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RESEARCH PAPER

The mixed-lineage kinase 1–3 signalling pathway regulates stress response in cardiac myocytes via GATA-4 and AP-1 transcription factors

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Background and purpose: The mixed-lineage kinases (MLKs) act upstream of mitogen-activated protein kinases, but their role in cardiac biology and pathology is largely unknown.
Experimental approach: We investigated the effect of a MLK1–3 inhibitor CEP-11004 on G protein-coupled receptor agonist-induced stress response in neonatal rat cardiac myocytes in culture.
Key results: CEP-11004 administration dose-dependently attenuated phenylephrine and endothelin-1 (ET-1)-induced c-Jun N-terminal kinase activation. MLK inhibition also reduced ET-1- and phenylephrine-induced phosphorylation of p38 mitogen-activated protein kinase. In contrast, phenylephrine-induced extracellular signal-regulated kinase phosphorylation was further up-regulated by CEP-11004. ET-1 increased activator protein-1 binding activity 3.5-fold and GATA-binding protein 4 (GATA-4) binding activity 1.8-fold, both of which were attenuated with CEP-11004 administration by 59% and 63% respectively. Phenylephrine induced activator protein-1 binding activity by 2.6-fold, which was decreased by 81% with CEP-11004 administration. Phenylephrine also induced a 3.7-fold increase in the transcriptional activity of B-type natriuretic peptide (BNP), which was attenuated by 41% with CEP-11004 administration. In agreement, MLK inhibition also reduced hypertrophic agonist-induced secretion of immunoreactive atrial natriuretic peptide and BNP.
Conclusions and implications: These results showed that inhibition of the MLK1–3 signalling pathway was sufficient for suppressing the activity of key nuclear effectors (GATA-4 and activator protein-1 transcription factors) in cardiac hypertrophy, and attenuated the agonist-induced atrial natriuretic peptide secretion and activation of BNP gene transcription.
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Keywords: MLK; CEP-11004; GPCR agonists; cardiac hypertrophy; GATA-4; AP-1

Abbreviations: ANP, atrial natriuretic peptide; AP-1, activator protein-1; BNP, B-type natriuretic peptide; DMEM, Dulbecco's modified Eagle's medium; Elk-1, ETS-like gene-1 transcription factor; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; FBS, fetal bovine serum; GATA-4, GATA-binding protein 4; GPCR, G protein-coupled receptor agonist; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MLK, mixed-lineage kinase; ZAK, zipper sterile α-motif kinase

Introduction

An increase in haemodynamic load induces cardiac gene expression via activation of ion channels and activation of paracrine/autocrine mechanisms. A number of signalling pathways have been implicated in the regulation of stress-induced cardiomyocyte growth, including G protein-coupled receptors agonist (GPCR), receptor tyrosine kinases, protein kinase C, calcineurin and members of the mitogen-activated protein kinase (MAPK) signalling cascade (Heineke and Molkentin, 2006). The GPCR agonists, such as angiotensin II, endothelin-1 (ET-1) and α-adrenoceptor agonists such as phenylephrine, activate intracellular pathways by binding to 7-transmembrane-spanning receptors coupled to heterotrimeric G proteins of the G_q class. This G_q signalling has been shown to be a critical transducer of cardiac hypertrophy, as expression of a peptide that blocks G_q signalling completely abolishes pressure overload-induced cardiac hypertrophy (Akhter *et al.*, 1998). G_q signals to MAPKs, which consist of a hierarchy of kinases that ultimately phosphorylate and activate one of the three main terminal effector kinases: p38 MAPKs, c-Jun N-terminal kinases (JNKs) or extracellular

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signal-regulated kinases (ERKs) (Sugden, 2003b). These terminal effector kinases are each regulated by reversible dual phosphorylation of adjacent Tyr and Thr residues (Sugden and Clerk, 1998; Kyriakis and Avruch, 2001).

The mixed-lineage kinases (MLKs) are a family of serine/threonine protein kinases, which act upstream of MAPKs. They can be divided into three subgroups, MLK1–4, dual-leucine-zipper bearing kinases and zipper sterile α-motif kinase (ZAK) (see Gallo and Johnson, 2002). Phosphorylation of MLKs has been shown to activate MAPK kinases (MKKs) 3, 4, 6 and 7. MKK4 and MKK7 activate the JNK pathway, whereas MLK3 has been shown to activate p38 MAPK through MKK3 and MKK6 (see Kyriakis and Avruch, 2001). Indolocarbazole analogues CEP-1347 and CEP-11004 are ATP competitive inhibitors of the MLK family of kinases, which inhibit the activation of the JNK pathway and, consequently, the cell death in many cell culture and animal models of neuronal cell death (Ganguly *et al.*, 2004; Wang *et al.*, 2004; Mishra *et al.*, 2007). The possible importance of the JNK pathway in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases provides a rationale for the use of CEP-1347 and CEP-11004 for the treatment of these diseases. Although CEP-1347 has already been in clinical trials for treatment of Parkinson's disease, almost nothing is known about the role of MLKs in cardiac biology or pathology.

