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Biochemical and Biophysical Research Communications 327 (2005) 136–142
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CaMKII activates ASK1 and NF-κB to induce cardiomyocyte hypertrophy

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Received 27 November 2004
Available online 9 December 2004

Abstract

Ca^{2+}/calmodulin-dependent protein kinase (CaMK) is an important downstream target of Ca^{2+} in the hypertrophic signaling pathways. We previously showed that the activation of apoptosis signal-regulating kinase 1 (ASK1) or NF-κB is sufficient for cardiomyocyte hypertrophy. Infection of isolated neonatal cardiomyocytes with an adenoviral vector expressing CaMKIIδ3 (AdCaMKIIδ3) induced the activation of ASK1, while KN93, an inhibitor of CaMKII, inhibited phenylephrine-induced ASK1 activation. Overexpression of CaMKIIδ3 induced characteristic features of in vitro cardiomyocyte hypertrophy. Infection of cardiomyocytes with an adenoviral vector expressing a dominant negative mutant of ASK1 (AdASK(KM)) inhibited the CaMKIIδ3-induced hypertrophic responses. Overexpression of CaMKIIδ3 increased the κB-dependent promoter/luciferase activity and induced IκBα degradation. Coinfection with AdCaMKIIδ3 and AdASK(KM), and pre-incubation with KN93 attenuated CaMKIIδ3- and phenylephrine-induced NF-κB activation, respectively. Expression of a degradation resistant mutant of IκBα inhibited CaMKIIδ3-induced hypertrophic responses. These results indicate that CaMKIIδ3 induces cardiomyocyte hypertrophy mediated through ASK1-NF-κB signal transduction pathway.

Keywords: Cardiomyocyte hypertrophy; Ca^{2+}; CaMKII; CaM kinase II; ASK1; NF-κB; GPCR agonist

A variety of pathophysiological stimuli, such as myocardial infarction and hypertension, lead to an increase in cardiac workload and elevated mechanical stress. Hypertrophic growth is an adaptive response of the heart to such hemodynamic overload. In response to hypertrophic signals, cardiomyocytes activate a cellular response characterized by an increase in size of individual ventricular cardiomyocytes, sarcomere assembly, and re-expression of genes normally expressed in the embryonic ventricles [1].

Numerous studies indicate that an increase in intracellular Ca^{2+} concentration is a primary stimulus for the hypertrophic responses [2,3]. There are substantial evidences suggesting that the Ca^{2+}-binding protein, calmodulin, may be a key regulator of cardiac hypertrophy [2,4]. The multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaMK) and calcineurin are well-known

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.12.002

downstream effectors of the actions of Ca^{2+} and calmodulin. CaMK subfamily comprises CaMKI, -II, and -IV. All three subtypes have been reported to be involved in cardiac hypertrophy in vitro and in vivo [5–8]. There are four different, highly conserved genes coding for CaMKII, called α, β, γ, and δ [9]. The gene products are further divided into splicing variants. The δ2 (also termed δ_C) and δ3 (also termed δ_B) isoforms of CaMKII were cloned from rat hearts and δ3 was found to be the predominant isoform of CaMKII in the heart [10,11]. The δ3 isoform localizes to nucleus, while the δ2 isoform localizes to the cytoplasm [5,10]. Transgenic study of CaMKIIδ2 indicates its role in excitation–contraction coupling [12]. On the other hand, transgenic mice overexpressing CaMKIIδ3 in hearts exhibited cardiac hypertrophy and dilatation [8], suggesting that CaMKIIδ3 plays an important role in cardiac hypertrophy. However, a downstream signaling pathway of CaMKIIδ3 to the development of cardiac hypertrophy remains to be elucidated.

