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Differential roles of MAPKs and MSK1 signalling pathways in the regulation of c-Jun during phenylephrine-induced cardiac myocyte hypertrophy

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Abstract Gq-protein-coupled receptor (GqPCR) signalling is associated with the induction of cardiac myocyte hypertrophy, which is characterized by an increase in expression of immediate early genes via activation of pre-existing transcription factors. Here, we explore the role of MSK1 and MAPK signalling pathways in the regulation of the immediate early gene c-*jun*. The results provide further support for the role of MSK1 in cardiac myocyte hypertrophy and indicate that PE activates distinct signalling mechanisms which culminate with a complex activation of c-*jun*. ERK1/2 and JNKs are the principal kinases responsible for phosphorylation of c-Jun, whereas c-*jun* mRNA and protein up-regulation by PE is mediated by multiple signalling pathways that include MSK1, ERK1/2, p38-MAPK and JNKs. These signalling mechanisms seem to be critical to the phenotypic changes of cardiac myocytes in response to hypertrophic stimulation.

Keywords Adult cardiac myocytes · Hypertrophy · Immediate early gene · MSK1 · MAP kinases · c-Jun

Introduction

Myocardial hypertrophy is an important adaptational response that allows the heart to maintain or increase its haemodynamic output when there is a requirement for increased workload and it is characterized by changes in myocardial phenotype and gene expression. These include a transient expression of immediate early genes (e.g. c-*jun,* c-*fos* and *egr-1*), recapitulation of "fetal" pattern of gene expression, up-regulation of genes encoding constitutively expressed contractile proteins and increased cell size. The mechanisms that regulate hypertrophy are not fully understood but it is likely that the response is mediated by autocrine/paracrine effects of locally released neurohumoral factors such as the vasoactive peptide endothelin-1 (ET-1) and α_1-adrenergic agonists [1].

A number of studies have shown that the three best-characterized mitogen-activated protein kinase (MAPK) subfamilies [extracellular signal-regulated kinases 1/2 (ERK1/2), the c-Jun N-terminal kinases (JNKs) and p38-MAPKs] are activated by hypertrophic Gq-protein-coupled receptor (GqPCR) agonists in isolated perfused hearts, neonatal or adult rat cardiac myocytes [2–6], and they have all been implicated in the hypertrophic response [1]. However, the hypertrophic effect of MAPKs is still controversial and their downstream effectors for cardiac hypertrophy remain to be fully determined. We have previously reported that mitogen and stress activated kinase 1 (MSK1), a well-known target of MAPKs [7], is activated in response to phenylephrine (PE) in adult rat cardiac myocytes and that this effect requires activation of both ERK1/2 and p38-MAPK [6]. Furthermore, we have demonstrated that these signalling pathways contribute to transcriptional changes associated with hypertrophy by activating the transcription factor CREB (cAMP responsive element binding protein) and up-regulating atrial natiuretic factor (ANF) [8].

c-Jun protein can homodimerize or heterodimerize with other Jun, Fos or ATF members to form transcriptionally active activator protein-1 (AP-1) complexes [9]. The c-*jun* promoter region contains potential binding sites for several

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transcription factors including SP1, CTF (CCAAT Transcription Factor) and AP-1 itself [10]. A key regulatory component in the post-translational function of the c-Jun is the phosphorylation on residues Ser-63 and Ser-73, located within its N-terminal transactivation domain. N-terminal phosphorylation potentiates its transactivation properties and correlates with the transcriptional activation of AP-1 promoters [10]. Although ERK1/2 was shown originally to phosphorylate c-Jun in vitro at Ser-63 and Ser-73, it subsequently became clear that c-Jun is preferentially phosphorylated at these sites by JNKs [11]. However, the molecular mechanisms acting as upstream mediators of c-Jun activation in cardiac myocytes have not been fully characterized.

The aim of the present study was to explore further the signalling pathways involved in c-Jun regulation in adult rat cardiac myocyte hypertrophy induced by PE. Our results point to a differential role of MAPKs and MSK1 pathways in this response. ERK1/2 and JNKs are the principal kinases responsible for phosphorylation of c-Jun, whereas c-*jun* mRNA and protein up-regulation by PE is mediated by multiple signalling pathways that include MSK1, ERK1/2, p38-MAPK and JNKs. These signalling mechanisms seem to be critical to the phenotypic changes of cardiac myocytes in response to hypertrophic stimulation.

Materials and methods

Animals

Male Wistar rats weighing between 250 and 300 g were used in this study and received proper care in compliance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the US National Academy of Sciences and published by the National Institute of Health. Animals were anaesthetized with sodium pentothione (100 mg/kg body weight) intraperitoneally.

Isolation of adult rat cardiac myocytes and culture

Ventricular myocytes were isolated from adult male Wistar rats by cardiac retrograde aortic perfusion and collagenase treatment as described previously [8]. Cells were transferred to M199 culture medium, supplemented with 100 U penicillin/streptomycin, 25 mM HEPES and 10% fetal bovine serum and plated at a density $2 \times 10^3/mm^2$ in culture dishes precoated with laminin. Four hours after plating, dishes were washed with M199 medium to remove nonattached cells, and attached myocytes were incubated in serum-free medium for 24 h in a 5% CO_2 humidified incubator at 37°C, prior to experimentation. Myocytes were exposed to PE (100 μM) for various times (5–60 min). When necessary, 10 min prior to this treatment, cells were exposed to protein kinase inhibitors: SB903580 (1 μM), PD98059 (10 μM), SP600125 (10 μM) and Ro318220 (5 μM). The optimal concentration of inhibitors was chosen according to our previous studies [6, 8] and preliminary experiments.