To address whether MLKs could serve as a potential target for inhibiting the stress response in cardiac myocytes, we studied the effects of CEP-11004 on activation of downstream targets in response to exposure to GPCR agonists. Our results demonstrated that MLK inhibition had contrasting effects on GPCR-induced activation of MAPK pathways and attenuated the GPCR-induced stress response in cardiac myocytes.

Methods

Oligonucleotides and plasmids

Oligonucleotides were from Sigma-Genosys (Cambridgeshire, UK). Rat B-type natriuretic peptide (BNP) promoter-driven reporter plasmid was constructed by subcloning p(Δ–534/+4)BNP in pGL3 Basic background (Promega, Madison, WI, USA), referred here as –534rBNP. The double-stranded synthetic oligonucleotides for electrophoretic mobility shift assay (EMSA) were as follows (overhangs are in italics) 5′-*TGTG*TCTGATAAATCAGAGATAACCCCACC-3′ and 5′-*GGAA*GTGTTTTTGATGAGTCA*CCCCA*-3′ for the -90 GATA (rBNP-90 GATA) and -373 activator protein (AP)-1 (rBNP-373 AP-1) binding sites of the rat BNP promoter respectively.

Cell culture

All animal care and experimental procedures were approved by the Animal Use and Care Committee of the University of Oulu. Neonatal rat ventricular myocytes were prepared from 2–4-day-old Sprague-Dawley rats (Kerkelä *et al.*, 2002). Transfection, when designated, was performed on the second day of the cell culture. Plasmid transfection was carried out by exposing the cells to 3 μL of FuGENE 6 and 1.5 μg of DNA per millilitre of complete serum free medium for 6 h. To control the transfection efficiency, luciferase reporter plasmids were cotransfected with RSV-β-gal plasmids at the ratio of 2:1. Subsequently, the cells were washed twice with Dulbecco's modified Eagle's medium (DMEM)/F-12 and cultured thereafter in complete serum free medium as described above. The reporter gene activities were measured by using Luciferase Assay System (Promega) and Luminescent β-galactosidase Detection Kit II (BD Biosciences Clontech, Palo Alto, CA, USA) with Luminoskan RS luminometer (ThermoLabsystems, Vantaa, Finland). Luciferase activity levels were 14.1 ± 1.5 (average ± SEM)-fold higher than those of β-galactosidase activity. Both luciferase and β-galactosidase levels were at the linear range of the assay measurement scale.

Total protein extraction and Western blotting (immunoblotting)

The cells were lysed in ice-cold lysis buffer [20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton-X100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4] supplemented with 20 $\mu g \cdot mL^{-1}$ leupeptin, 2 $\mu g \cdot mL^{-1}$ pepstatin, 20 $\mu g \cdot mL^{-1}$ aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, 1 mM dithiothreitol (DTT), 6 $\mu g \cdot mL^{-1}$ L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) and 6 $\mu g \cdot mL^{-1}$ 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK). The lysate was cleared by 10 min centrifugation at +4°C, and the supernatant was transferred in a new tube as total protein extract. Total protein extracts were boiled in Laemmli buffer, resolved by SDS-PAGE and transferred to Optitran BA-S 85 nitrocellulose membranes (Schleicher & Schuell GmbH, Dassel, Germany). The membranes were blocked in 5% non-fat milk and then incubated with the appropriate antibody in 1% milk in Tris-buffered saline – 0.05% Tween 20. Antibody concentration in the dilution varied from 1:500 to 1:2000, depending on the signal strength. Antibody binding was detected with HRP-linked anti-rabbit or anti-mouse IgG at a 1:2000 dilution and revealed using ECLPlus reagents as described by the manufacturer.

c-Jun N-terminal kinase assay

Phospho c-Jun kinase assay was performed with SAPK/JNK non-radioactive assay kit (Cell Signalling Technology Inc.) according to the manufacturer's protocol. Briefly, a c-Jun fusion protein linked to agarose beads was used to pull down JNK enzyme from 250 μg of total protein extracts. The pellet was suspended in kinase buffer supplemented with 100 μM ATP and incubated for 30 min at 30°C. The reaction was terminated by boiling in SDS sample buffer. Phospho-c-Jun (Ser63) antibody was then used to measure JNK activity by immunoblotting.

Nuclear protein extraction and EMSA

Nuclear extracts from cardiac myocytes were prepared as described (Pikkarainen *et al.*, 2003a). Cells were washed and scraped with phosphate-buffered saline. After centrifugation, cells were resuspended in 400 μL of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, supplemented with 1 mM Na_3VO_4, 50 mM NaF, 20 $\mu g \cdot mL^{-1}$ leupeptin, 20 $\mu g \cdot mL^{-1}$ pepstatin, 20 $\mu g \cdot mL^{-1}$ aprotinin, 2 mM