We identified apoptosis signal-regulating kinase 1 (ASK1) and NF-κB as novel signaling intermediates involved in cardiomyocyte hypertrophy [13,14]. While ablation of ASK1 had no effect on pressure overload-induced hypertrophy [15], it attenuated angiotensin II-induced cardiac hypertrophy [16]. This suggests that ASK1 is involved in cardiac hypertrophy, although parallel signaling pathways also exist in the development of cardiac hypertrophy.

Sagasti et al. [17] reported that *nsy-1*, which encodes a homologue of mammalian ASK1, appears to act downstream of the CaMKII *unc-43* in *Caenorhabditis elegans*. NSY-1 associates with UNC-43, suggesting that UNC/CaMKII activates the NSY-1 MAP kinase signaling pathway. Thus, we can hypothesize that CaMKII leads to cardiac hypertrophy via ASK1-NF-κB signaling pathway. In this study, we examined whether CaMKII-ASK1 signaling pathway is involved in cardiomyocyte hypertrophy. We demonstrated that CaMKII mediates cardiomyocyte hypertrophy via ASK1 and NF-κB activation.

Materials and methods

Primary culture of neonatal ventricular myocytes. Ventricular myocytes from 1- to 2-day-old Wistar rats were prepared and cultured in serum-free Dulbecco's modified Eagle's medium (DMEM) as described previously [13,15]. In this study, phenylephrine (100 μmol/L) was used to stimulate cardiomyocytes.

Recombinant adenovirus vectors. A replication-defective E1 and E3 adenoviral vector expressing either a mutated form of human IκBα (AdIκBα32/36A; Ser32 and Ser36 to Ala) [13], a dominant negative form of ASK1 (AdASK(KM); Lys709 to Met) [18] or bacterial β-galactosidase (AdLacZ) [13] was described previously. Adenovirus, AdCaMKIIδ3, expressing CaMKIIδ3 [19] was constructed by means of homologous recombination in HEK293 cells using the adenovirus-based plasmid JM17 [20]. Cardiomyocytes were infected with the recombinant adenovirus vectors at a multiplicity of infection of 50–100 plaque-forming units per cell for 1 h. Subsequently, the cells were cultured in serum-free DMEM for an additional 24 h prior to treatment. Expression of CaMKIIδ3, ASK1, and IκBα was assessed by Western blotting using 20 μg cell lysates along with an anti-CaMKII, anti-ASK1, and anti-IκBα antibody (Santa Cruz Biotechnology), respectively. The probed protein was detected with a secondary antibody by an ECL plus or ECL advance Western blotting detection kit (Amersham Biosciences).

Luciferase reporter gene assay. Cardiomyocytes plated on 22-mm-diameter culture dishes were exposed to 5 μg lipofectin with the luciferase reporter construct possessing consensus NF-κB-binding sites (κB-Luc; 1.67 μg) [21] according to the manufacturer's instruction (Life Technologies). Myocytes were incubated in OPTI-MEM (Life Technologies) for 6 h, then adenoviral infection or phenylephrine stimulation was performed and the media were changed to serum-free DMEM for 24 h. Myocytes were assayed for luciferase activity with a dual-luciferase reporter assay kit (Promega). Activity was normalized to an internal co-transfected constitutive control (*Renilla* luciferase expressing vector, pRL-CMV, Promega) and expressed as *n*-fold stimulation relative to control.

Assessment of hypertrophic responses. For the assessment of protein synthesis, cardiomyocytes were stimulated with phenylephrine or viral infection for 48 h in the medium supplemented with [^{3}H]leucine (1 μCi/ml) [13]. The number of cells was not changed by any intervention used in this study. Atrial natriuretic factor (ANF) expression was examined on cardiomyocytes grown on gelatin-coated glass coverslips with the aid of rabbit anti-rat ANF polyclonal antiserum (Phoenix Laboratories) and fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibodies (Amersham Biosciences) [13]. The F-actin was detected using rhodamine-conjugated phalloidin (Molecular Probes) [13].