RNA preparation, cDNA synthesis and reverse transcriptase-PCR

The expression of endogenous c-*jun* mRNA was determined by reverse transcription of total RNA followed by PCR analysis. Total RNA was extracted using TRI Reagent, according to the manufacturer's instructions. The RNA was resuspended in 0.1% (v/v) diethylpyrocarbamate-treated water and its concentration determined by absorbance at 260 nm. For cDNA synthesis, 1 μg of total RNA was denatured in the presence of 6.75 μg random hexamers in a reaction volume of 13.5 μl at 65°C for 5 min. Reverse transcription was performed with M-MuLV Reverse Transcriptase, first strand buffer, dithiothreitol and deoxy-nucleotide triphosphates (dNTPs). The first strand reaction was incubated at 37°C for 1 h. Termination of the reaction was achieved by inactivation of the reverse transcriptase at 70°C for 5 min. PCR for c-*jun* was performed using *Taq* DNA polymerase with sense 5′-ATG ACTGCAAAGATGGAAACG-3′ and antisense 5′-ATT CTGGCTATGCAGTTCAG-3′ based on the published rat *c-jun* sequence (EMBL accession number **X17215**). After a 10 s denaturation at 94°C, PCR was carried out for 25 cycles (94°C for 30 s, 59°C for 50 s and 72°C for 50 s), and then a final extension was done at 72°C for 4 min. PCR for GAPDH was performed for 22 cycles using the following primers: sense 5′-ACCACAGTCCATGCCATCA C-3′ and antisense 5′-TCCACCACCCTGTTGCTGTA-3′. PCR products were separated on a 2% (w/v) agarose gel supplemented with ethidium bromide (EtBr) at a final concentration of 100 μg/l and bands were captured under UV illumination.

Preparation of whole cell extracts

For western blot analysis of kinases, cardiac myocytes were lysed in ice-cold buffer containing 20 mM β-glycerophosphate, 20 mM HEPES pH 7.5, 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 10 mM benzamidine 5 mM DTT, 0.3 mM PMSF, 0.2 mM leupeptin, 0.01 mM E64 and 1% (v/v) Triton X 100. Samples were extracted on ice (10 min) and centrifuged (4°C, 5 min, 10,000×g). The supernatants (whole cell extracts) were retained and the protein concentration was determined using the BioRad Bradford assay.

Preparation of nuclear extracts

For c-Jun analysis, nuclear extracts were prepared as described previously [12]. Briefly, cells were resuspended in nuclear extraction buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.3 mM Na_3VO_4, 5 mM DTT, 0.01 mM E64, 0.3 mM PMSF, 0.2 mM leupeptin) and incubated on ice for 10 min. Samples were centrifuged (4°C, 5 min, 10,000×*g*) and the supernatants were discarded. Pellets were washed in nuclear extraction buffer A containing 0.1% Nonidet P40, resuspended in nuclear extraction buffer B (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.3 mM Na_3VO_4, 5 mM DTT, 0.01 E64, 0.3 mM PMSF, 0.2 mM leupeptin) and extracted on ice for 1 h with occasional vortex mixing. Samples were centrifuged (4°C, 5 min, 10,000×*g*) and the protein concentration in the supernatant was determined using the BioRad Bradford assay.

Immunoblotting

Samples were boiled with 0.33 vol of SDS-polyacrylamide gel electrophoresis sample buffer (10% SDS (w/v), 13% glycerol (v/v), 300 mM Tris–HCl, pH 6.8, 130 mM dithiothreitol and 0.2% bromophenol blue (w/v). Proteins were separated by SDS-PAGE on 10% acrylamide, 0.275% (w/v) bis-acrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm). Nonspecific binding sites were blocked (30 min at room temperature) with 5% (w/v) nonfat milk powder in TBST Buffer [20 mM Tris–HCl pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20]. Membranes were incubated (overnight, 4°C) with primary antibodies diluted in TBST Buffer containing 1% (w/v) bovine serum albumin, then washed in TBST Buffer (3 × 5 min, room temperature). Primary antibodies were used at the following dilutions: phospho-JNKs 1/1000; JNKs 1/500; phospho-MSK1 1/1000; phospho-c-Jun 1/500; c-Jun 1/1000. Membranes were incubated (60 min, room temperature) with horseradish peroxidase-conjugated secondary antibodies (1/5000) in TBST Buffer containing 1% (w/v) nonfat milk powder, and were then washed in TBST Buffer (3 × 5 min, room temperature). Bands were detected by enhanced chemiluminescence and blots were quantified by scanning densitometry.

Planimetry

Myocytes were either left untreated or exposed to PE (24 h) in the absence or presence of inhibitors. Digital planimetry was used to measure cardiac myocyte area (Scion Image Beta 4.02, Scion Corporation USA), and 100 myocytes were measured for each condition for three independent experiments.