benzamidine, 0.5 mM PMSF, 1 mM DTT, 3 μg·mL^{-1} TPCK and 3 μg·mL^{-1} TLCK) with gentle pipetting and allowed to swell on ice for 15 min. Cell membranes were then lysed by adding 25 μL of 10% Nonidet P-40 per sample and vortexing vigorously for 15 s followed by a 30 s centrifugation (16 600× *g*). The supernatant was removed, and the pellet containing the nuclear fraction was then resuspended in 25 μL of ice-cold buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA and 1 mM EGTA, with similar supplements as in buffer A) and rocked on ice for 15 min. The samples were centrifuged for 5 min at 16 600× *g*. The supernatant was saved as the nuclear fraction. The entire procedure described above was carried out at 4°C. Nuclear protein concentrations were colorimetrically measured (BioRad Laboratories). Double-stranded oligonucleotide probes were sticky-end-labelled with [α^{32}P]-dCTP by Klenow fragment. Binding reactions contained 3–6 μg of nuclear protein and 2 μg of poly-(dI-dC)•(dI-dC) in a buffer consisting of 16 mM HEPES, pH 7.9, 120 mM NaCl, 1 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 0.7 mM EDTA, 0.3 mM EGTA, 8% glycerol, 0.02% Nonidet P-40, 0.25 mM PMSF and 1 μg·mL^{-1} of each aprotinin, leupeptin and pepstatin. Reaction mixtures were incubated at room temperature with a labelled probe for 20 min followed by gel-electrophoresis (250 V, for 1.5–2 h) on 5% polyacrylamide gel. Gels were dried for 60 min and the DNA/protein complexes were exposed with PhoshorImager screens (Amersham Biosciences Inc.). The screens were scanned with Molecular Imager FX Pro Plus and analysed with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Radioimmunoassay for atrial natriuretic peptide (ANP) and BNP
Secretion levels of ANP and BNP were measured from samples of cell culture incubation medium with radioimmunoassay as previously described (Pikkarainen *et al*., 2003b)

Statistical analysis
All the results are expressed as means ± SEM. To determine the statistical difference between two groups, Student's *t*-test was used. For multiple comparisons, data were analysed with one-way analysis of variance followed by a least significant difference *post hoc* test. Differences at or above the 95% level were considered statistically significant.

Materials
CEP-11004 MLK1–3 inhibitor was a gift from Cephalon Inc. (West Chester, PA, USA). ET-1 and phenylephrine were from Sigma-Aldrich Co. (St. Louis, MO, USA). Specific antibodies against Phospho-p44/42 MAPK, SAPK/JNK, p44/42 MAPK, p38 MAPK and non-radioactive SAPK/JNK Assay Kit, as well as HRP-linked Anti-Rabbit IgG and Anti-Mouse IgG were purchased from Cell Signalling Technology Inc. (Beverly, MA, USA). Phospho-p38 antibody was obtained from Chemicon International (Temecula, CA, USA). Specific antibody against phospho-JNK (G-7, sc-6234) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FuGENE 6 transfection reagent was from Roche Molecular Biochemicals (Indianapolis, IN, USA). ECL Plus reagents, poly-(dI-dC)•(dI-dC) and Hyperfilm MP were obtained from Amersham Biosciences Inc. (Bucks, UK). Cell culture reagents were from Sigma-Aldrich Co. Receptor nomenclature follows Alexander *et al.* (2008)

Results

Mixed-lineage kinase 1–3 inhibition by CEP-11004 has opposing effects on mitogen-activated protein kinase activity
Mixed-lineage kinase 1–3 acts upstream of the MAPKs, and previous studies have implicated MLKs in the activation of ERK, JNK and p38 pathways (Chadee and Kyriakis, 2004a; Chadee *et al*., 2006). We first sought to determine the effect of CEP-11004 administration on the activity of the MAPKs in cultured neonatal rat ventricular myocytes. Cell were stimulated with hypertrophic agonists ET-1 or phenylephrine, both known to activate all three main MAPK pathways – ERK, JNK and p38 MAPK – in this experimental system (Yue *et al*., 2000; Liang *et al*., 2001; Sugden, 2003a). As observed in previous studies, JNK was activated 15 min after treatment with ET-1 or phenylephrine as measured by non-radioactive kinase assay employing c-Jun fusion protein as a substrate (data not shown). Administration of CEP-11004 dose-dependently inhibited ET-1- or phenylephrine-induced phosphorylation of c-Jun, and sub-maximal inhibition was seen with 100 nM CEP-11004 (Figure 1). We therefore chose to use the 100 nM concentration in the subsequent studies. To further study the specificity of MLK inhibition we assessed the effect of CEP-11004 on ET-1- or phenylephrine-induced p38 MAPK and ERK activation by using Western blot analysis. Phosphorylation of p38 MAPK and ERK was studied 15 min after treatment with ET-1 or phenylephrine. Administration of CEP-11004 completely blocked both ET-1- or phenylephrine-induced activation of p38 MAPK (Figure 2). In contrast, CEP-11004 significantly potentiated ERK phosphorylation induced by either ET-1 or phenylephrine (Figure 3).

MLK1–3 inhibition modulates the GPCR agonist-induced activation of central nuclear effectors of MAPK signalling pathways
To study the effect of MLK1–3 inhibition on potential nuclear targets of MAPKs, DNA binding activity of AP-1 and GATA-binding protein 4 (GATA-4) transcription factors were studied. Gel mobility shift analyses revealed that 1 h ET-1 treatment significantly increased both AP-1 and GATA-4 DNA binding activities, which were reduced by CEP 11004 (Figure 4). Phenylephrine-induced AP-1 binding activity was also markedly activated after 1 h. This effect was completely abolished by CEP-11004 (Figure 4). CEP-11004 treatment slightly increased the phenylephrine-induced GATA-4 DNA binding activity, but that was not statistically significant (data not shown). Administration of CEP-11004 alone had no significant effect on either AP-1 or GATA-4 DNA binding activity (data not shown). The specificity of GATA-4 and AP-1 binding was confirmed by competition and supershift analysis (Figure S1). Interestingly, CEP-11004 treatment had no effect on GPCR agonist-induced ETS-like gene-1 transcription factor (Elk-1) binding activity or nuclear factor κ-B binding activity (data not shown).

