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Role of protein turnover in the activation of p38 mitogen-activated protein kinase in rat pinealocytes

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Abstract

Differences in the time profiles of activation between p38MAPK and p42/44MAPK by norepinephrine (NE) in rat pinealocytes suggest involvement of mechanisms other than the phosphorylation cascades in their activation. In the present study we investigated whether protein turnover played a role in regulating p38MAPK activation in the rat pineal gland. NE stimulation caused an increase in MAPK kinase3/6 (MKK 3/6) and p38MAPK phosphorylation that occurred in the absence of changes in the mRNA or protein levels of p38MAPK or MKK3/6. The stimulatory effect of NE on phosphorylated MKK3/6 and p38MAPK, but not phosphorylated p42/44MAPK, was blocked by treatment with actinomycin or cycloheximide, indicating a requirement of transcription and translation in activation of the p38MAPK but not the p42/44MAPK pathway. Moreover, inhibition of proteasomes by clasto-lactacystin β-lactone or Z-Leu-Leu-Leu-CHO (MG132) selectively increased basal and NE-stimulated phosphorylated MKK3/6 and p38MAPK levels without affecting the mRNA or protein levels of MKK3 or p38MAPK. In contrast, the effect of proteasomal inhibition on NE-stimulated p42/44MAPK phosphorylation was inhibitory. Treatment with MG132 also reduced the decline in the phosphorylated levels of NE-stimulated MKK3/6 and p38MAPK that normally follows β-adrenergic blockade. Together, our results indicate that p38MAPK but not p42/44MAPK activation in the rat pineal gland is tightly coupled to protein synthesis and degradation. The synthesis of an activator upstream of MKK3/6 is required for the NE-activation of p38MAPK.

Keywords: p38MAPK; p42/44MAPK; Proteasome; Norepineprhine; Pineal

1. Introduction

In the rat pineal gland, the synthesis of melatonin is tightly regulated by the nightly release of norepinephrine (NE) from the sympathetic nerve terminals [1,2]. NE stimulates both α_1- and β_1-adrenergic receptors resulting in a 100-fold increase in intracellular cAMP level [1,3]. Through transcriptional and post-translational mechanisms, the main consequence of this cAMP elevation is an increase in arylalkylamine-*N*-acetyltransferase (AA-NAT) activity, the rate-controlling enzyme in melatonin biosynthesis [2,4,5]. Besides induction of AA-NAT activity, NE stimulation also increases the expression of other proteins including mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) [6,7] and transcription factors such as Fos-related antigen 2 and induced cAMP early repressor [8,9], as well as activates additional signaling pathways such as MAPKs via phosphorylation cascades [10,11]. Some of these NE-stimulated cellular events are involved in the regulation of AA-NAT activity [2,5,9,12].

MAPK cascades have been implicated in the regulation of a variety of cellular processes such as growth, differentiation, secretion and metabolism [13–16]. There are three major subgroups of MAPKs: p42/44MAPK, p38MAPK and

Abbreviations: AA-NAT, arylalkylamine-*N*-acetyltransferase; Actin, actinomycin; c-lact, clasto-lactacystin β-lactone; Cyclo, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; JNK, c-Jun amino terminal kinase; MAPK, mitogen-activated protein kinase; MKP-1, MAPK phosphatase 1; MKK, MAPK kinase; NE, norepinephrine; p-MAPK, phosphorylated MAPK; MG132, Z-Leu-Leu-Leu-CHO; PKA, protein kinase A

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c-Jun amino-terminal kinase (JNK) [14]. Similar protein kinase cascades are involved in the activation process that results in the phosphorylation of MAPKs. Activation of p42/44MAPK involves phosphorylation of the enzyme by MAPK kinase (MEK or MKK)1 and MEK2 [17,18], and members of the p38MAPK families are phosphorylated by MKK3, MKK4 and MKK6 [19–21], while JNK by MKK7 [22,23].

Activation of MAPKs in the rat pineal gland is under adrenergic control. Phosphorylated levels of p42/44MAPK and p38MAPK as well as MEK1/2 and MKK3/6, are increased at night [24–26] and these increases are blocked by propranolol, a β-adrenergic blocker [24,26]. However, pineal cell culture studies indicated that different signaling mechanisms are involved in the NE activation of p42/44MAPK versus p38MAPK and their time profiles of activation also differ. Whereas cGMP/protein kinase G is the main signaling pathway that activates p42/44MAPK [10,27], the cAMP/protein kinase A (PKA) pathway is the principle mechanism involved in the activation of p38MAPK [24]. Moreover, the NE-stimulated phosphorylated p42/44MAPK response is limited to 1 h with a peak response occurring within 15 min. This differs from the phosphorylated p38MAPK response that is detectable at 2 h and is sustained for at least 4 h [24,27]. This difference in the respective time profiles of activation may signify differential requirements for protein synthesis. Therefore, the objective of the present study is to investigate the role of protein turnover in adrenergic activation of the p38MAPK pathway. Our results show that in the rat pineal gland, activation of p38MAPK, but not p42/44MAPK, is tightly coupled to both protein synthesis and degradation.