Immune complex kinase assay of ASK1. After incubation for 24 h in serum-free DMEM, phenylephrine, H_2O_2 (200 μmol/L) or ionomycin (100 nmol/L) stimulation or viral infection was initiated, and cell lysates were prepared after 10 min of drug stimulation or 8 h of viral infection. The activity of ASK1 was measured by an immune complex kinase assay as described previously [13].

Statistical analysis. Data are expressed as means ± SE. Differences between experimental groups were evaluated for statistical significance using one-way ANOVA followed by Bonferroni's post test. P values <0.05 were considered to be statistically significant.

Results

CaMKIIδ3 induced ASK1 activation in cardiomyocytes

Many features of pathological hypertrophy can be reproduced in isolated neonatal cardiomyocytes by treatment with a variety of agents including phenylephrine, angiotensin II, and endothelin-1 [22]. These cells respond to an increase in protein synthesis, sarcomeric organization, and re-expression of embryonic genes such as ANF. By using this experimental model, we examined the relationship of CaMKIIδ3 and ASK1 in the development of cardiomyocyte hypertrophy.

Initially, we examined the ability of CaMKIIδ3 to activate ASK1 in neonatal cardiomyocytes. Eight hours after infection of cardiomyocytes with an adenoviral vector expressing CaMKIIδ3 (AdCaMKIIδ3), the whole cell extracts were immunoprecipitated with an

anti-ASK1 antibody. An ASK1 kinase assay was performed using MKK6 as a substrate (Fig. 1). Infection of cardiomyocytes with AdCaMKIIδ3 induced overexpression of CaMKIIδ3 protein. The expression of CaMKIIδ3 resulted in the activation of ASK1, whereas infection of a control adenoviral vector expressing bacterial β-galactosidase, AdLacZ, had no effect on ASK1 activation. The protein levels of ASK1 were not different among groups (Fig. 1A). Then, we investigated whether phenylephrine-induced ASK1 activation was mediated through CaMKII signaling pathway. Whereas phenylephrine activated ASK1, pre-treatment with KN93, a specific inhibitor of CaMKII, abolished phenylephrine-induced ASK1 activation (Fig. 1B).

