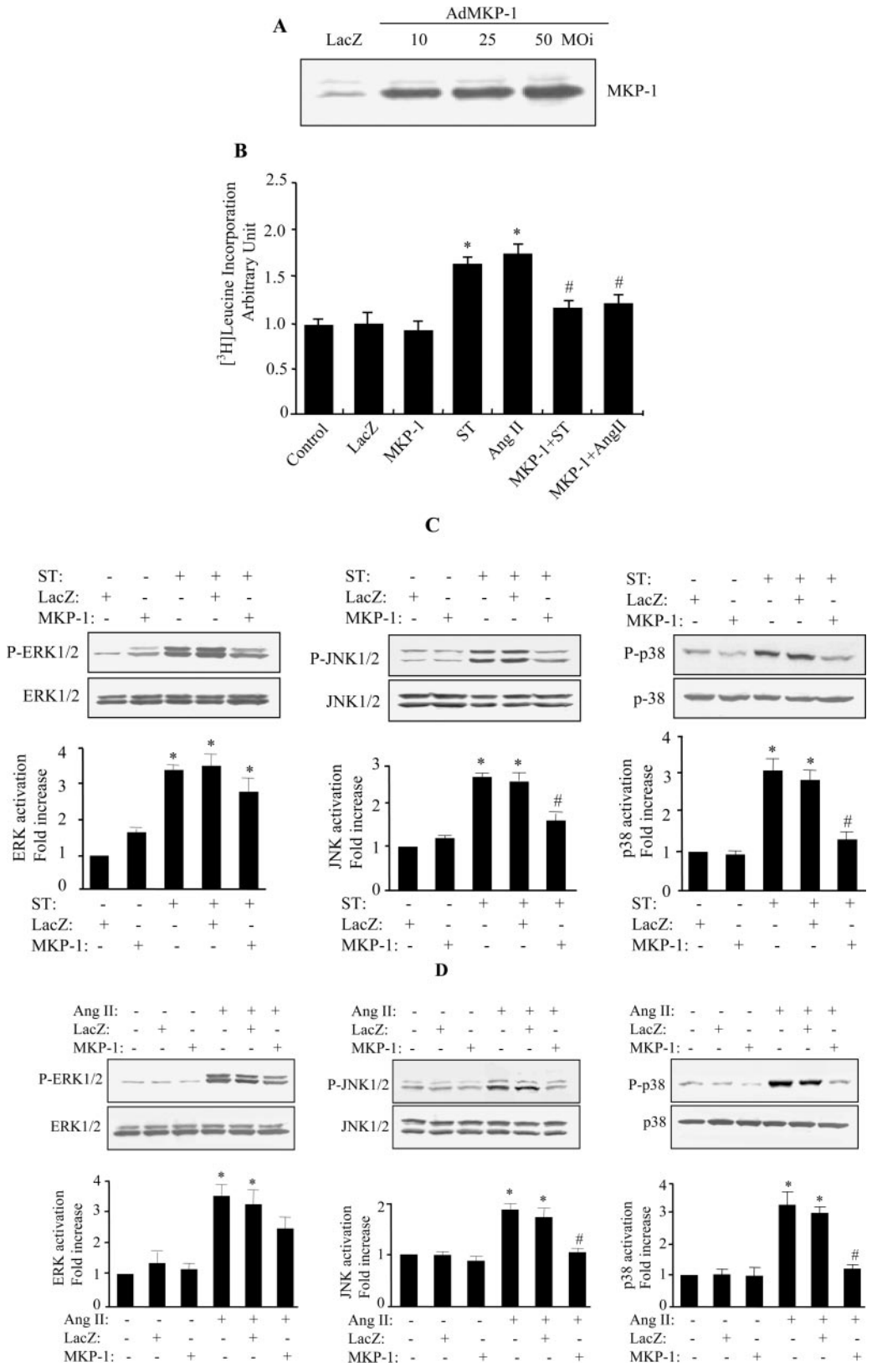

FIG. 8. **Overexpression of MKP-1 inhibits the activation of MAP kinases and protein synthesis.** *A*, cardiomyocytes were infected with AdLacZ or AdMKP-1 adenovirus at different MOIs, as indicated, and after 24 h, cell lysates (50 μg) were subjected to SDS-PAGE. The overexpression of MKP-1 was detected using anti-MKP-1 antibody. *B*, cardiomyocytes were infected with 50 MOI of AdLacZ or AdMKP-1, and after 24 h, subjected to cyclic stretch or exposed to Ang II for an additional 24 h. [^{3}H]Leucine incorporation was performed as described in Fig. 1*C*. Each bar represents the mean ± S.E. of six separate experiments. *, $p < 0.05$ *versus* control and #, $p < 0.05$ *versus* stretch or Ang II. After infection with AdMKP-1, cardiomyocytes were subjected to cyclic stretch for 8 min (*C*) or exposed to Ang II for 3 min (ERK, p38) or 40 min (JNK) (*D*). The phosphorylation of MAP kinases was determined using specific phospho-ERK1/2, -JNK, or -p38 antibodies. Membranes were reprobed for total ERK1/2, JNK1, or p38, as a control for equal protein loading. Phosphorylated levels of ERK1/2, JNK1/2, or p38 were quantified by densitometric scanning, and normalized to the level of total ERK1, JNK, or p38 (*C* and *D*, *bottom panel*). Data represent average fold increase of controls from three independent experiments (mean ± S.E.). *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* stretch or Ang II.