2. Materials and methods

2.1. Materials

Cycloheximide, NE, propranolol, and antibodies against p38MAPK (M0800), phosphorylated p38MAPK (p-p38MAPK; M8177) and phosphorylated p42/44MAPK (p-p42/44MAPK; M8159) were obtained from Sigma Chemical Co. (St. Louis, MO). Actinomycin, clasto-lactacystin β-lactone (c-lact) and MG 132 were obtained from Calbiochem Corp. (San Diego, CA). Antibodies against phosphorylated MKK3/6 (p-MKK3/6; 9231) and MKK3 (9232) were from Cell-Signaling Technology (Beverly, MA). Antibodies against MKK3/6 (SC-13069) were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of the purest grades available commercially.

2.2. Preparation of cultured pinealocytes and drug treatment

Sprague–Dawley rats (male; weighing 150 g) were obtained from the University of Alberta animal unit and housed under a lighting regimen providing 12 h of light every 24 h with lights on at 0600 h. For pinealocyte cell culture preparation, animals were sacrificed 3 h after the onset of light, and pineal glands were removed and stored in ice-cold PBS until trypsinization. All procedures were reviewed and approved by the health sciences animal and welfare committee of the University of Alberta (Edmonton, Alberta). Pinealocytes were prepared from the rat pineal glands by trypsinization as described previously [7,28]. The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and maintained at 37 °C for 24 h in a gas mixture of 95% air and 5% CO_2 before experiments. Aliquots of pinealocytes (5×10^5 cells/0.5 ml) were treated with drugs which had been prepared in concentrated solutions in water or DMSO for the duration indicated. Treated cells were collected by centrifugation (2 min, 12,000 × *g*). Pinealocyte total RNAs were isolated and purified by RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instruction. Samples for Western blot analysis were solubilized in 1× sample buffer made by combining equal volumes of 2× sample buffer and homogenization buffer by boiling for 5 min and stored until electrophoresis. The homogenization buffer contained 20 mM Tris–HCl, 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulphonyl fluoride, 1 μg/ml each of aprotinin, leupeptin and pepstatin, 1 mM sodium orthovanadate and 1 mM sodium fluoride (pH 7.5).

2.3. RT-PCR analysis

First strand cDNA was synthesized from the isolated RNA using an Omniscript reverse-transcriptase kit (Qiagen Inc., Valencia, CA) with an oligo-dT primer as per the manufacturer's instructions. A 3 μl of a 1:10 dilution of cDNA was used as template for PCR reactions. PCR was performed in a 29.3 μl reaction mixture containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 1.25 U Taq polymerase (Perkin-Elmer, Cetus) and 1 μM each of the two primers. All PCR reactions were performed as follows: denaturing for 1 min at 94 °C, annealing for 1 min at 63 °C, and extension for 1 min at 72 °C. Initial denaturing and final extension were both 5 min in duration. Cycle numbers varied slightly between cell preparations, but in general, 22 cycles were used to amplify *gapdh*, and 25 cycles to amplify both *mkk3* and *p38mapkα*. All reaction sets included water blanks as negative controls. Amplified products were separated on ethidium bromide-stained 1.5% agarose gels. In all cases, *gapdh* mRNA levels were also measured from the samples to demonstrate uniformity of sample preparation and loading. PCR products were confirmed by sequencing. Primers used are as follows: *mkk3*: upstream primer: 5′-ATG GAC ACG TCC CTG GAT AA-3′; downstream primer: 5′-CCC TTC TGA TTC AGC TCA GG-3′. *p38mapkα*: upstream primer: 5′-GAG CTG TTG ACC GGA AGA AC-3′; downstream primer: 5′-TCA TCA TCA GGG TCG TGG TA-3′.