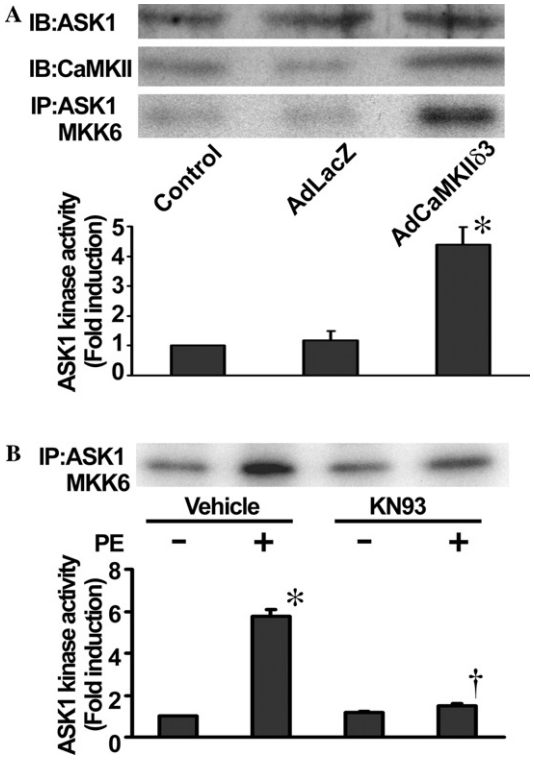

Fig. 1. CaMKIIδ3 activates ASK1. (A) Cardiomyocytes were infected with AdLacZ or AdCaMKIIδ3. After 8 h of infection, cell lysates were subjected to Western blot analysis with anti-ASK1 (upper panel) or anti-CaMKII antibody (middle panel) or incubated with anti-ASK1 antibody. ASK1 activity was measured by immune complex kinase assay with His-MKK6 as a substrate (lower panel). Density is expressed as *n*-fold induction relative to uninfected control. Values represent means ± SE of data for three experiments performed in triplicate. $^{*}P < 0.05$ versus control. (B) Cardiomyocytes were incubated for 1 h with either vehicle or 0.5 μmol/L KN93. Myocytes were then treated with or without 100 μmol/L phenylephrine. Ten minutes after the treatment, cell lysates were extracted. ASK1 activity was measured and density is expressed as *n*-fold induction relative to untreated cells pre-incubated with vehicle. $^{*}P < 0.05$ versus untreated cells pre-incubated with vehicle; $^{\dagger}P < 0.05$ versus phenylephrine treated cells pre-incubated with vehicle.

Involvement of CaMKII in cardiomyocyte hypertrophy

We examined whether AdCaMKIIδ3 can induce biochemical and morphological responses of cardiomyocyte hypertrophy. Infection with AdCaMKIIδ3 increased [^{3}H]leucine uptake into cardiomyocytes, while AdLacZ had no effect on [^{3}H]leucine uptake (Fig. 2A). We also analyzed the cytoskeletal organization in cardiomyocytes by phalloidin staining and the expression of ANF by immunohistochemical staining using an antibody against the ANF protein. Both uninfected cells and cells infected with AdLacZ showed a reticular actin organization lacking sarcomeric formation, whereas infection with AdCaMKIIδ3 significantly induced the reorganization of the actin cytoskeleton into a sarcomeric structure. The ratio of myocytes with well-organized sarcomere (more than 2/3 of cell area [23]) was 12.2 ± 1.7% in uninfected cells and 14.0 ± 2.1% in AdLacZ-infected cells, whereas it was 71.4 ± 3.4% in AdCaMKIIδ3-infected cells (Fig. 2B). The percentage of myocytes expressing the ANF protein was increased by infection with AdCaMKIIδ3

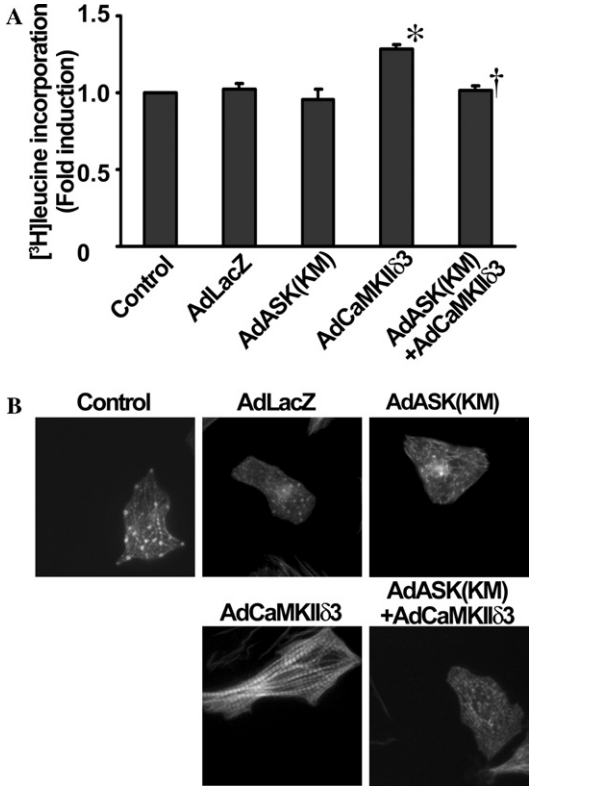

Fig. 2. ASK1 is required for CaMKIIδ3-induced cardiomyocyte hypertrophy. Cardiomyocytes were infected with AdLacZ, AdCaMKIIδ3, or AdASK(KM) alone or co-infected with AdCaMKIIδ3 and AdASK(KM). (A) [^{3}H]Leucine incorporation. Values show means ± SE of data for three experiments performed in triplicate. $^{*}P < 0.05$ versus control; $^{\dagger}P < 0.05$ versus AdCaMKIIδ3. (B) Organization of sarcomere structure.

compared with uninfected cells or cells infected with AdLacZ (AdCaMKIIδ3; 64.1 ± 6.8%, Uninfected; 11.0 ± 3.2%, and AdLacZ; 13.1 ± 3.7%). Thus, CaMKIIδ3 was able to induce morphological and biochemical features of in vitro cardiomyocyte hypertrophy.