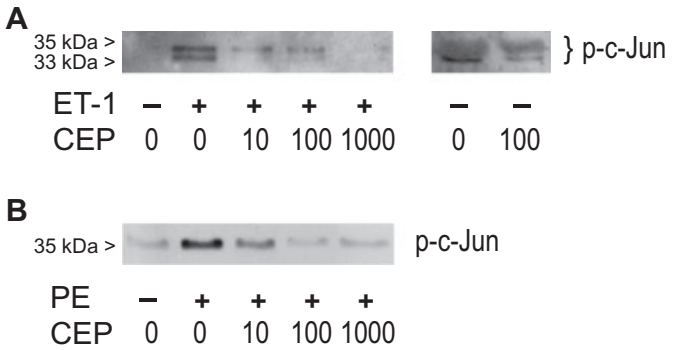

Figure 1 Effect of CEP-11004 administration on c-Jun phosphorylation. Effect of CEP-11004 on c-Jun N-terminal kinase (JNK) activity was studied by measuring the phosphorylation of JNK's main target, c-Jun as described in *Methods*. Myocytes were treated with hypertrophic agonists; either endothelin-1 (ET-1) or phenylephrine (PE) alone or in combination with CEP-11004, for 15 min and phospho-c-Jun (p-c-Jun) formation was measured by Western blot analysis. (A) CEP-11004 dose-dependently decreases ET-1-induced phosphorylation of c-Jun. Sub-maximal inhibition is seen with 100 nM of CEP-11004. CEP-11004 treatment alone has no effect on basal c-Jun phosphorylation. (B) CEP-11004 dose-dependently attenuates PE-induced increase in c-Jun phosphorylation. (A,B) Panels are representative of three separate experiments.

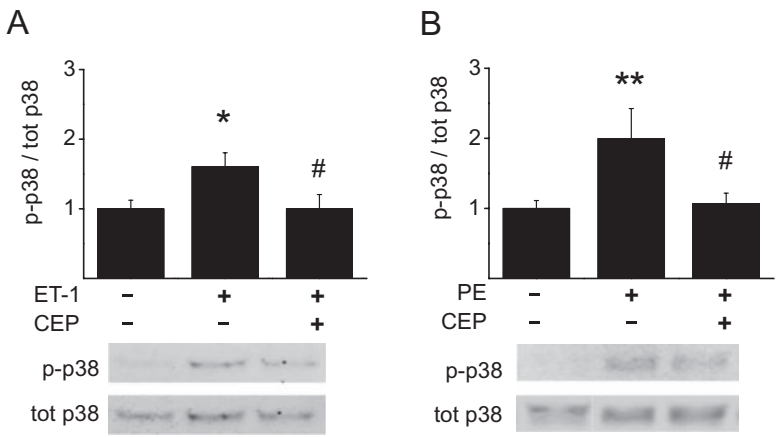

Figure 2 The effect of CEP-11004 administration on p38 mitogen-activated protein kinase (MAPK) phosphorylation. The effect of mixed-lineage kinase (MLK) inhibition on p38 MAPK activity was studied by measuring the phosphorylation of p38 MAPK by Western blot analysis. Myocytes were pretreated with 100 nM CEP-11004 or vehicle for 4 h before treatment with endothelin-1 (ET-1) or phenylephrine (PE) alone or in combination with CEP-11004 for 15 min. (A) Activation of p38 MAPK by ET-1 is completely blocked by MLK1–3 inhibition with CEP-11004. (B) Administration of CEP-11004 abolishes PE-induced activation of p38 MAPK. Representative Western blots are shown below. Results are the mean ± SE, $n = 7$–11. $^{*}P < 0.05$; $^{**}P < 0.01$ versus non-treated control; $^{\#}P < 0.05$ versus ET-1 or PE treatment.

MLK inhibition attenuates the GPCR agonist-induced secretion of hypertrophic markers

Atrial natriuretic peptide and BNP are well-established phenotypic markers of cardiac hypertrophy and have previously been shown to be targets for GATA-4 and AP-1 (Ruskoaho, 1992; Omura *et al.*, 2002; Pikkarainen *et al.*, 2003b). Myocytes were subjected to either ET-1 or phenylephrine for 24 h. Inhibition of MLK1–3 by CEP-11004 significantly attenuated ET-1-induced secretion of BNP and completely blocked that of ANP (Figure 5). Similarly, CEP-11004 attenuated the phenylephrine-induced increase in BNP and ANP secretion (Figure 5). MLK inhibition alone had no significant effect on the release of either natriuretic peptide (Figure 5).