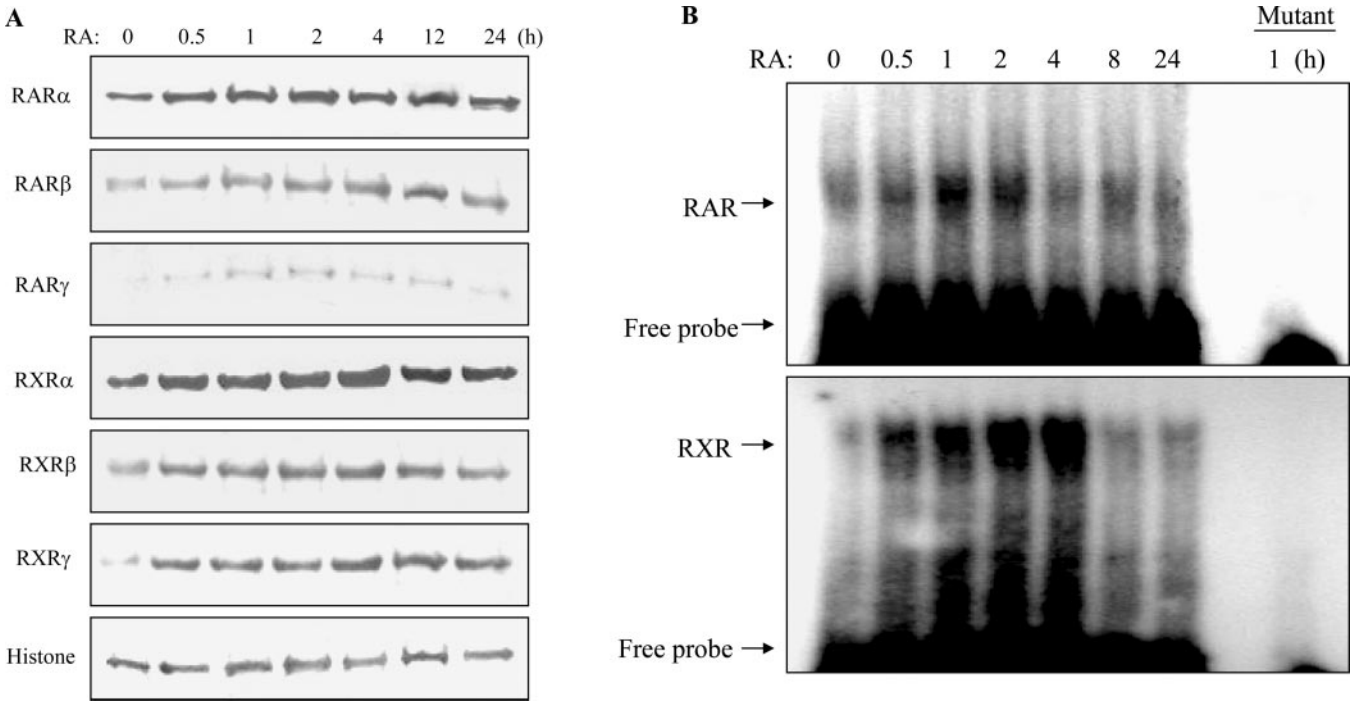

FIG. 9. **RA induces expression and activation of RARs and RXRs.** *A*, cardiomyocytes were stimulated by RA for the times indicated. Equal amounts of extracted nuclear proteins (20 μg) were separated on 10% SDS-PAGE, transferred to nitrocellulose membrane, and the expression of RARs and RXRs detected using anti-RARα, -β, -γ, and -RXRα, -β, and -γ antibodies. Another set of nuclear proteins was Western blotted using anti-histone antibody, to verify equal loading. *B*, gel mobility shift assay was performed using nuclear extracts from RA-treated cardiomyocytes. Nuclear extracts (5 μg) were incubated with the ^{32}P-labeled RAR or RXR oligonucleotide for 20 min at 25 °C and resolved by native 4% TAE polyacrylamide gel electrophoresis. A mutant-labeled RAR or RXR oligonucleotide was used as a negative control (*last lane*).

specific expression, differential regulation in response to various stimuli, distinct subcellular localization, and substrate specificity (66). MKP-1 and MKP-2 are widely expressed in various tissues, including heart, where they regulate inactivation of p38, JNK1/2, and ERK1/2 (32–34, 45–47). Various stimuli, such as growth factors, stress, phorbol ester, and vasoactive peptides, have been reported to induce MKP-1 and MKP-2 mRNA expression (45, 64, 67, 68). Studies have shown that MKP-1 limits the cardiac hypertrophic response, *in vitro* and *in vivo*, via dephosphorylation and inactivation of MAP kinases (34–36). RA induces expression of MKP-1 in non-small cell lung cancer cells, a mouse embryonic P19 stem cell line, rat fibroblast cell line NRK49F, and mesangial cells. The expression of MKP-1 is differentially regulated at transcriptional or translational levels in different cell lines (37, 38, 69). Conversely, RA has no effect on the expression