ASK1 is required for CaMKIIδ3-induced cardiomyocyte hypertrophy

Then, to determine the involvement of ASK1 in the CaMKIIδ3-induced cardiomyocyte hypertrophy, we infected cardiomyocytes with an adenovirus expressing a dominant negative form of ASK1 (AdASK(KM)) along with AdCaMKIIδ3. The increase in [^{3}H]leucine uptake induced by AdCaMKIIδ3 was suppressed by co-infection with AdASK(KM). Infection with AdASK(KM) alone had no effect on [^{3}H]leucine uptake (Fig. 2A). Co-infection of cardiomyocytes with AdASK(KM) led to the elimination of the enhancement of the sarcomere organization induced by AdCaMKIIδ3 (15.1 ± 0.8%), while infection with AdASK(KM) alone had no effect on cell morphology (11.4 ± 2.0%) (Fig. 2B). Inhibition of the CaMKIIδ3-induced ASK1 activation by co-infection with AdASK(KM) eliminated the increases in ANF protein (17.8 ± 4.2%). These findings suggested that the activation of ASK1 is involved in morphological and biochemical hypertrophic responses induced by CaMKIIδ3.

CaMKIIδ3-induced NF-κB activation

We examined whether NF-κB is involved as a transcription factor in CaMKIIδ3-induced cardiomyocyte hypertrophy. Isolated cardiomyocytes were transiently transfected with a luciferase reporter construct containing consensus NF-κB-binding sites. As seen in Fig. 3A, infection of myocytes with AdCaMKIIδ3 led to increased luciferase activity. To confirm the activation of NF-κB by CaMKIIδ3, we examined the degradation of IκBα using an anti-IκBα antibody for Western blot analysis. IκBα was degraded in cells infected with AdCaMKIIδ3 (Fig. 3B).

Next, to determine the involvement of ASK1 in the CaMKIIδ3-induced NF-κB activation, we examined the effect of AdASK(KM) on CaMKIIδ3-induced NF-κB activation. Overexpression of ASK(KM) significantly inhibited the activation of κB-dependent luciferase (Fig. 3A) and the degradation of IκBα (Fig. 3B) induced by CaMKIIδ3.

Then, we examined the involvement of CaMKIIδ3 in phenylephrine-induced NF-κB activation. Pre-treatment with KN93 abolished phenylephrine-induced NF-κB activation (Fig. 3C). KN93 eliminated phenylephrine-induced IκBα degradation (Fig. 3D). These findings indicate that CaMKIIδ3 activates NF-κB mediated through ASK1 activation.