Administration of CEP-11004 affects the phenylephrine-induced transcriptional activity of the BNP promoter

Previous studies have indicated that the proximal 5′-flanking region of 534 bp is sufficient to confer induction by ET-1 and phenylephrine (Thuerauf and Glembotski, 1997; Pikkarainen *et al.*, 2003a) and contains consensus binding sites for several transcription factors associated with cardiac transcription factors, including GATA-4 and AP-1 (Tokola *et al.*, 2001). To study whether the changes observed in the natriuretic peptide secretion reflected altered transcriptional activity of BNP, cardiac myocytes were transfected with rat (Δ-534bp/+8bp) BNP promoter-driven luciferase reporter constructs. Exposure to phenylephrine for 24 h resulted in 3.7-fold increase in the transcriptional activity of the BNP promoter. Importantly, CEP-11004 reduced phenylephrine-induced BNP reporter activity by 41% without affecting the reporter activity when administered alone (Figure 6).

Discussion

The MLK family comprises seven different MLKs, which are clustered in three subfamilies on the basis of domain

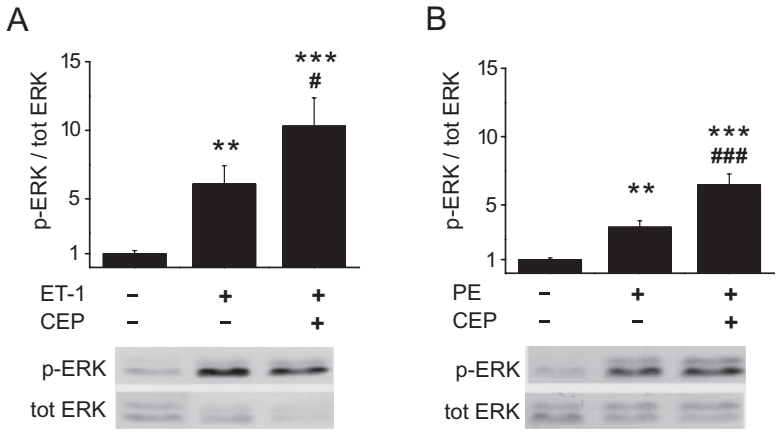

Figure 3 The effect of mixed-lineage kinase inhibition on extracellular signal-regulated kinase (ERK) phosphorylation. Phosphorylation of ERK was measured by Western blot analysis to study the effect of mixed-lineage kinase inhibition on ERK activity. Myocytes were pretreated with 100 nM CEP-11004 or vehicle for 4 h before treatment with endothelin-1 (ET-1) or phenylephrine (PE) alone or in combination with CEP-11004 for 15 min. (A) ET-1 induces an increase in ERK phosphorylation, which is further enhanced by administration of CEP-11004. (B) PE exposure induces ERK phosphorylation, which is further increased by treatment of cell with CEP-11004. Representative Western blots are shown below. Results are the mean ± SE, $n = 7$–11. $^{**}P < 0.01$; $^{***}P < 0.001$ versus non-treated control; $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ versus ET-1 or PE treatment.