Materials

Phenylephrine, dimethyl sulphoxide, dithiothreitol (DTT), leupeptin, trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane (E64) and phenyl methyl sulphonyl fluoride (PMSF) were obtained from Sigma Chemical Co (St. Louis, MO, USA). Collagenase type II was from Biochrome KG (Germany), Fetal Bovine Serum was from PAA Laboratories GmBH (Pasching, Austria) and laminin was from Becton Dickinson Hellas (Athens, Greece). SB203580, PD98059, Ro318220 and SP600125 were obtained from Calbiochem (La Jolla, CA, USA). TRI Reagent was from Ambion (Ambion Europe Ltd), M-MuLV Reverse Transcriptase was from Finnzymes (Finnzymes Oy, Finland) and *Taq* DNA polymerase was obtained from NEB (New England Biolabs UK Ltd). Bradford protein assay reagent was from Bio-Rad (Hercules, California, USA). Nitrocellulose (0.45 μm) was obtained from Schleicher & Schuell (Keene N.H. 03431, USA). Prestained molecular mass markers were from New England Biolabs (Beverly, MA, USA). Rabbit polyclonal antibodies to phospho-MSK1 (Thr581) phospho-c-Jun (Ser63) and c-Jun were obtained from Cell Signaling (Beverly, MA, USA). Secondary antibodies were from DAKO (High Wycombe, Buckinghamshire, UK) and western blotting chemiluminescence reagent kit was from Chemicon (Chemicon International, Inc). Primers for c-Jun and GAPDH were synthesized by the Laboratory of Microchemistry, IMBB (Crete, Greece). General laboratory reagents were from Sigma Chemical Co (St. Louis, MO, USA) or Merck (Darmstadt, Germany). X-OMAT AR film was purchased from Eastman Kodak Company (New York, USA).

Statistics

Data are presented as mean ± S.E. of *n* independent experiments. Statistical analyses (ANOVA with Tukey's Multiple Comparison Test or two-tailed Student's *t*-test where appropriate) were performed using Graph Pad Prism (Graph Pad Software, San Diego, CA, USA) with significance taken as being established at $P < 0.05$.

Results

PE promotes up-regulation of c-jun mRNA through MAPKs and MSK1

c-*jun* is an immediate early gene which is rapidly and transiently up-regulated during cardiac myocyte hypertrophy [1, 13]. To further investigate the potential role of MSK1 in the hypertrophic response of cardiac myocytes,

we examined the regulation of c-*jun* transcription during PE-induced hypertrophy. Accumulation of endogenous c-*jun* mRNA was measured by semi-quantitative ratiometric RT-PCR. As shown in Fig. 1a, c-*jun* mRNA was detectable under basal conditions and it was significantly increased by PE within 20 min. Maximum levels (≈4.5-fold relative to control) were attained at 30 min, declining thereafter and reaching basal levels by 60 min. To investigate which kinases may regulate c-*jun* transcription, we examined the effect of a range of protein kinase inhibitors, which have been shown to block the activation of ERK1/2, p38-MAPK, JNKs or MSK1 in response to PE in adult cardiac myocytes [6, 8, Markou and Lazou, unpublished data]. Inhibition of p38-MAPK with SB203580 (1 μM), ERK1/2 with PD98059 (10 μM), or JNKs with SP600125 (10 μM) depressed the increase of c-*jun* mRNA by 54%, 53% and 60%, respectively (Fig. 1b). Furthermore, Ro318220 (5 μM), a potent inhibitor of MSK1 activity, markedly reduced the PE-stimulated c-*jun* up-regulation by 44%. Similar results were obtained when H89, another widely used inhibitor of MSK1, was used (data not shown). None of the above inhibitors had any effect on c-*jun* expression when tested alone in the absence of PE. These results demonstrate that the up-regulation of c-*jun* mRNA in response to PE is mediated by activation of JNKs, ERK1/2, p38-MAPK and MSK1 signalling pathways.

PE-induced phosphorylation of MSK1 is independent of JNKs activation

Given that JNKs are considered to be the principal mediators of c-Jun phosphorylation [11, 14], we determined the activation of these kinases by PE in our experimental model. Maximum phosphorylation levels of JNKs (≈3-fold relative to control) were attained at 10–15 min after the onset of stimulation with a subsequent decline to basal after 60 min (Fig. 2a). The phosphorylation of JNKs by PE was not affected by Ro318220 (5 μM), PD98059 (10 μM) or SB203580 (1 μM) (Fig. 2b). On the other hand, SP600125 significantly reduced phosphorylation of JNKs consistent with previous studies [15].

We have previously demonstrated that MSK1 is a downstream target of ERK1/2 and p38-MAPK in cardiac myocytes exposed to PE [6]. Although both Thr581 and Ser360 residues of MSK1 have been shown to be targets of ERKs and p38-MAPK in other cell systems, phosphorylation of only Thr581 in response to PE was detected in adult cardiac myocytes and this was sufficient for the activation of the kinase [6]. In order to investigate whether JNKs are involved in this response, we examined the effect of SP600125 (10 μM) on MSK1 phosphorylation by immunoblotting with an antibody selective for phosphorylated MSK1(Thr581) (Fig. 3). Pretreatment of cardiac