of MKP-1 in rat aortic smooth muscle cells (70). These data indicate that the regulation of MKPs by RA is a cell specific response. Our data showed that the protein expression of MKP-1 and MKP-2 was dose- and time-dependently up-regulated by RA. The up-regulation was observed from 4 h of treatment with RA, peaked at 24 h, and was sustained to 48 h. The up-regulation of MKP-1 and MKP-2 was blocked by either inhibition of RNA synthesis or protein synthesis, suggesting that RA regulates the expression of MKPs at both the transcriptional and translational level in cardiomyocytes. It has been reported that RA inhibits H_2O_2 and serum-induced phosphorylation of JNK, via up-regulation of MKP-1 (37, 38). It is notable that RA suppresses the activation of MAP kinases through up-regulation of MKPs in neonatal cardiomyocytes. Several of our findings support this latter possibility as follows: First, the inhibition of MAP kinases by RA was attenuated by vanadate, a potent inhibitor of protein-tyrosine phosphatases, including MKP-1 and MKP-2 (64, 71, 72), and the inhibitory effect of RA on increased protein synthesis was also reversed by vanadate. Second, overexpression of MKP-1 in cardiomyocytes significantly inhibited cyclic stretch- and Ang II-induced phosphorylation of JNK and p38, and the increase in protein synthesis. The phosphorylation of ERK1/2 was only partly inhibited by overexpression of MKP-1, indicating that other MKPs may also have contributed to the suppression of MAP kinases by RA. Third, RA treatment induced up-regulation of MKP-1 and MKP-2 in cardiomyocytes, the time course of which is correlated with the inhibitory effect of RA on the activation of MAP kinases. These findings suggested that up-regulation of MKP-1 and MKP-2 had an important role in the suppression of MAP kinase activities by RA.