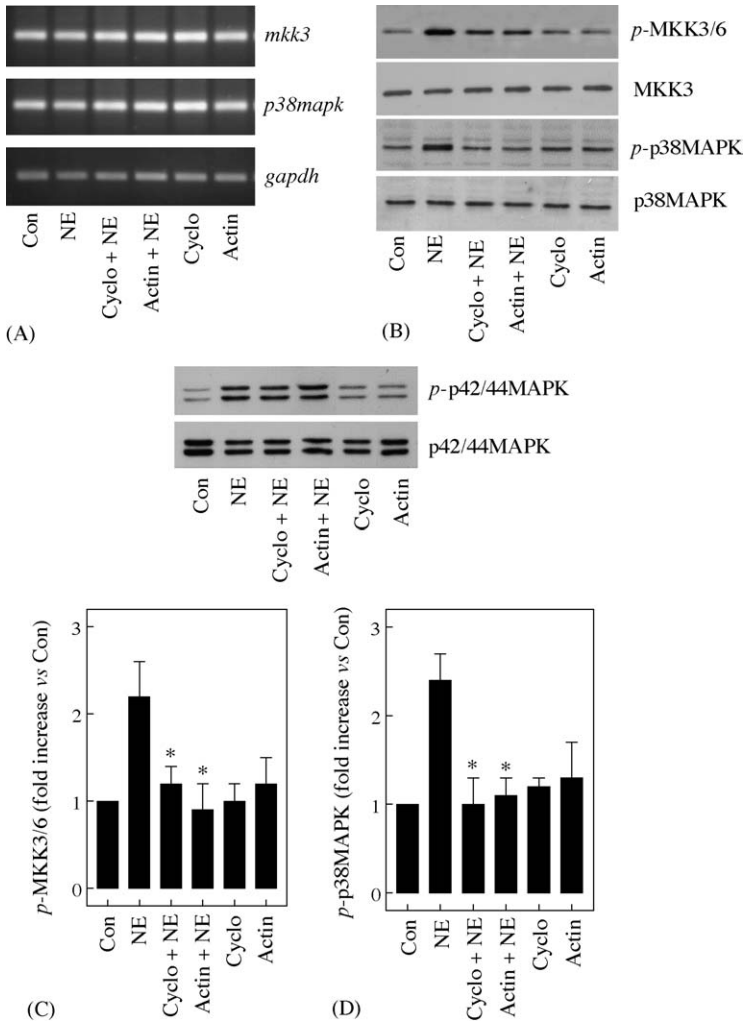

Fig. 1. Effects of transcription and translation inhibitors on NE-mediated p38MAPK activation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated for 2 h with NE (3 μM), cycloheximide (15 μg/ml; Cyclo), actinomycin (15 μg/ml; Actin) or NE (3 μM) in the presence of cycloheximide (15 μg/ml) or actinomycin (15 μg/ml) as indicated. The cells were then collected by centrifugation and prepared for RT-PCR or Western blot analysis as described in Section 2. (A) RT-PCR analysis of *mkk3* and *p38mapk* mRNA expressions in pinealocytes treated as indicated, *gapdh* was included to demonstrate loading consistency. (B) Immunoblots of p-MKK3, MKK3, p-p38MAPK and p38MAPK in pinealocytes treated as indicated. (C) Immunoblots of p42/44MAPK and p-p42/44MAPK in pinealocytes treated with drugs as indicated for 15 min. The blots presented are representative of at least three separate experiments. (D) Graphical summary from experimental replicates of densitometric values from Western blots for p-MKK3 and p-p38MAPK. *Significantly different from control ($p < 0.05$).

Sequences of the *gapdh* primers used were previously described [29].

2.4. Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli [30] using 10% acrylamide in the presence of 1 mg/ml sodium dodecyl sulfate (Mini-Protein II gel system, Bio-Rad, Hercules, CA). Following electrophoresis, gels were equilibrated for 15 min in transfer buffer (25 mM Tris, 190 mM glycine and 20% methanol). Proteins were transferred onto polyvinylidene difluoride membranes (1 h, 100 V), which were then incubated with a blocking solution [5% dried skim milk in 100 mM Tris (pH 7.5) with 140 mM NaCl and 0.01% Tween-20] for a minimum of 1 h. The blots were then incubated overnight at 4 °C with diluted specific antisera as indicated. After washing twice with the blocking solution, the blots were then incubated with diluted horseradish peroxidase-conjugated second antibodies (Bio-Rad) for 1 h at room temperature. They were then washed again extensively and developed using enhanced chemiluminescence (Amersham Pharmacia Biotech).

2.5. Results presentation and statistical analysis

For quantitation of RT-PCR analyses, gel images were acquired with Kodak 1D software on a Kodak 2000R imaging station (Eastman Kodak Co., Rochester, NY). For analyses of Western blots, exposed films were scanned and band densitometry of acquired images was performed with Kodak 1D software. Densitometric values were normalized to % maximal and presented as the mean ± S.E.M. from at least three independent experiments.