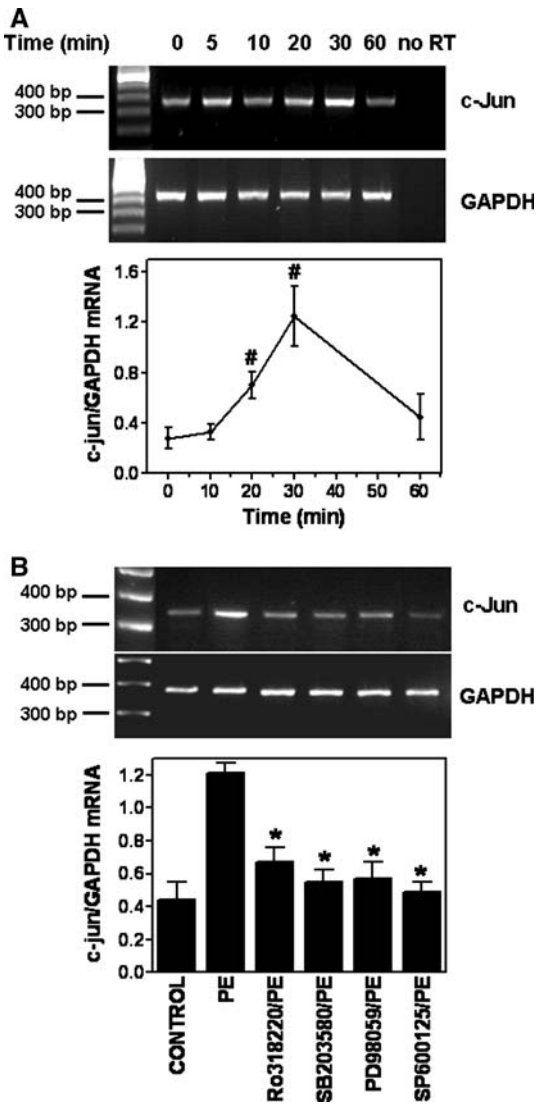

Fig. 1 Up-regulation of *c-jun* mRNA by PE is attenuated by inhibitors of ERK1/2, p38-MAPK, JNKs and MSK1. **a** Cardiac myocytes were exposed to PE (100 μM) for the times indicated. **b** Cardiac myocytes were either not exposed to inhibitors or pretreated for 10 min with SB203580 (1 μM), PD98059 (10 μM), SP600125 (10 μM) or Ro318220 (5 μM). Then they were incubated in the presence or absence of PE (100 μM) for 30 min. Total RNA was extracted and *c-jun* mRNA ((**a** and **b**) upper panels) or GAPDH mRNA ((**a** and **b**) middle panels) were amplified by RT-PCR. PCR products were analysed by ethidium bromide/agarose gel electrophoresis. Markers were run in the first lane and the positions of the 400 and 300 kb markers are indicated on the left. Lanes in which no reverse transcriptase (No RT) was incubated in the reaction are also shown. Agarose gels were analysed by scanning densitometry and the results are presented as means ± S.E. for three independent experiments ((**a** and **b**) lower panels). $^{\#}P < 0.05$ relative to control values (ANOVA with Tukey's multiple comparison test). * $P < 0.05$ relative to cells stimulated with PE (unpaired two-tailed Student's *t*-test)

myocytes with SP600125 had no significant effect on the PE-stimulated phosphorylation of MSK1 suggesting that PE activates MSK1 and JNKs through independent

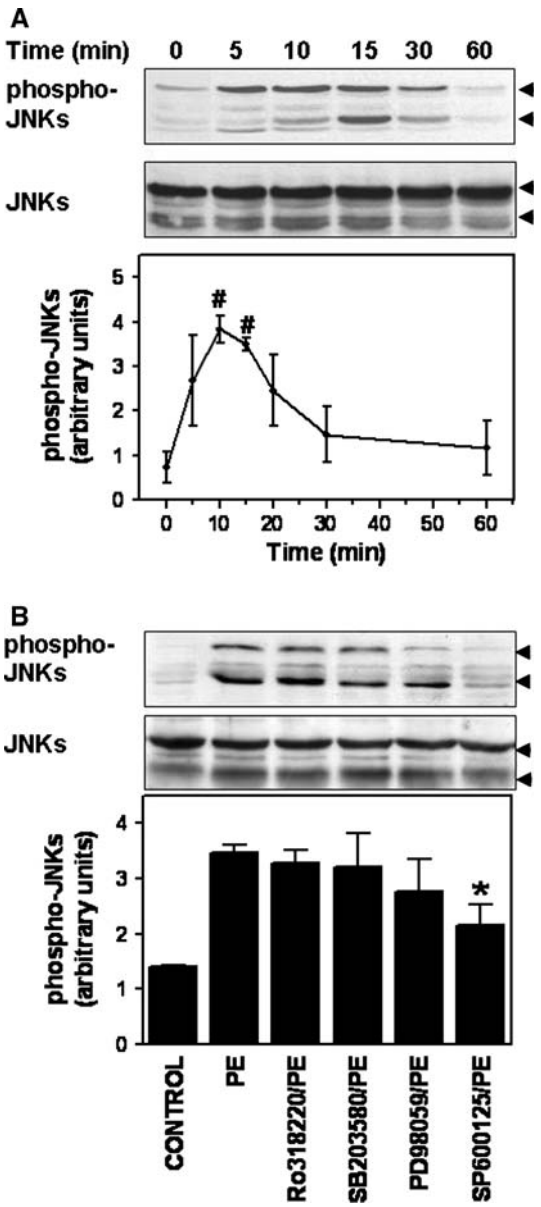

Fig. 2 Phosphorylation of JNKs by PE. **a** Cardiac myocytes were unstimulated or exposed to PE (100 μM) for the times indicated. **b** Cardiac myocytes were either not exposed to inhibitors or pretreated for 10 min with Ro318220 (5 μM), SB203580 (1 μM), PD98059 (10 μM) or SP600125 (10 μM). Then, they were incubated in the presence or absence of PE (100 μM) for 15 min. Whole cell extracts were subjected to SDS/PAGE and immunoblotted with antibodies against phospho-JNKs ((**a** and **b**) upper panels) or total JNKs to verify equal protein loading ((**a** and **b**) middle panels). Blots were quantified by scanning densitometry and plotted ((**a** and **b**) lower panels). The results are presented as means ± S.E. for three to five independent experiments. $^{\#}$ $P < 0.05$ relative to control values * $P < 0.05$ relative to cells stimulated with PE (ANOVA with Tukey's multiple comparison test)

signalling pathways and JNKs are not involved in the activation of MSK1. In addition, we tested again the specificity of Ro318220. Corroborating previous results