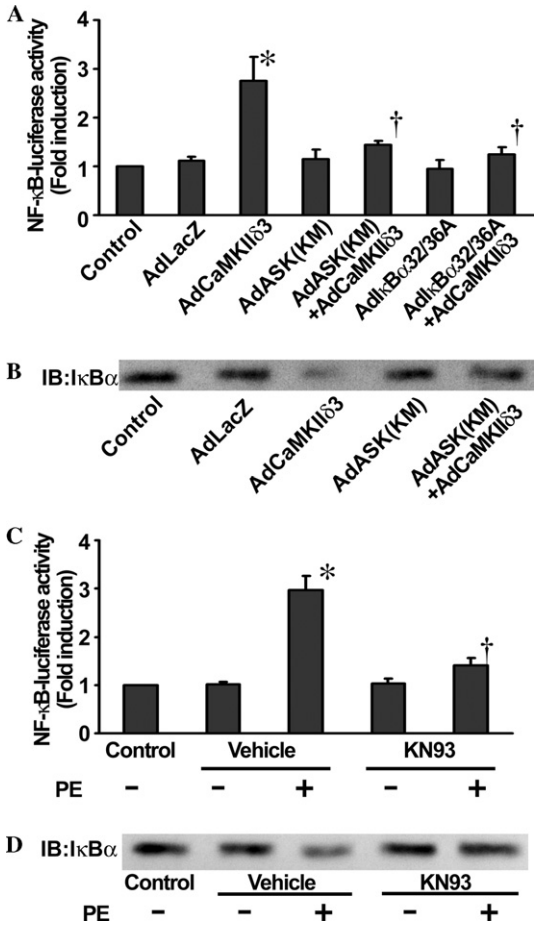

Fig. 3. CaMKIIδ3 activates NF-κB mediated through ASK1. (A,B) Involvement of ASK1 in CaMKIIδ3-induced NF-κB activation. (A) κB-dependent reporter assay. Cardiomyocytes, transfected with κB-luciferase construct, were infected with AdLacZ, AdCaMKIIδ3, AdASK(KM), AdIκBα 32/36A alone or co-infected with AdCaMKIIδ3 and AdASK(KM) or AdCaMKIIδ3 and AdIκBα 32/36A. Luciferase activity is expressed as *n*-fold induction relative to untreated control. Values represent means ± SE of data for three experiments performed in triplicate. $^*P < 0.05$ versus control; $^\dagger P < 0.05$ versus AdCaMKIIδ3. (B) IκBα immunoblotting. (C,D) Involvement of CaMKII in phenylephrine-induced NF-κB activation. Cardiomyocytes were incubated for 1 h with either no drug, vehicle or 0.5 μmol/L KN93. Myocytes were then treated with or without phenylephrine. Twenty-four hours after treatment, measurement of κB-luciferase activities (C) and IκBα immunoblotting (D) were performed as described above. $^*P < 0.05$ versus control; $^\dagger P < 0.05$ versus phenylephrine treated cells pre-incubated with vehicle.

CaMKIIδ3-induced cardiomyocyte hypertrophy is mediated through NF-κB

We examined the role of NF-κB activation in the CaMKIIδ3-induced hypertrophy using an adenovirus expressing a degradation-resistant form of IκBα (AdIκBα 32/36A). The effectiveness of AdIκBα 32/36A in blocking CaMKIIδ3-induced activation of NF-κB was assessed in a reporter gene assay. The CaMKIIδ3-induced luciferase activation was reduced to near

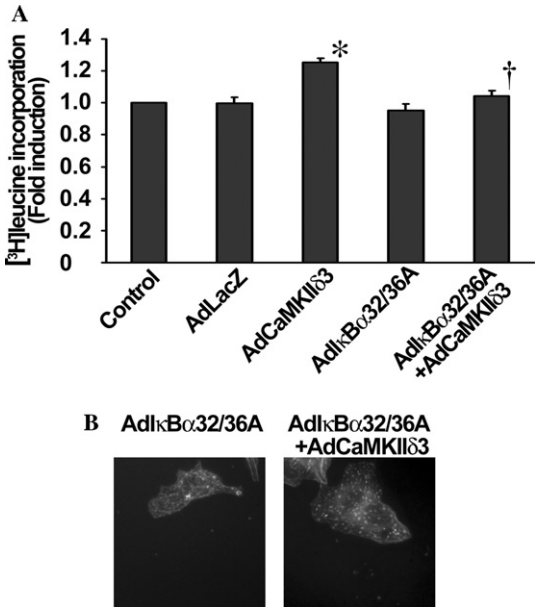

Fig. 4. CaMKIIδ3-induced cardiomyocyte hypertrophy is mediated through NF-κB. Cardiomyocytes were infected with AdLacZ, AdCaMKIIδ3, or AdIκBα 32/36A alone or co-infected with AdCaMKIIδ3 and AdIκBα 32/36A. (A) [^{3}H]Leucine incorporation. Values represent means ± SE of data for three experiments performed in triplicate. *$P < 0.05$ versus control; †$P < 0.05$ versus AdCaMKIIδ3. (B) Organization of sarcomere structure.