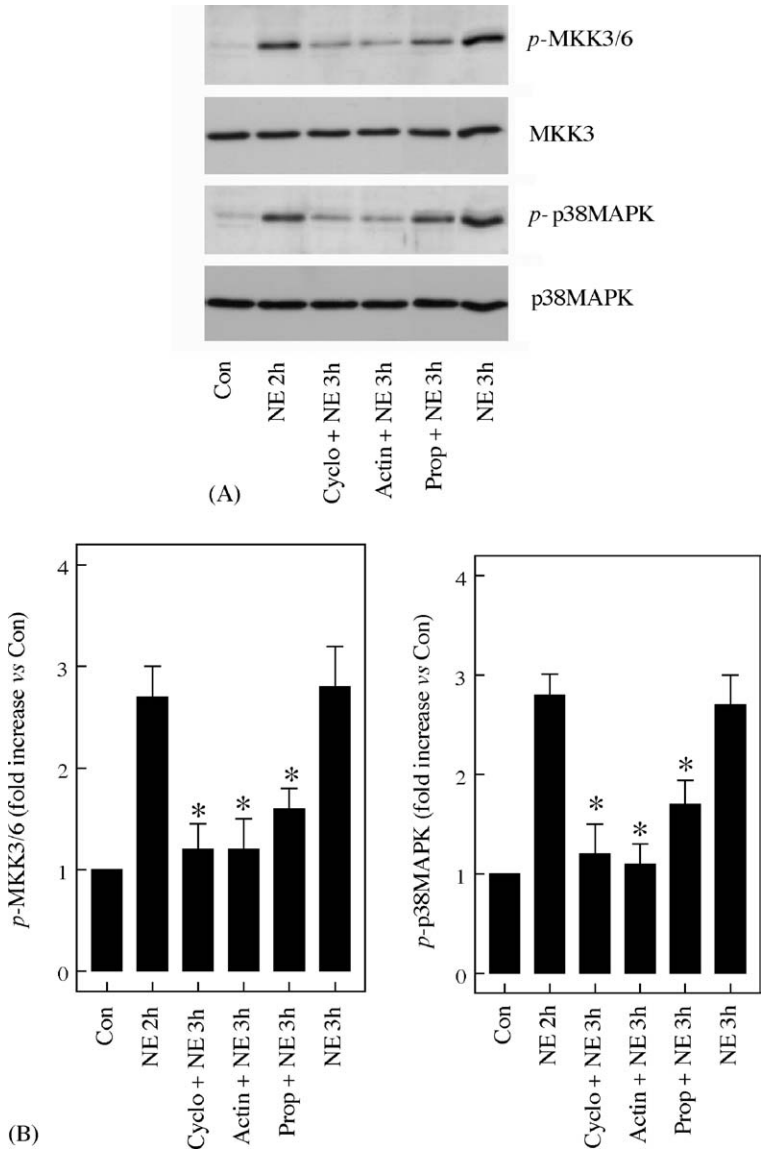

Fig. 2. Effects of addition of transcription or translation inhibitors on p38MAPK activation during NE stimulation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with NE (3 μM) for 2 h before addition of cycloheximide (15 μg/ml; Cyclo), actinomycin (15 μg/ml; Actin) or propranolol (10 μM; Prop) and incubated for an additional 1 h. The cells were then collected by centrifugation and prepared for Western blot analysis as described in Section 2. (A) Immunoblots of p-MKK3, MKK3, p-p38MAPK and p38MAPK in pinealocytes treated as indicated. The blots presented are representative of at least three separate experiments. (B) Graphical summary from experimental replicates of densitometric values from Western blots for p-MKK3 and p-p38MAPK. *Significantly different from NE treatment ($p < 0.05$).

Statistical analysis involved either a paired *t*-test or ANOVA with the Newman–Keuls test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Differential effects of inhibitors of transcription and translation on the activation of p38MAPK versus p42/p44MAPK by NE

To assess whether protein synthesis plays a role in the activation of p38MAPK, the effects of inhibitors of transcription and translation respectively, actinomycin and cycloheximide, on NE-stimulated p-MKK3/6 and p-p38MAPK levels were determined. Treatment with NE (3 μM) for 2 h increased levels of p-MKK3/6 and p-p38MAPK by more than two-fold (Fig. 1B and D). Pretreatment with cycloheximide (15 μg/ml) or actinomycin (15 μg/ml) for 5 min, while having no effect on the mRNA (Fig. 1A) or protein levels of MKK3/6 or p38MAPK, abolished the effects of NE on p-MKK3/6 and p-p38MAPK (Fig. 1B and D). In contrast, neither cycloheximide nor actinomycin had an effect on the NE-stimulated increase in p-p42/44MAPK levels at 15 min (Fig. 1C). These results suggest that protein synthesis is required for the activation of p38MAPK, but not the p42/44MAPK signaling pathway in the rat pineal gland.

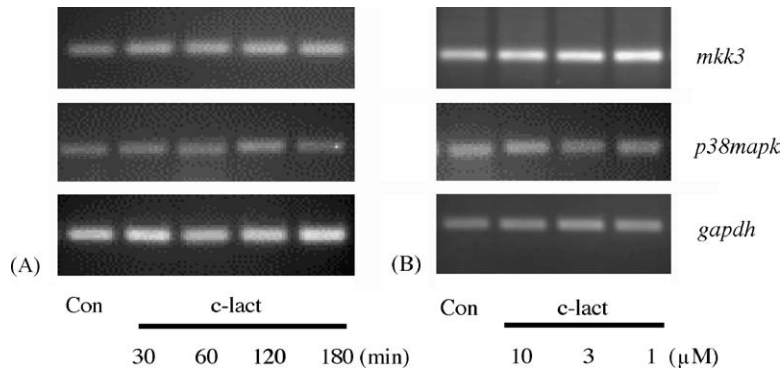

Fig. 3. Effects of c-lact on *mkk3* and *p38mapk* mRNA levels. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with (A) c-lact (3 μM) for different time periods or (B) c-lact (1–10 μM) for 2 h as indicated. The cells were then collected by centrifugation and prepared for RT-PCR analysis of *mkk3* and *p38mapk* mRNA expressions as described in Section 2, *gapdh* was included to demonstrate loading consistency. The blots presented are representative of at least three separate experiments.

3.2. Additions of inhibitors of transcription and translation on p38MAPK activation during NE stimulation

To determine whether protein synthesis is required to maintain the NE-stimulated p38MAPK response, pinealocytes were stimulated with NE (3 μM) for 3 h with addition of cycloheximide (15 μg/ml) or actinomycin (15 μg/ml) at 2 h of treatment. Because continuous β-adrenergic stimulation is required to maintain the p38MAPK response, addition of propranolol (10 μM) after 2 h was included for comparison [24]. There was no significant difference between the levels of p-MKK3/6 and p-p38MAPK at 2 and 3 h after NE stimulation (Fig. 2). However, addition of cycloheximide or actinomycin 2 h after NE treatment caused a significant decline in the levels of p-MKK3/6 and p-p38MAPK (Fig. 2). One hour after addition of cycloheximide or actinomycin, p-MKK3/6 and p-p38MAPK were similar to basal levels (Fig. 2).