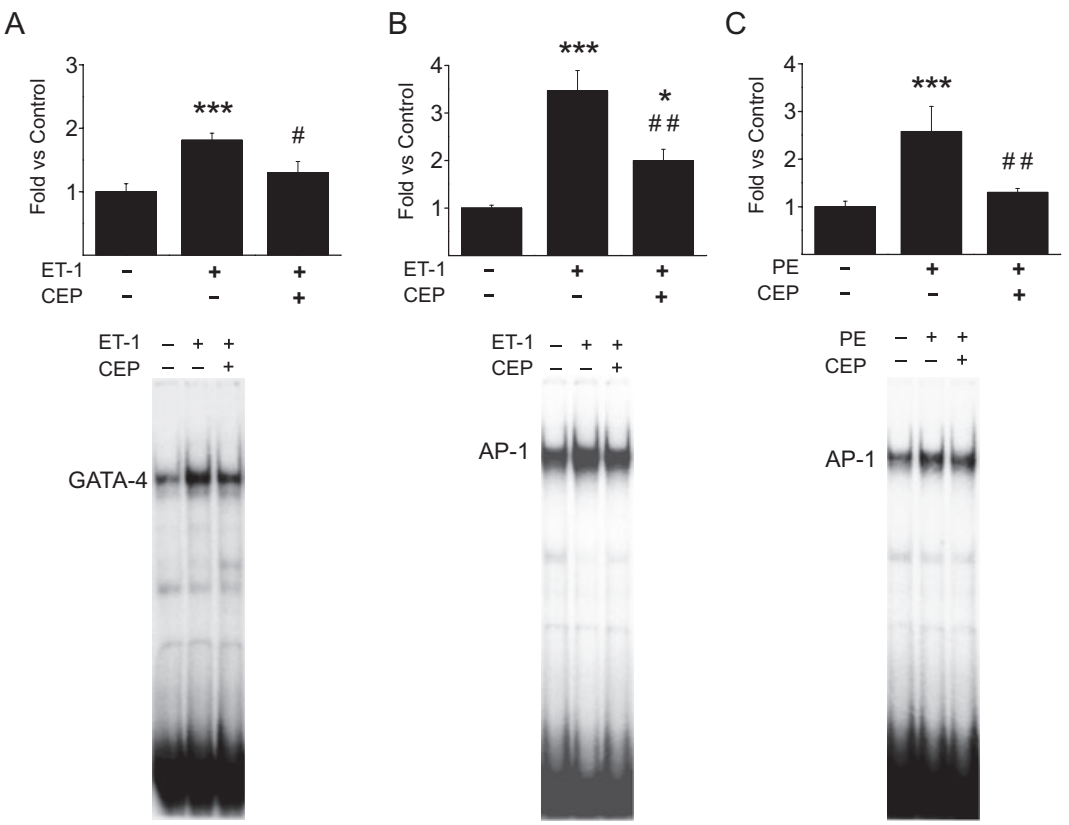

Figure 4 The effect of mixed-lineage kinase inhibition on transcription factor GATA-4 or activator protein-1 (AP-1) DNA binding activity. Myocytes were pretreated with 100 nM CEP-11004 or vehicle for 4 h prior to treatment with endothelin-1 (ET-1) or phenylephrine (PE) alone, or in combination with CEP-11004 for 1 h. The nuclear proteins were extracted and binding reactions containing 3 μg of extracts were probed with radiolabelled rBNP-90 GATA or rBNP-373 AP-1 probe and subjected to electrophoretic mobility shift assay (EMSA) as described in *Methods*. (A) ET-1-induced GATA-4 transcription factor DNA binding activity is significantly inhibited by CEP-11004. (B) CEP treatment inhibits ET-1-induced increase in AP-1 transcription factor DNA binding activity. (C) PE-induced AP-1 binding activity is significantly attenuated by CEP-11004 treatment. Representative EMSAs are shown below. Results are the mean ± SE, $n = 7$–16. $^{*}P < 0.05$; $^{***}P < 0.001$ versus non-treated control; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ versus ET-1 or PE treatment.

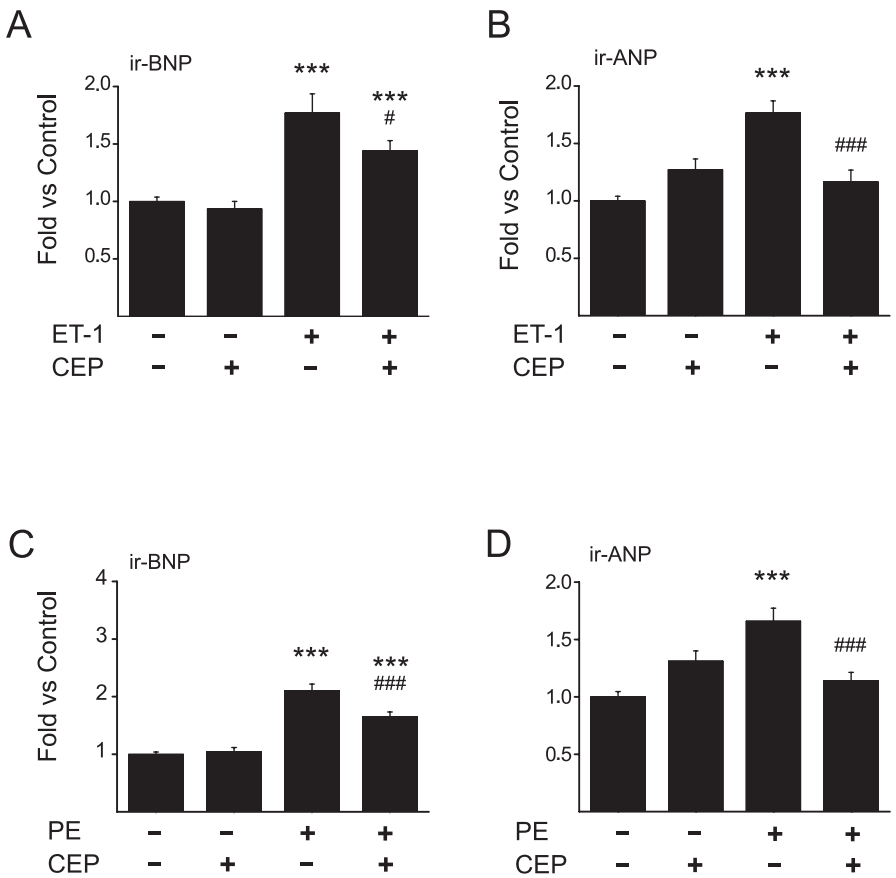

Figure 5 The effect of mixed-lineage kinase inhibition on atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) secretion. Myocytes were pretreated with 100 nM CEP-11004 or vehicle for 4 h before exposure to the hypertrophic agonists endothelin-1 (ET-1) or phenylephrine (PE) for 24 h. The natriuretic peptide levels in the incubation medium were measured by radioimmunoassay. (A) CEP-11004 treatment reduces the ET-1-induced secretion of BNP. (B) ET-1-induced ANP secretion is attenuated by treatment with CEP-11004. (C) The increase in BNP secretion stimulated by PE is attenuated by treatment with CEP-11004. (D) CEP-11004 treatment reduces the PE-induced increase in ANP secretion. Results are the mean ± SE, $n = 11–22$. ***$P < 0.001$ versus non-treated control; $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ versus ET-1 or PE treatment.