The transcriptional regulatory effects mediated by RA are influenced at multiple levels, such as specificity of the ligand-receptor interaction, and temporal and spatial variations in receptor expression. Previous studies have shown that retinoid receptors are required at early stages of cardiac development, support cell proliferation, and control the shape of ventricular myocytes (6, 7). Both RXRs and RARs may participate in the mediation of these functions (6, 7, 73). In the post-development period, the retinoid receptor is involved in preserving the normal differentiated phenotype of cardiomyocytes, by antagonizing the effect of various hypertrophic stimuli (11–13). However, very little is known about the regulation of retinoid receptor expression in response to RA in neonatal cardiomyocytes. In the adult rat heart, RXRγ is predominantly expressed, compared with RXRα and RXRβ. The positive regulation of RXRβ and negative regulation of RXRγ by thyroid hormone is observed in adult rat heart (74). RXRγ though not detectable in embryonic heart, is expressed in neonatal cardiomyocytes and developmentally regulated during the postnatal period (75). All of the retinoid receptors were expressed and induced by RA in neonatal cardiomyocytes. RARα, RXRα, and RXRγ were the most abundant receptors, whereas RARγ was weakly expressed. Both RAR and RXR were transcriptionally activated by RA. It has been shown that RARs and RXRs have different

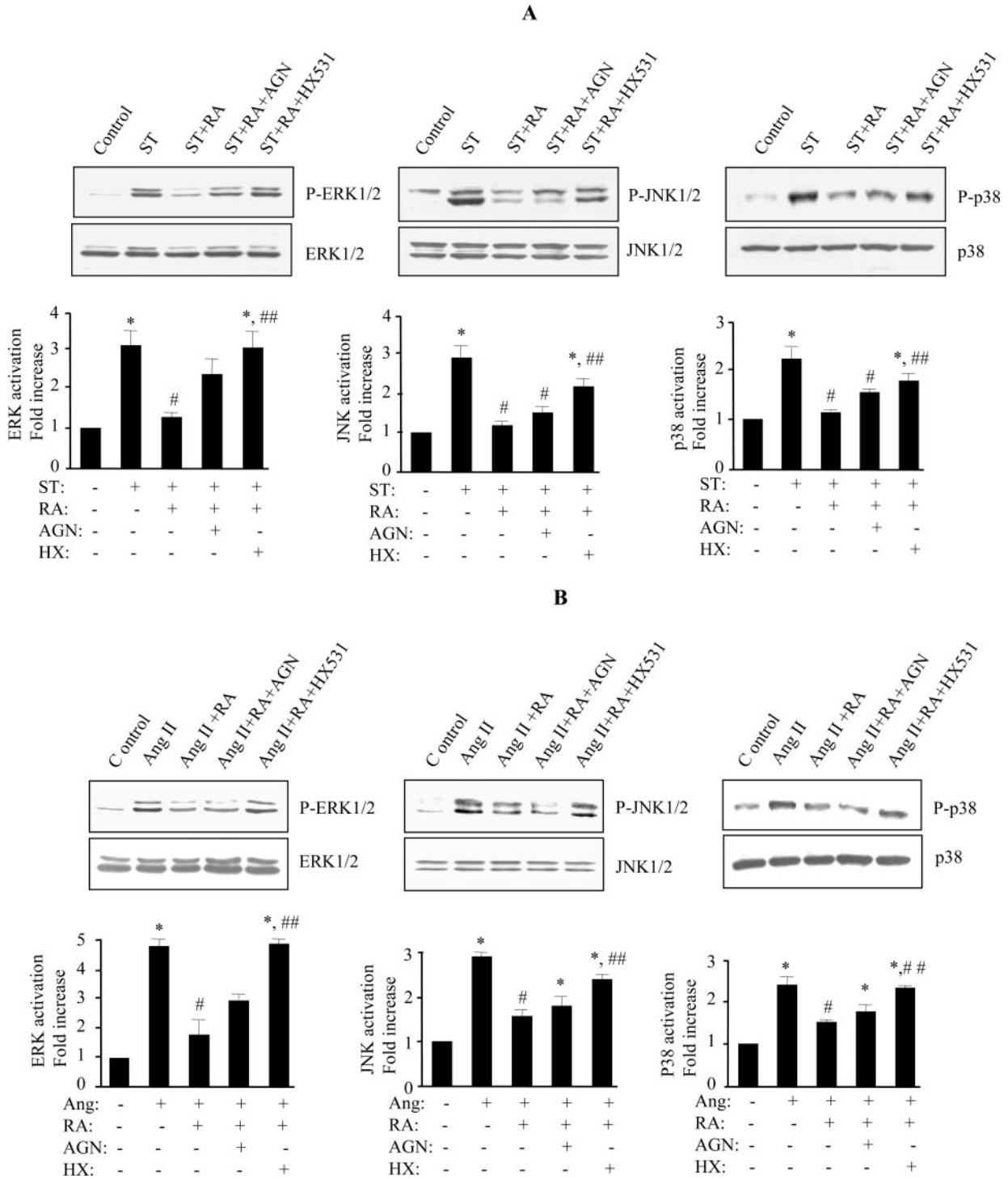

FIG. 10. **The role of RAR and RXR in mediating the activation of MAP kinases.** To determine the role of RAR and RXR in regulation of the activation of MAP kinases, a specific RAR antagonist AGN193109 (AGN, 1 μM), and RXR antagonist HX531 (1 μM) was used. Cells were pretreated with or without AGN193109 or HX531 for 1 h, treated with or without RA (5 μM) for 24 h, and subjected to stretch (*A*) or exposed to Ang II (*B*). The phosphorylation of ERK1/2, JNK1/2, and p38 