3.3. Differential effects of proteasomal inhibition on activation of p38MAPK versus p42/44MAPK

To determine whether proteasomal proteolysis contributes to the regulation of p38MAPK activation in the rat pineal glands, time course and concentration response studies of c-lact on p38MAPK activation were performed. Treatment with c-lact (3 μM) for 30–180 min or c-lact (1–10 μM) for 2 h had no effect on the mRNA levels (Fig. 3). Similar results were obtained with the structurally unrelated proteasome inhibitor MG132 (data not shown). However, c-lact caused significant time- and concentration-dependent increases in the levels of p-MKK3/6 and p-p38MAPK (Fig. 4B) without having an effect on the total protein levels of MKK3 or p38MAPK (Fig. 4A). Treatment with MG132 (1 μM) for 30–180 min or MG132 (0.1–10 μM) for 2 h also caused time- and concentration-dependent increases in the levels of p-MKK3/6 and p-p38MAPK (Fig. 5B) without having an effect on the total protein levels of MKK3 or p38MAPK (Fig. 5A). At 2 h of treatment, MG132 (1 μM) or c-lact (10 μM) increased the levels of p-MKK3/6 or p-p38MAPK by about three-fold (Figs. 4B and 5B, right panel). In comparison, treatment with MG132 (1 μM) or c-lact (10 μM) for up to 2 h caused a significant decline in the levels of p-p42/44MAPK at 2 h (Fig. 6B and C). This occurred in the absence of changes in the protein levels of p42/44MAPK (Fig. 6A).

3.4. Effects of MG132 and cycloheximide on the propranolol-mediated decline in p38MAPK activation by NE

To determine the role of protein degradation in the decline of the NE-stimulated p-MKK3/6 and p-p38MAPK levels following β-adrenergic blockade, proteasome inhibitors were used to block the decline caused by propranolol. Pinealocytes were treated with NE (3 μM) for 3 h and addition of propranolol (3 μM) at 2 h of treatment abolished the NE stimulated p-MKK3/6 and p-p38MAPK levels within 1 h as reported previously [24] (Fig. 7). Treatment with MG132 (1 μM) had an enhancing effect on the NE-mediated increase in p-MKK3/6 and p-p38MAPK levels (Fig. 7B). In the presence of MG132 (1 μM), the propranolol-mediated decline in NE stimulated p-MKK3/6 and p-p38MAPK levels was not observed (Fig. 7B). Treatment with MG132 (1 μM) was also effective in reversing the cycloheximide-mediated decline in NE-stimulated p-MKK3/6 and p-p38MAPK levels (Fig. 8B). None of the above treatments had an effect on MKK3/6 and p38MAPK protein levels (Figs. 7A and 8A).

4. Discussion

Previously, we have shown that NE induces a time- and dose-dependent increase in p38MAPK activation in rat

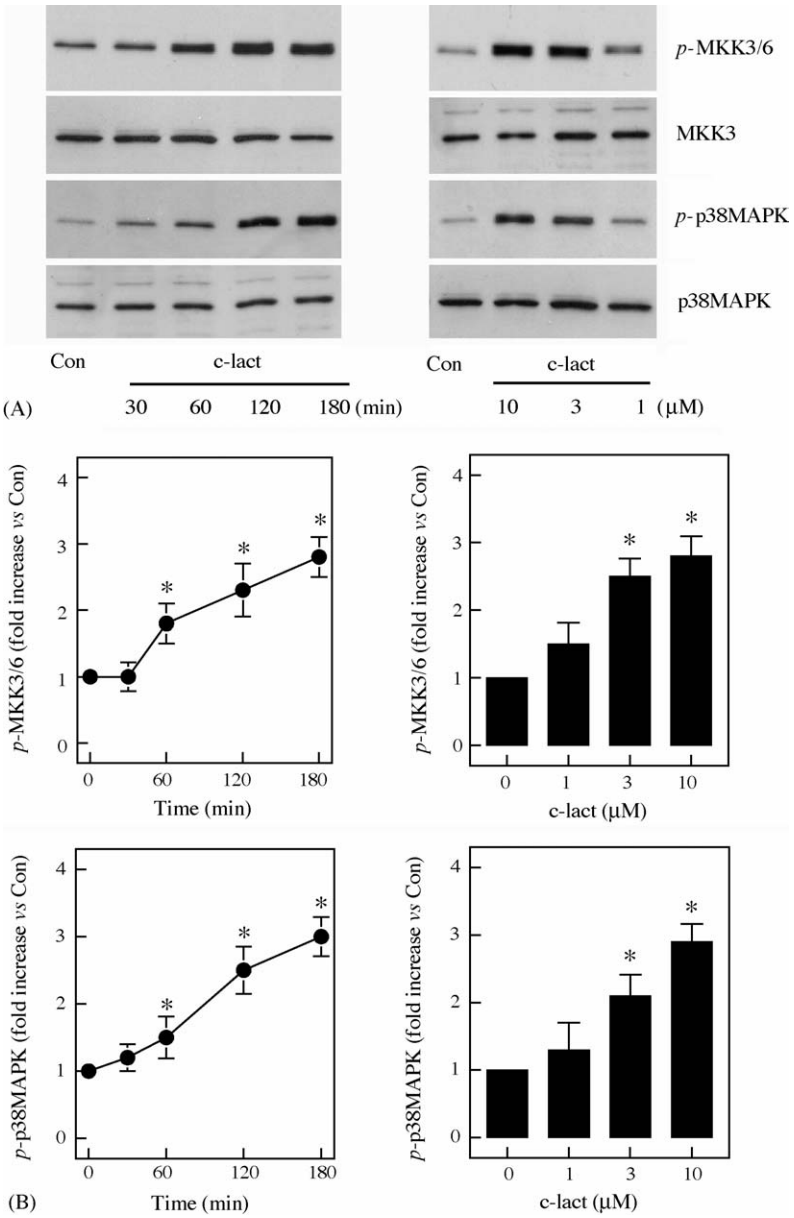

Fig. 4. Effects of c-lact on phosphorylated levels of *mkk3* and *p38mapk*. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with c-lact (3 μM) for 30–180 min or c-lact (1–10 μM) for 2 h as indicated. The cells were then collected by centrifugation and prepared for Western blot analysis as described in Section 2. (A) Immunoblots of p-MKK3, MKK3, p-p38MAPK and p38MAPK in pinealocytes treated as indicated. The blots presented are representative of at least three separate experiments. (B) Graphical summary from experimental replicates of densitometric values from Western blots for p-MKK3 and p-p38MAPK. *Significantly different from control ($p < 0.05$).