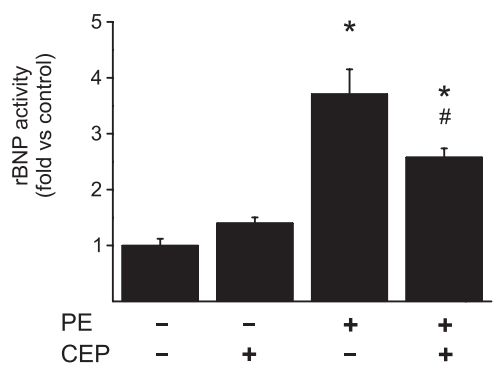

Figure 6 The effect of mixed-lineage kinases 1–3 inhibition on rat B-type natriuretic peptide (BNP) promoter transcription. Luciferase reporter construct containing (Δ-534 bp/+8 bp) of the rat BNP promoter was cotransfected with RSV-β-Gal-plasmid into neonatal rat ventricular myocytes. Myocytes were incubated for 4 h with 100 nM CEP-11004 or vehicle before 24 h treatment with 50 μM phenylephrine (PE) alone or in combination with CEP-11004. CEP-11004 significantly inhibits the PE-induced activation of the BNP promoter (rBNP), but has no effect on the basal transcriptional activity. Results are the mean ± SE, $n = 8–10$. *$P < 0.001$ versus non-treated control; $^{\#}P < 0.01$ versus PE treatment.

arrangements and sequence homology within their catalytic domains: MLK1–4, the dual-leucine-zipper bearing kinases and ZAK, also referred to as MLK7 (Gallo and Johnson, 2002). Among these, MLK3 has been shown to be expressed throughout postnatal development in rat ventricles at protein level (Kim *et al.*, 1998), and ZAK mRNA can be found in human heart (Bloem *et al.*, 2001). However, it is unclear to which extent the other MLKs are expressed in cardiac tissue. Furthermore, the data available about the functional role of MLKs in the heart is limited to ZAK. Overexpression of MLK7 activates protein synthesis and fetal gene expression in cultured cardiomyocytes and cardiac-specific overexpression of MLK7 results in cardiac hypertrophy and fibrosis with impaired cardiac function in transgenic mice (Bloem *et al.*, 2001; Christe *et al.*, 2004). The present study is the first one to investigate the role of MLK1–3 in cardiac hypertrophy or biology. Our results demonstrate the distinct effects of MLK1–3 inhibition on the GPCR agonist-induced activity of JNK and p38 MAPK pathways compared with that of the ERK pathway, leading to reduced activation of GATA-4 and AP-1 transcription factors, the key nuclear effectors in cardiac hypertrophy.

In vitro kinase screening has shown CEP-11004 to be a fairly selective inhibitor of MLK1–3 with IC_{50} values ranging from 31 ± 17 to 89 ± 14 nM, over 10-fold less than for protein kinase C and more than 100-fold less than for NGF high-affinity receptor tyrosine kinase (Murakata *et al.*, 2002). In human neuroblastoma SH-SY5Y cells, CEP-11004 at 100 nM totally inhibited 1-methyl-4-phenylpyridinium ion (MPP^+)-induced phosphorylation of JNK (Murakata *et al.*, 2002). Our results in cultured neonatal rat cardiac myocytes are consistent with these findings, as both ET-1- and phenylephrine-induced phosphorylation of c-Jun, the main target of JNK, were inhibited by 100 nM CEP-11004. Inhibition of MLKs by 300 nM CEP-11004 was shown to block lipopolysaccharide-stimulated activation of JNK and p38 MAPK in cultured human THP-1 monocytes (Ciallella *et al.*, 2005). In agreement with these observations, in the present study CEP-11004 attenuated the GPCR agonist-induced p38 MAPK activation, albeit with at a lower concentration.

Although MLKs were initially thought to be specific for the JNK pathway (Kyriakis and Avruch, 2001), several studies have shown that MLK3, and possibly some other MLKs, are able to activate p38 MAPK pathway via MKK3/MKK6 in mammalian cells (see Gallo and Johnson, 2002). Silencing of MLK3 by using RNA interference strategy showed that MLK3 is required for mitogen- and cytokine-induced activation of all three MAPK pathways, including ERK, in several continuous cell lines as well as in primary endothelial cells (Chadee and Kyriakis, 2004b). On the other hand, MLK3 has been shown to have a non-catalytic function in maintaining the $G\alpha_q$/p63RhoGEF complex, thereby limiting $G\alpha_q$ signalling to Rho (Swenson-Fields *et al.*, 2008). This provides a plausible explanation for the findings by Brancho *et al.* (2005), who noted an increase in p38 phosphorylation upon tumour necrosis factor-α stimulation in MLK3 null cells. Interestingly, phosphorylation of MLK3 reduces its affinity for the $G\alpha_q$/p63RhoGEF complex (Swenson-Fields *et al.*, 2008). Further, MLK3-mediated activation of JNK induces a self-stimulatory loop leading to increased phosphorylation of MLK3 (Schachter *et al.*, 2006). It is therefore plausible that MLK3 inhibition by a competitive antagonist of ATP would also result in reduced MLK3 phosphorylation, thereby affecting also its non-catalytic functions.