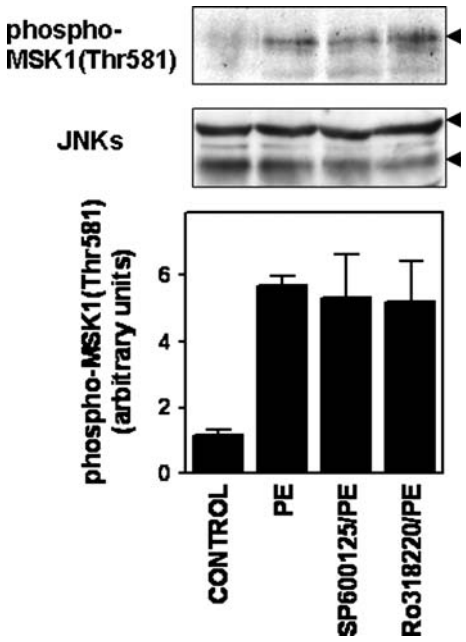

Fig. 3 JNKs are not involved in the phosphorylation of MSK1 by PE. Cardiac myocytes were either left untreated or they were pretreated with SP600125 (10 μM) or Ro318220 (5 μM) for 10 min. Then, they were incubated in the presence or absence of PE (100 μM) for 15 min. Cell extracts were immunoblotted for phospho-MSK1(Thr581) (upper panel) or JNKs (middle panel). Blots were quantified by scanning densitometry and plotted (lower panel). The results are presented as means ± S.E. for three independent experiments

[8], Ro318220 had no effect on the phosphorylation of MSK1 (Fig. 3) indicating that it does not decrease the activity of any upstream protein kinase to a level where it would become rate limiting for the phosphorylation and activation of MSK1.

Effect of MAPKs or MSK1 inhibition on c-Jun protein expression and phosphorylation

The effect of PE stimulation on c-Jun protein expression was assessed by immunoblotting, as shown in Fig. 4a. Although c-Jun protein is detectable under basal conditions, PE induced a ≈4.5-fold increase in its expression. A maximum increase in c-Jun protein levels was observed at 30 min and this was sustained for 60 min. Activation of c-Jun is regulated by post-translational phosphorylation in the N-terminus. We, thus, examined the phosphorylation of c-Jun in response to PE by immunoblotting with antibodies selective for phosphorylated c-Jun(Ser63). PE induced substantial increase in c-Jun phosphorylation (Fig. 4b), which was detected at 10 min after the onset of stimulation, maximized at 15 min and reached basal levels by 60 min. To determine that the increased c-Jun phosphorylation observed was not an indirect effect of increased

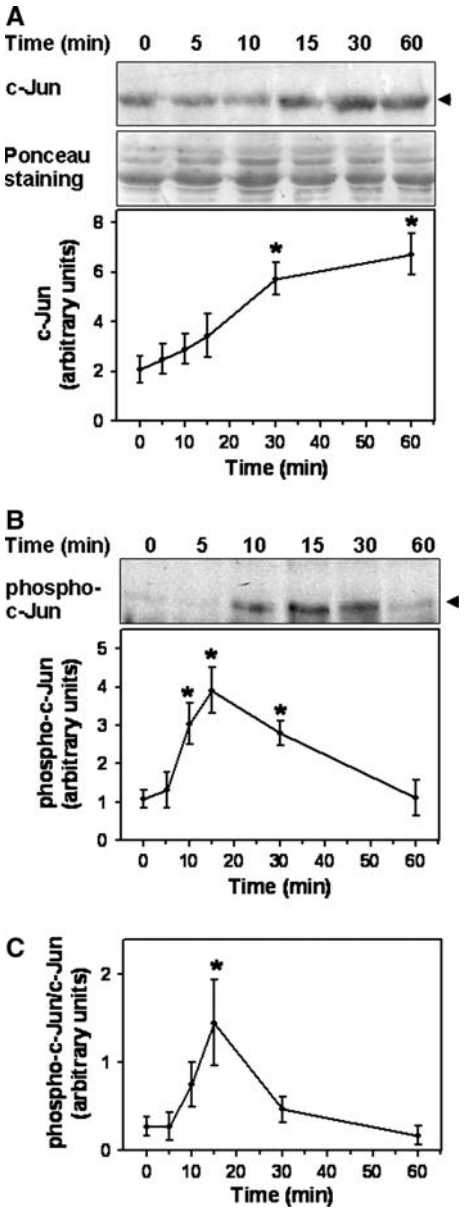

Fig. 4 PE promotes phosphorylation of c-Jun(Ser-63) and up-regulation of c-Jun protein. Cardiac myocytes were unstimulated or exposed to PE (100 μM) for the times indicated. Nuclear extracts were subjected to SDS/PAGE and immunoblotted for total c-Jun ((**a**) upper panel) or phosphorylated c-Jun(Ser63) ((**b**) upper panel). Equal loading was verified by Ponceau S staining ((**a**) middle panel). Blots were quantified by scanning densitometry ((**a** and **b**) lower panels). The relative ratio of phosphorylated to total c-Jun, as measured by scanning densitometry, was plotted (**c**). The results are presented as means ± S.E. for three independent experiments. * $P < 0.05$ relative to control values (ANOVA with Tukey's multiple comparison test)

protein levels, we calculated the relative ratio of phosphorylated to total c-Jun as measured by scanning densitometry (Fig. 4c). The phospho-c-Jun(Ser63)/c-Jun ratio increased with a maximum ($\approx$4 fold) at 15 min after stimulation indicating that PE indeed promotes c-Jun phosphorylation.