pinealocytes and that PKA is the main signaling pathway involved [24]. Because NE stimulation causes parallel increases in p-MKK3/6 and p-p38MAPK levels, p38MAPK activation by NE likely occurs upstream of MKK3/6 [24]. Whereas NE-stimulated activation of p42/44MAPK is rapid and transient [10,27], the response of p38MAPK activation to the identical stimulus is delayed and sustained [24]. In the present study, we have discovered that protein turnover likely accounts for the difference in the activation time profile of p38MAPK versus p42/44MAPK, and that only NE-mediated p38MAPK activation requires protein synthesis. Taken together, our results indicate involvement of mechanisms other than the phosphorylation cascade in NE-activation of p38MAPK in rat pinealocytes.

Our findings that inhibitors of transcription and translation can block the phosphorylation of MKK3/6 and p38MAPK induced by NE suggest de novo protein synthesis is required for the activation of the p38MAPK signaling pathway in the rat pineal gland. Requirement for protein synthesis likely explains the late nocturnal activation of MKK3/6 and p38MAPK that occurs several hours

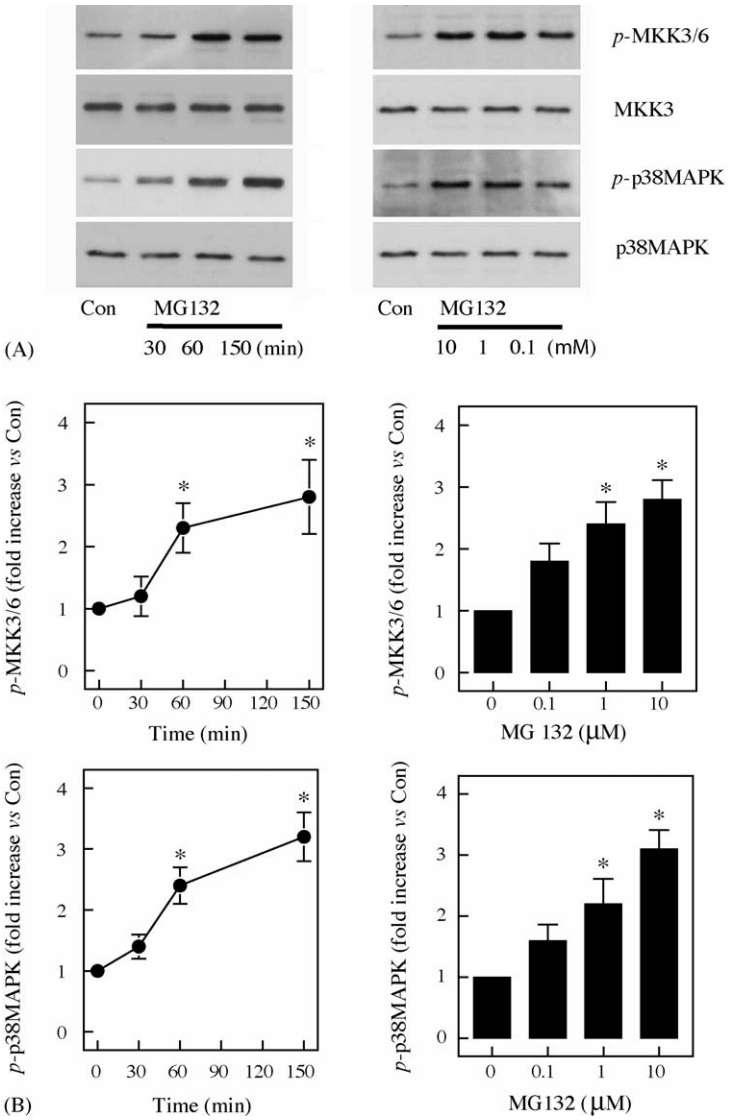

Fig. 5. Effects of MG132 on phosphorylated levels of *mkk3* and *p38mapk*. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with MG132 (3 μM) for 30–180 min or MG132 (0.1–10 μM) for 2 h as indicated. The cells were then collected by centrifugation and prepared for Western blot analysis as described in Section 2. (A) Immunoblots of p-MKK3, MKK3, p-p38MAPK and p38MAPK in pinealocytes treated as indicated. The blots presented are representative of at least three separate experiments. (B) Graphical summary from experimental replicates of densitometric values from Western blots for p-MKK3 and p-p38MAPK. *Significantly different from control ($p < 0.05$).