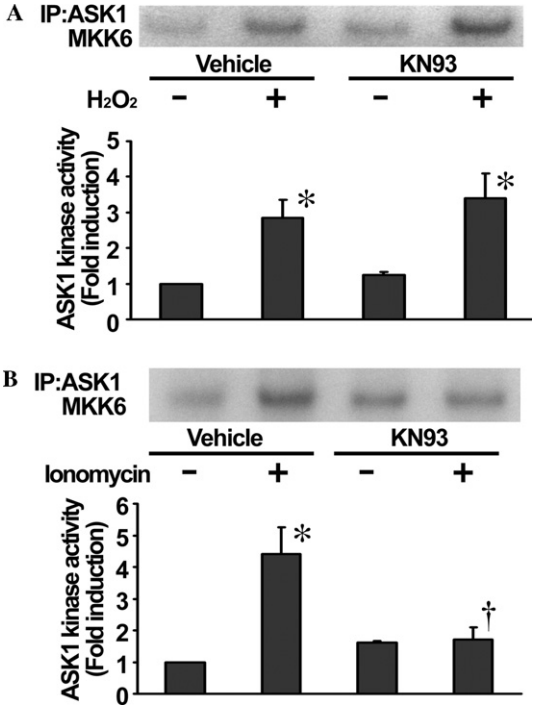

Fig. 5. Involvement of CaMKII in Ca^{2+}- or H_2O_2-induced ASK1 activation. (A) Cardiomyocytes were incubated for 1 h with either vehicle or 0.5 μmol/L KN93. Myocytes were then treated with or without 200 μmol/L H_2O_2. Ten minutes after the treatment, cell lysates were extracted. ASK1 activity was measured and density is expressed as *n*-fold induction relative to untreated cells pre-incubated with vehicle. *$P < 0.05$ versus untreated cells pre-incubated with vehicle. (B) Cardiomyocytes were incubated for 1 h with either vehicle or KN93. Myocytes were then treated with or without 100 nmol/L ionomycin. Ten minutes after the treatment, ASK1 activity was measured and density is expressed as *n*-fold induction relative to untreated cells pre-incubated with vehicle. *$P < 0.05$ versus untreated cells pre-incubated with vehicle; †$P < 0.05$ versus ionomycin treated cells pre-incubated with vehicle.

control levels in the cells infected with AdIκBα32/36A (Fig. 3A). Co-infection with AdIκBα32/36A eliminated CaMKIIδ3-induced increase in [^{3}H]leucine uptake (Fig. 4A), enhancement of sarcomeric reorganization (15.1 ± 0.8%) (Fig. 4B), and increase in the percentage of myocytes expressing the ANF protein (15.3 ± 3.4%), but infection with AdIκBα 32/36A alone had no effect on the hypertrophic responses (sarcomere; 11.2 ± 1.7%, ANF; 9.5 ± 2.4%). These findings suggest that the activation of NF-κB is involved in morphological and biochemical hypertrophic responses induced by CaMKIIδ3.

Ca^{2+}- and H_2O_2-dependent ASK1 activation

H_2O_2 activates ASK1 through inactivation of the intrinsic inhibitor thioredoxin [18]. We examined the involvement of CaMKIIδ3 in H_2O_2-induced ASK1 activation. Pre-incubation of cardiomyocytes with KN93 had no effect on H_2O_2-induced ASK1 activation (Fig. 5A). On the other hand, an increase in intracellular Ca^{2+} induced by Ca^{2+} inophore ionomycin treatment caused ASK1 activation, and pre-incubation with KN93 abolished the Ca^{2+}-induced ASK1 activation (Fig. 5B). These findings suggest that CaMKII is involved in Ca^{2+}-induced ASK1 activation, but not in H_2O_2-induced ASK1 activation.