In order to investigate the signalling pathways that regulate c-Jun protein expression, cardiac myocytes were pretreated with inhibitors of kinases, as mentioned above, and stimulated with PE for 30 min. As shown in Fig. 5a, SB203580 or PD98059 suppressed the increase in c-Jun protein by 55% and 54%, respectively. Pretreatment of cardiac myocytes with SP600125 or Ro318220 also reduced c-Jun protein levels (64% and 63%, respectively, Fig. 5a). In a parallel set of experiments, we examined the

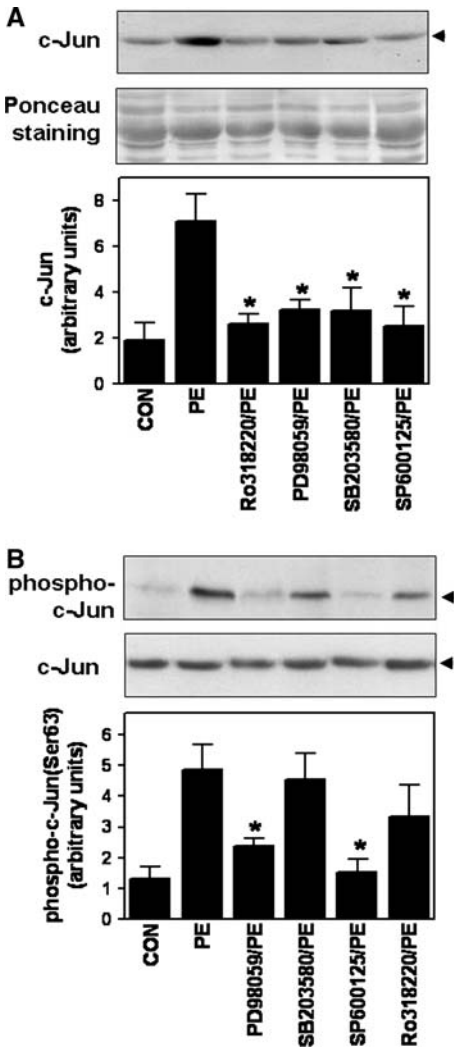

Fig. 5 Effect of MAPKs or MSK1 inhibition on c-Jun protein expression and phosphorylation. Cardiac myocytes were either not exposed to inhibitors or pretreated for 10 min with SB203580 (1 μM), PD98059 (10 μM), SP600125 (10 μM) or Ro318220 (5 μM). Then, they were incubated in the presence or absence of PE (100 μM) for 30 min (**a**) or 15 min (**b**). Nuclear extracts were subjected to SDS/PAGE and immunoblotted for total c-Jun ((**a**) upper panel; (**b**) middle panel) or phosphorylated c-Jun(Ser-63) ((**b**) upper panel). Equal loading was verified by Ponceau S staining ((**a**) middle panel). Blots were quantified by scanning densitometry ((**a** and **b**) lower panels). The results are presented as means ± S.E. for four independent experiments. * $P < 0.05$ relative to cells stimulated with PE (unpaired two-tailed Student's *t*-test)

effect of these inhibitors on c-Jun phosphorylation induced by PE (Fig. 5b). Because PE promotes both phosphorylation and protein up-regulation of c-Jun, the effect of inhibitors was tested at 15 min when phosphorylation was maximum (Fig. 4c) whereas no significant effect on protein expression was expected (Fig. 4a). Ro318220 (5 μM) or SB203580 (1 μM) had no effect on c-Jun phosphorylation, whereas pretreatment with PD98059 (10 μM) and SP600125 (10 μM) resulted in a substantial inhibition (51% and 68%, respectively) (Fig. 5b, upper and lower panel). As expected, none of the inhibitors had an effect on c-Jun protein levels at this time point (Fig. 5b, middle panel). In addition, none of the inhibitors had any effect on c-Jun phosphorylation or expression when tested alone in the absence of PE (data not shown). Collectively, these results demonstrate that JNKs and ERK1/2 mediate c-Jun phosphorylation whereas JNKs, ERK1/2, p38-MAPK and MSK1 are involved in the up-regulation of c-Jun protein.

Effect of MAPK or MSK1 inhibition on PE-stimulated changes in cardiac myocyte morphology

We have previously shown that inhibition of MSK1 results in the attenuation of phenylephrine-induced ANP up-regulation in adult cardiac myocytes [8]. In order to determine whether MSK1 is involved in the morphological changes associated with hypertrophy, cardiac myocytes were exposed to PE (100 μM) for 24 h in the absence or presence of protein kinase inhibitors and the cell area was quantified by digital planimetry. PE promoted a 40% increase in cell area when compared with control values (Fig. 6a–c and g). Consistent with previous studies implicating ERK1/2 and p38-MAPK cascade in the hypertrophic response [4, 16, 17], the effect of PE was significantly reduced by PD98059 (10 μM) and SB203580 (1 μM) (Fig. 6c, d and g). Furthermore, pre-treatment of cardiac myocytes with Ro318220 (5 μM) resulted in a significant decrease in cell area (Fig. 6e and g). Interestingly, the morphological changes induced by PE were also inhibited in cardiac myocytes treated with the JNK inhibitor SP600125 (10 μM) (Fig. 6f and g). These results, taken together, provide evidence for the involvement of MSK1 and JNKs in hypertrophic cell growth induced by PE in adult rat cardiac myocytes.