after onset of darkness in the intact pineal glands and the slow onset of activation after NE treatment in cultured pineal cells [24]. Continuous protein synthesis is also required to maintain the MKK3/6 → p38MAPK signaling pathway in the activated state after stimulation. This is based on the observation that addition of cycloheximide or actinomycin after the pathway has been activated causes a decline in phosphorylated p38MAPK to the basal level within 1 h. Together, these results suggest protein synthesis plays a pivotal role in the mechanism through which NE regulates the MKK3/6 → p38MAPK signaling in the rat pineal gland. However, the protein being synthesized is neither MKK3/6 nor p38MAPK since no difference in protein or mRNA level is observed after treatment with NE, cycloheximide or actinomycin. A lack of effect of these inhibitors on the protein levels of MKK3/6 and p38MAPK also suggests a slow turnover rate of these proteins in the rat pineal gland since their mechanisms of activation involve phosphorylation of existing protein rather than synthesis of new protein [24]. Although the identity of the protein whose synthesis is required as part of the activation mechanism of p38MAPK is not known, parallel increases in p-MKK3/6 and p-p38MAPK suggest that this protein likely exerts its effect upstream of MKK3/6.

The critical role of protein turnover in regulating the MKK3/6 → p38MAPK signaling pathway is also demonstrated by the use of proteasome inhibitors. Treatment with either MG132 or c-lact alone is effective in activating the

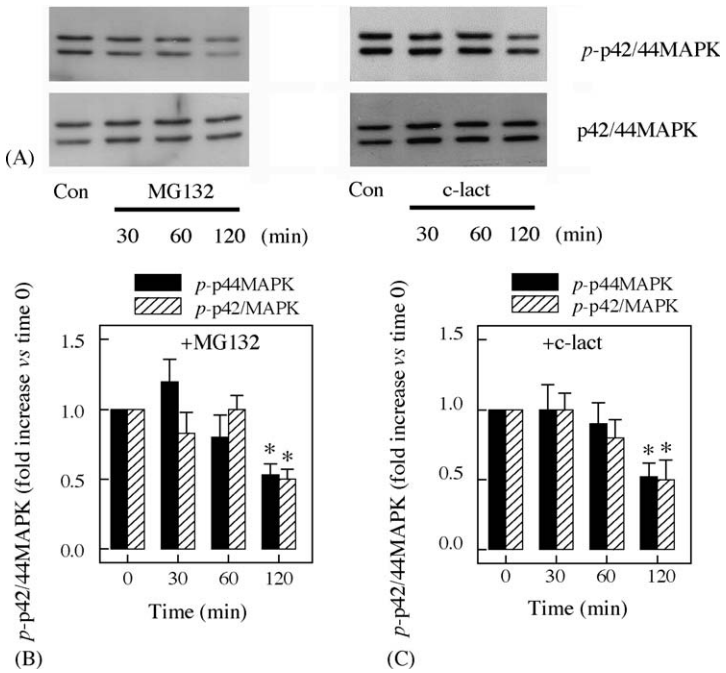

Fig. 6. Effects of MG132 and c-lact on p42/44MAPK activation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with MG132 (10 μM) or c-lact (10 μM) for different time periods as indicated. The cells were then collected by centrifugation and prepared for Western blot analysis as described in Section 2. (A) Immunoblots of p-42/44MAPK and p42/44MAPK in pinealocytes treated as indicated. The blots presented are representative of at least three separate experiments. (B) Graphical summary from experimental replicates of densitometric values from Western blots for p-42/44MAPK.

MKK3/6 → p38MAPK pathway without affecting total MKK3 and p38MAPK protein levels. Moreover, parallel increases in p-MKK3/6 and p-p38MAPK after treatment with proteasome inhibitors also indicate a site of action upstream of MKK 3/6, similar to that of cycloheximide or actinomycin. Taken together, it is likely that the same protein is being manipulated by inhibitors of transcription, translation and proteasomes to mediate both an activating and an inhibiting effect on the MKK3/6 → p38MAPK pathway.

We have previously shown that continuous β-adrenergic stimulation is required during the sustained phase of the NE-stimulated p38MAPK activation [24]. Results from studies with the proteasome inhibitor, MG132, indicate that proteasomal inhibition can reverse the β-adrenergic blockade-mediated decline in both p-MKK3/6 and p-p38MAPK. Thus the decline in activation of the p38MAPK signaling pathway following β-adrenergic blockade is due to proteasomal degradation of an upstream activating factor. In other tissues, MKP-1 has been suggested as a critical negative regulator of MAPKs, and p38MAPK is the preferred substrate [31,32]. In pinealocytes, although *mkp-1* is induced by NE [6,7], our result is against the contribution of this phosphatase to p38MAPK activation because blockade of proteasomes alone is sufficient to prevent the propranolol-mediated decline of p38MAPK activation. In contrast, based on the time profiles of *mkp-1* induction and activation of p42/44MAPK by NE [6], this phosphatase could be involved in the decline of p-p42/44MAPK observed at 2 h after treatment with proteasome inhibitors because these inhibitors produce rapid accumulation of MKP-1 in rat pinealocytes [our unpublished observations].

Activation of p38MAPK by proteasome inhibitors has been reported previously [33–35] but the precise mechanism that links proteasome inhibition to p38MAPK activation remains unclear. MAPK activation has been suggested as part of the cellular response to stress induced by proteasomal inhibition [33]. In rat pinealocytes, proteasomal inhibition is only effective in activating p38MAPK but has no effect on p42/44MAPK activation. Moreover, proteasomal inhibition is equally effective in abolishing the decline of p38MAPK activation induced by cycloheximide, a protein synthesis inhibitor. These results suggest that the activation state of p38MAPK signaling pathway reflects a balance between synthesis and degradation of an upstream activating protein. It remains speculative whether this is the same protein that mediates the effects of NE on p38MAPK.