Discussion

CaMK subfamily has been reported to be involved in cardiac hypertrophy in vitro and in vivo [5–8]. Myocyte enhancer factor-2 (MEF2) transcription factor is supposed to be a downstream target for CaMKI and CaMKIV signaling in the hypertrophic hearts [7]. On the other hand, the signaling pathways through which CaMKII induces cardiac hypertrophy have remained to be elucidated. In *C. elegans*, the *nsy-1* encodes a homologue of the human ASK1 and appears to act downstream of UNC-43, CaMKII, to execute a lateral signaling decision required for asymmetric olfactory neuron fates by repressing str-2 expression in AWC cells [17]. In this study, we demonstrated that CaMKIIδ3-induced cardiomyocyte hypertrophy mediated through ASK1 in mammalian cells.

The mechanism whereby CaMKIIδ3 activates ASK1 remains to be determined. Recently, Takeda et al. [24]

have shown that CaMKII is directly associated with ASK1 to phosphorylate ASK1. Phosphorylation site of ASK1 by CaMKII is not Thr845 of ASK1 that is essential for ASK1 activation, but at sites other than Thr845 in HEK-293 cells. KN93 inhibited Ca^{2+}-induced ASK1 phosphorylation, indicating that phosphorylation of ASK1 by CaMKII may be an important step to initiate the phosphorylation at Thr845, which might be phosphorylated by ASK1 itself or undefined protein kinase. However, Zhang et al. [8] showed that protein phosphatase 2A is upregulated in the hearts of their CaMKIIδ3 transgenic mice. High protein phosphatase 2A activity may affect MAPK signaling including ASK1. Thus, we cannot exclude the possibility that CaMKIIδ3 activates ASK1 mediated through protein phosphatase 2A activation.

We have reported that the Ca^{2+}-sensitive tyrosine kinase Pyk2 and the small GTP-binding protein Rac1 are also involved in G-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy mediated through reactive oxygen species (ROS) production and ASK1 activation [13,25,26]. ASK1 is present as an inactive complex with thioredoxin, and ROS oxidizes thioredoxin, thus dissociating from ASK1 and leading to the activation of ASK1 [18]. Our results indicate that CaMKII activity is required for Ca^{2+}-induced ASK1 activation, but not ROS-induced activation in agreement with recent report on mouse embryonic fibroblasts [24]. Further investigation will be necessary to establish the interaction between Ca^{2+}- and ROS-mediated signaling pathways to lead to ASK1 activation in the development of cardiac hypertrophy.

Although ASK1 has been reported to inhibit interleukin-1-induced NF-κB activity in HEK-293 cells [27], we have shown that NF-κB is activated by ASK1, leading to cardiomyocyte hypertrophy in isolated neonatal cardiomyocytes [13]. We demonstrated here that CaMKIIδ3-induced NF-κB activation, while the inhibition of NF-κB signaling resulted in the attenuation of CaMKIIδ3-induced cardiomyocyte hypertrophy. Furthermore, inhibition of ASK1 resulted in the attenuation of CaMKIIδ3-induced NF-κB activation. Thus, CaMKIIδ3 regulates hypertrophic responses mediated through ASK1-NF-κB activation.

In conclusion, CaMKIIδ3 is involved in cardiomyocyte hypertrophy. ASK1 and NF-κB are located downstream of CaMKIIδ3 in the signaling pathway to the development of cardiomyocyte hypertrophy.

Acknowledgments

We thank Dr. Eishichi Miyamoto of Kumamoto University for the gift of the CaMKIIδ3 cDNA and Dr. Hidenori Ichijo of University of Tokyo for AdASK(KM). This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to K.O.)

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