was determined, as described in Fig. 2. Phosphorylated levels of ERK1/2, JNK1/2, or p38 were quantified by densitometric scanning, and normalized to the level of total ERK, JNK, or p38 (*A* and *B*, *bottom panel*). Data represent average fold increase of controls from three independent experiments (mean ± S.E.). *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* stretch or Ang II; ##, $p < 0.05$ *versus* RA plus stretch or Ang II.

roles in regulating the inhibitory effect of RA on activation of JNK (38, 76). To determine the role of RAR and RXR in RA-induced suppression of the activation of MAP kinases in cardiomyocytes, specific receptor antagonists were used. Our data showed that antagonizing RARs, had a minor effect on the activation of MAP kinases. However, RXR antagonist significantly reversed the inhibited activation of MAP kinases induced by RA, indicating that RXR or the heterodimerization of RAR and RXR, is involved in regulating the activation of MAP kinases induced by cyclic stretch or Ang II. These data suggested that RA effects were mediated through both RAR- and RXR-regulated signaling pathways, resulting in suppression of the hypertrophic responses induced by cyclic stretch or Ang II.

Previous studies have shown that RA antagonizes the hypertrophic growth of cardiomyocytes. The results presented here were focused on the signaling mechanisms, which mediate the

inhibitory effects of RA on cardiac hypertrophy. This is the first report to demonstrate that hypertrophic stimuli-induced activation of MAP kinases is negatively regulated by RA, and that the up-regulated expression of MKP-1 has an important role in mediating the MAP kinase inhibition and antihypertrophic effect of RA, in cyclic stretch- or Ang II-stimulated neonatal cardiomyocytes. Further studies will be required to determine whether this mechanism is operable in *in vivo* models of cardiac hypertrophy.

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