Discussion

Hypertrophic growth of cardiac myocytes involves the coordinated activation of interconnected signalling modules and transcription factors, which result in sequential activation of immediate early and "fetal" genes. GqPCR-mediated signalling has been implicated in the development of hypertrophy in both hearts and isolated cardiac myocytes [1, 18]. Although there is still much debate and controversy, numerous studies have proposed that ERK1/2, JNKs or p38-MAPKs participate in this response [19]. We have also shown that MSK1, a downstream target of both ERK1/2 and p38-MAPK, is involved in the hypertrophic response by regulating CREB-dependent transcriptional changes [8]. In the present study, we explore the role of MSK1 and MAPKs in the regulation of the immediate early gene *c-jun*. In addition, we provide evidence that these pathways are involved in the changes of cardiac myocyte morphology associated with hypertrophy (Fig. 6c, e and g).

The pro-hypertrophic effects of PE have been shown to depend on the activation of c-Jun and subsequently AP-1, which in turn promotes cardiac myocyte growth and induction of a "fetal" gene program [18, 20, 21]. c-Jun has been shown to be regulated either by increased expression or by post-translational enhancement of its transcriptional activity, which includes phosphorylation of Ser63 and Ser73 by JNKs [10]. Phosphorylation of c-Jun (Ser63) clearly preceded the increase in *c-jun* mRNA and total c-Jun protein in adult cardiac myocytes (Fig. 1 and 4). Consistent with previous studies in neonatal cardiac myocytes and other cells [14, 22], this phosphorylation is mediated by ERK1/2 and JNKs (Fig. 5b). On the other hand, MSK1 and p38-MAPK do not contribute to this process (Fig. 5b). Thus, it seems that stimulation of adult cardiac myocytes with PE activates JNKs and ERK1/2, which phosphorylate endogenous basal c-Jun protein; the phosphorylated and, thus, activated form of c-Jun may subsequently induce its own transcription (Fig. 1).

The results of the present study implicate all three MAPK subfamilies and MSK1 in the up-regulation of c-*jun* mRNA in adult rat cardiac myocytes in response to the hypertrophic agonist PE (Fig. 1b). It is possible that MSK1 regulates *c-jun* transcription by activating transcription factors such as CREB [8] or ATF2 [23, 24]. The rat *c-jun* promoter contains CRE-/AP-1-like sites that have been shown to interact with CREB [25]. Inhibition of c-*jun* up-regulation observed with SB203580 or PD98059 in adult cardiac myocytes could be also attributed to inhibition of MSK1, which relies on both ERK1/2 and p38-MAPK for its activation [6]. However, we cannot exclude the possibility that ERK1/2 or p38-MAPK, independently of MSK1, regulates *c-jun* mRNA up-regulation through the activation of other transcription factors. Overall, these data suggest that the c-*jun* mRNA expression in adult rat cardiac myocytes is regulated by a complex network of signalling pathways which affect one or more regulatory elements on c-*jun* promoter and that the transcriptional response to PE most likely results from the temporal integration of each of these biochemical routes. The integration of numerous signals transduced by MAPKs for the regulation of c-*jun*