It appears that MLK3, independent of its kinase activity, is necessary for ERK activation, most likely by supporting the maintenance of the B-Raf/Raf-1 complex enabling B-Raf/Raf-1 transactivation (Wan *et al.*, 2004; Chadee *et al.*, 2006). Although the exact mechanism is not known, a previous study found that expression of active MLK3 rendered ERK resistant to activation by growth factors and mitogens (Shen *et al.*, 2003). This restriction in ERK activation was independent of activity of upstream activators of MEK–ERK pathway (i.e. Raf-1), but required active MLK3, and inhibition of JNK pathway signalling by dominant negative MKK4/MKK7, the JNK inhibitor I or by CEP-11004 reversed this restriction (Shen *et al.*, 2003). Thus, it is plausible that the observed increase in GPCR agonist-induced ERK activation during MLK3 inhibition by the ATP competitive antagonist CEP-11004 is due to the decrease in JNK activity, which results in attenuation of MLK3 activity thereby releasing the resistance in ERK signalling.

Activator protein-1 is a ubiquitous transcription factor, which consists of homo- and heterodimers of Jun, Fos, ATF (activating transcription factor) or MAF (musculoaponeurotic fibrosarcoma) family members (Angel and Karin, 1991; Eferl and Wagner, 2003). AP-1 is activated by environmental stress, radiation, cytokines and growth factors, stimuli that also activate JNKs (Gallo and Johnson, 2002; Eferl and Wagner, 2003). MLKs phosphorylate and activate MKKs, such as MKK4 and MKK7 (Kyriakis and Avruch, 2001), which, in turn, activate JNKs. Previously, both ERK and JNK pathways have been shown to positively regulate AP-1 binding activity in neonatal rat cardiac myocytes, whereas inhibition of p38 MAPK had no effect (Santalucia *et al.*, 2003; Shyu *et al.*, 2004). Our observation that GPCR agonist-induced increase in AP-1 binding activity was attenuated by CEP-11004, despite the coincident potentiation of ERK activity, emphasizes the role of JNK rather than ERK as a downstream effector of $G\alpha_q$ on AP-1 transcription factor activity.

GATA-4 is a cardiac specific transcription factor that is necessary for cardiac development and cardiomyocyte proliferation (Zeisberg *et al.*, 2005) and regulates cardiac hypertrophy in concert with nuclear factor of activated T-cells family members in the postnatal heart (Molkentin *et al.*, 1998). GATA-4 DNA binding activity is regulated by ERK and by p38 MAPK-mediated phosphorylation of serine residues of GATA-4 (Morimoto *et al.*, 2000; Liang *et al.*, 2001; Kerkelä *et al.*, 2002). The observed attenuation of GATA-4 binding activity by CEP-11004 in our study suggests a central role for p38 MAPK in $G\alpha_q$-mediated GATA-4 activation. Interestingly, in a previous study from our laboratory, CEP-11004 had no effect on left ventricular stretch-induced increase in GATA-4 DNA binding activity (Tenhunen *et al.*, 2004). Our findings concerning the observed trend for CEP-11004 to increase the phenylephrine-induced GATA-4 DNA binding activity are in agreement with previous findings that ERK activation is both sufficient to phosphorylate GATA-4 and necessary for phenylephrine-induced GATA-4 activation (Morimoto *et al.*, 2000; Liang *et al.*, 2001). We also found that CEP-11004 had no effect on GPCR agonist-induced activation of Elk-1 binding activity. Elk-1 is phosphorylated by ERK, JNK and p38 MAP kinase (Whitmarsh *et al.*, 1995; Price *et al.*, 1996), but it appears that in cardiomyocytes attenuation of JNK and p38 by CEP-11004 is not sufficient to affect Elk-1 activity, perhaps due to simultaneous increase in ERK activity.

In summary, we characterize for the first time the detailed effects of MLK1–3 inhibition in cardiac myocytes. Our results indicate that MLK1–3 are required for the GPCR agonist-induced activation of p38 MAPK and JNK pathways, whereas GPCR agonist-induced ERK activity is further potentiated by MLK1–3 inhibition. We show that MLK1–3 mediate the ET-1- and phenylephrine-induced activation of key nuclear effectors in cardiac hypertrophy, GATA-4 and AP-1, and expression and secretion of their targets, ANP and BNP. Whether the effects of MLK inhibition extend to other aspects of cardiac pathology provides an intriguing target for future studies.

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Conflicts of interest

The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Electrophoretic gel mobility shift assays depicting specific binding of GATA-binding protein 4 (GATA-4) and activator protein (AP)-1 to corresponding DNA binding sites in rat B-type natriuretic peptide (BNP) promoter. Nuclear protein extract from rat cardiomyocytes was incubated with double-stranded oligonucleotides containing binding sites for (A) GATA-4 and (B) AP-1. Competition analyses indicate that GATA-4 and AP-1 binding activities were inhibited by unlabelled oligonucleotides (lanes 3 and 4). An antibody against either GATA-4 or JunD was added in the reaction in the fifth lanes. No protein was present in the binding reaction in the first lanes. n.s., non-significant.

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