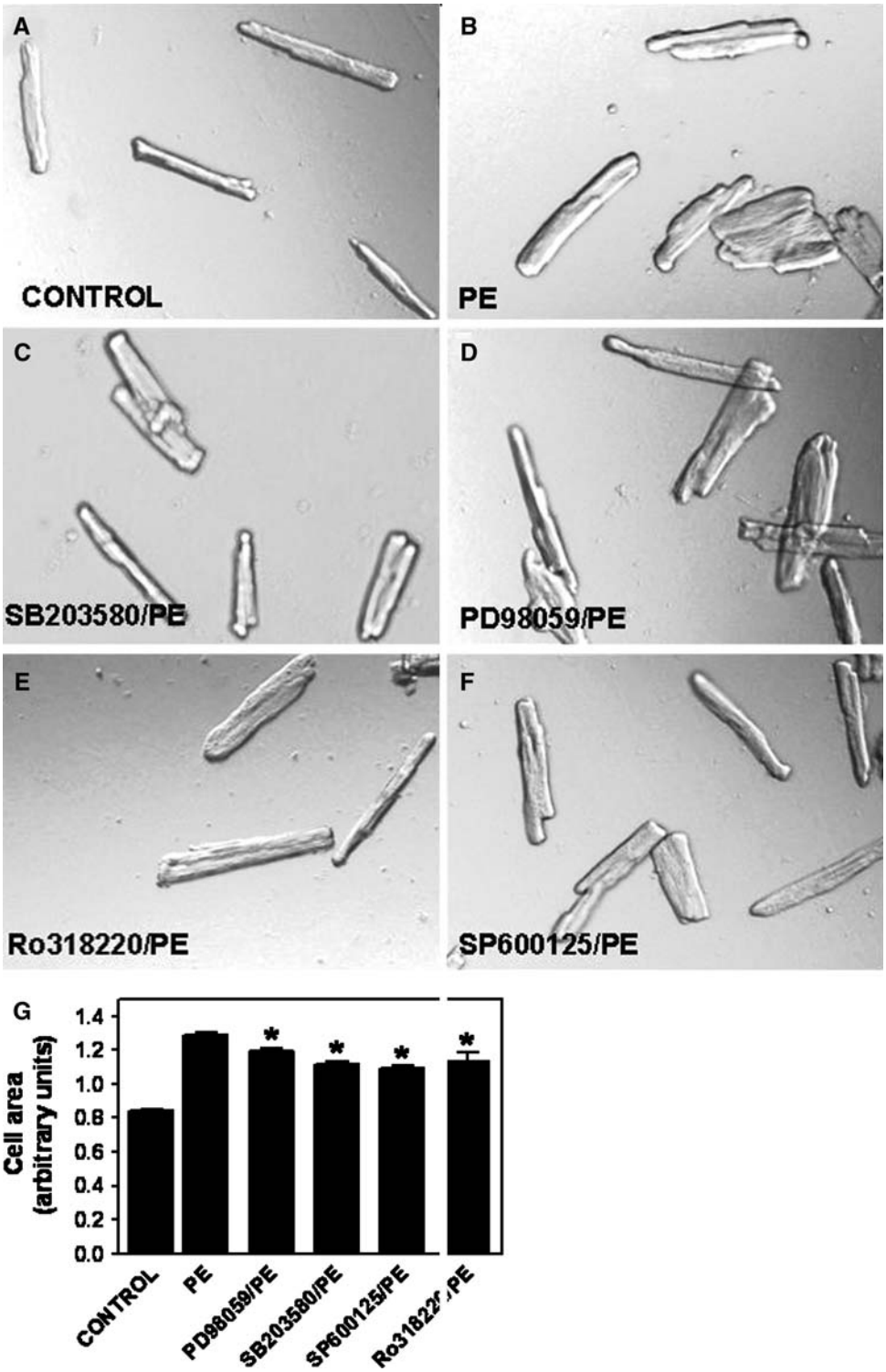

Fig. 6 Effect of MSK1 or MAPKs inhibition on PE-stimulated increase in cardiac myocyte area. Cells were either not treated (control) or pretreated for 10 min with SB203580 (1 μM), PD98059 (10 μM), SP600125 (10 μM) or Ro318220 (5 μM). Then, they were incubated in the absence or presence of PE (100 μM) for 24 h. **a–f** Representative images of cardiac myocytes. **g** Cell area was measured using Scion Image Software. Results are means ± S.E. for three independent experiments (each with $n = 100$), taking the mean size for each experiment as a single observation. * $P < 0.05$ relative to cells stimulated with PE (ANOVA with Tukey's multiple comparison test)

expression by GqPCR has been also reported in other cell systems [26]. On the other hand, ERK1/2 is the principal pathway regulating *c-jun* expression in neonatal cardiac myocytes following hypertrophic stimulation [22].

c-Jun protein levels are regulated by a complex mechanism that involves transcription, mRNA decay, translation and protein stability. Our data demonstrate that multiple signalling kinases that include ERK1/2, p38-MAPK, JNKs and MSK1 are involved in the PE-induced c-Jun protein up-regulation (Fig. 5a). However, the exact role of each kinase is not clear and awaits further investigation. Studies in cardiac myocytes have recently shown that a large

percentage of the c-*jun* mRNA pool is preserved in polysomes where it is stabilized by MNK1 (MAP kinase interacting kinase-1) [27]. This suggests that ERK1/2 and p38-MAPK are involved in promoting c-*jun* mRNA stability since MNK1 is a known substrate for these kinases [28]. c-Jun abundance is dependent on the regulation of protein stability conferred by phosphorylation mediated by JNKs, which decreases its ubiquitination [29]. The recent demonstration that the activation of JNKs by ET-1 is necessary to stabilize c-Jun for efficient up-regulation of c-Jun protein supports the importance of such mechanism in hypertrophying cardiac myocytes [22]. Accordingly, we showed here that the JNK inhibitor, besides inhibiting c-Jun phosphorylation, also attenuated the increase in c-Jun protein induced by PE (Fig. 5), suggesting that post-translational mechanisms might contribute to enhance c-Jun expression. However, the possibility that the increase of c-Jun protein content in hypertrophic cardiac myocytes is due to increased c-*jun* mRNA translation cannot be excluded.

In conclusion, the results of the present study expand our understanding of the signalling mechanisms underlying c-Jun regulation and provide further support for the role of MSK1 in adult cardiac myocyte hypertrophy. Our data would fit with a model where PE triggers the activation of ERK1/2, p38-MAPK, JNKs and MSK1. ERK1/2 and JNKs phosphorylate c-Jun to increase its transactivating activity and/or its protein stability. At the same time, ERK1/2 and p38-MAPK, directly or indirectly through MSK1, and JNKs contribute to *c-jun* mRNA and protein up-regulation. These signalling mechanisms seem to be critical to the phenotypic changes of cardiac myocytes in response to hypertrophic stimulation.

Limitations of the study

The results of the present study are entirely based on the use of pharmacological inhibitors. Cell permeant protein kinase inhibitors have been extensively used in order to delineate the physiological role of these enzymes. On the other hand, the specificity of some of these compounds has been questioned [30, 31]. Ro318220 was originally developed as a PKC inhibitor and it has been also shown to inhibit MSK1 among other kinases [30]. In our previous study, we have tested the specificity of Ro318220 against MSK1 in adult rat cardiac myocytes [8]. As it is also shown in Fig. 3, phosphorylation of MSK1 was not affected by Ro318220. On the other hand, GF109203X, a specific inhibitor of PKCs, completely inhibited MSK1 phosphorylation by PE [6]. Alternative methodologies to inhibit protein kinases such as overexpression of dominant-negative mutants, which are extensively used in studies of proliferating cells, cannot be easily applied to primary cultures of terminally differentiated cells such as adult cardiac myocytes. In addition, one would argue that overexpression of dominant-negative and constitutively active protein kinases can cause the specificity of signalling to break down and lead to erroneous conclusions being drawn. Future studies using dominant negative mutants of kinases, in particular MSK1, will undoubtedly enhance our understanding of its role in cardiac hypertrophy.

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