Established upstream kinases that can phosphorylate MKK3/6 in other cell types include MEKK3, MEKK4, MEKK5, transforming growth factor β-activating kinase and p21-activated kinase [36,37]. However, the identity of the protein that functions as MKK3/6 kinase in the rat pineal gland is not known. Nevertheless, our results suggest that this pineal protein, like AA-NAT, is tightly

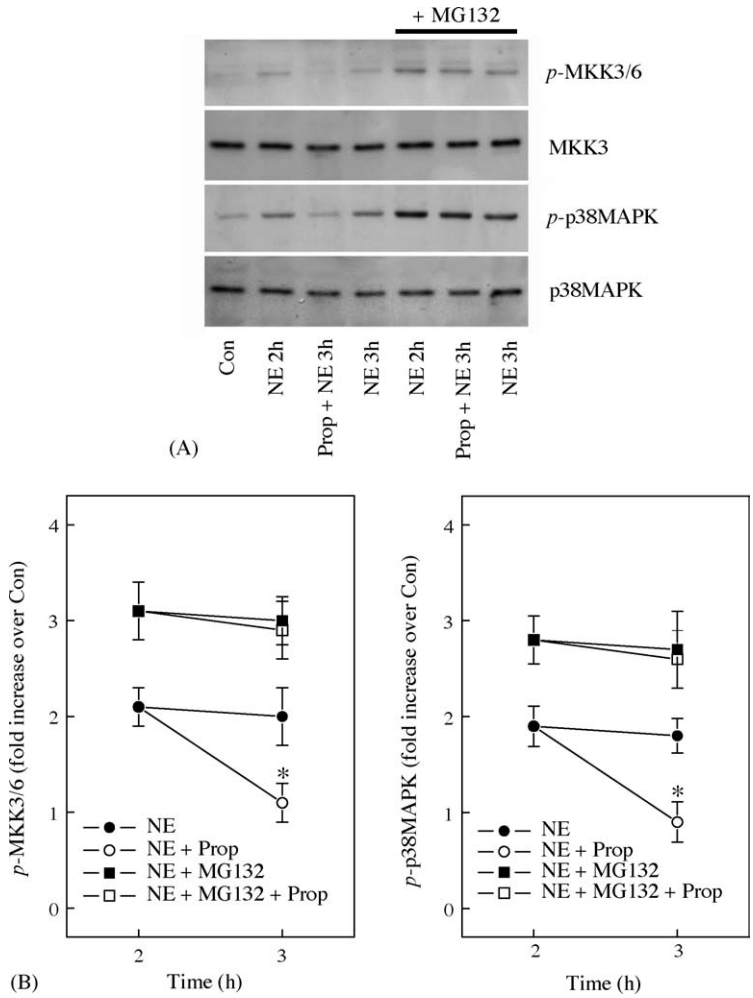

Fig. 7. Effects of MG132 on the inhibitory effect of propranolol on p38MAPK activation during NE stimulation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with NE (3 μM) or NE with MG132 (1 μM) for 2 h before addition of propranolol (10 μM; Prop) and incubated for an additional 1 h. The cells were then collected by centrifugation and prepared for Western blot analysis as described in Section 2. (A) Immunoblots of p-MKK3, MKK3, p-p38MAPK and p38MAPK from pinealocytes treated as indicated. The blots presented are representative of at least 3 separate experiments. (B) Graphical summary from experimental replicates of densitometric values from Western blots for p-MKK3 and p-p38MAPK. *Significantly different from NE treatment at 2 or 3 h ($p < 0.05$).

regulated by NE-mediated induction as well as proteasome-mediated degradation. However, without knowing the identity of the upstream kinase involved, it is possible that proteasome inihibitors can have a direct effect on the kinase activity instead of changing the protein level.

In regards to signaling mechanism, although Gαq11 or Gβγ is involved in G-protein coupled receptor-mediated activation of MAPK signaling pathways in other cell types [38–40], the signaling mechanism that regulates NE activation of p38MAPK in the rat pinealocyte is the cAMP/PKA pathway [24]. Based on our results, it will be important to determine if protein synthesis is also involved in adrenergic → cAMP-mediated p38MAPK activations in other cell types.

Our result is of special interest with respect to the transduction of environmental lighting information to the output of melatonin in the rat pineal gland [1,2]. The participation of p38MAPK in regulating melatonin synthesis is suggested by our earlier studies, which show that inhibition of this signaling pathway during NE stimulation amplifies AA-NAT induction and melatonin synthesis in rat pinealocytes [12]. Moreover, the enhancing effect of p38MAPK inhibition on melatonin synthesis can only be observed at least 3 h after NE stimulation. In the rat pineal gland, activation of the p38MAPK signaling pathway occurs several hours after onset of darkness. This endogenous time profile of p38MAPK activation, besides suggesting a requirement for synthesis of an upstream activator of this pathway, also supports its role in limiting the amplitude and duration of the melatonin response as demonstrated in our cell culture studies. Therefore, activation of the p38MAPK pathway in the rat pineal gland likely help shapes the nocturnal melatonin signal.

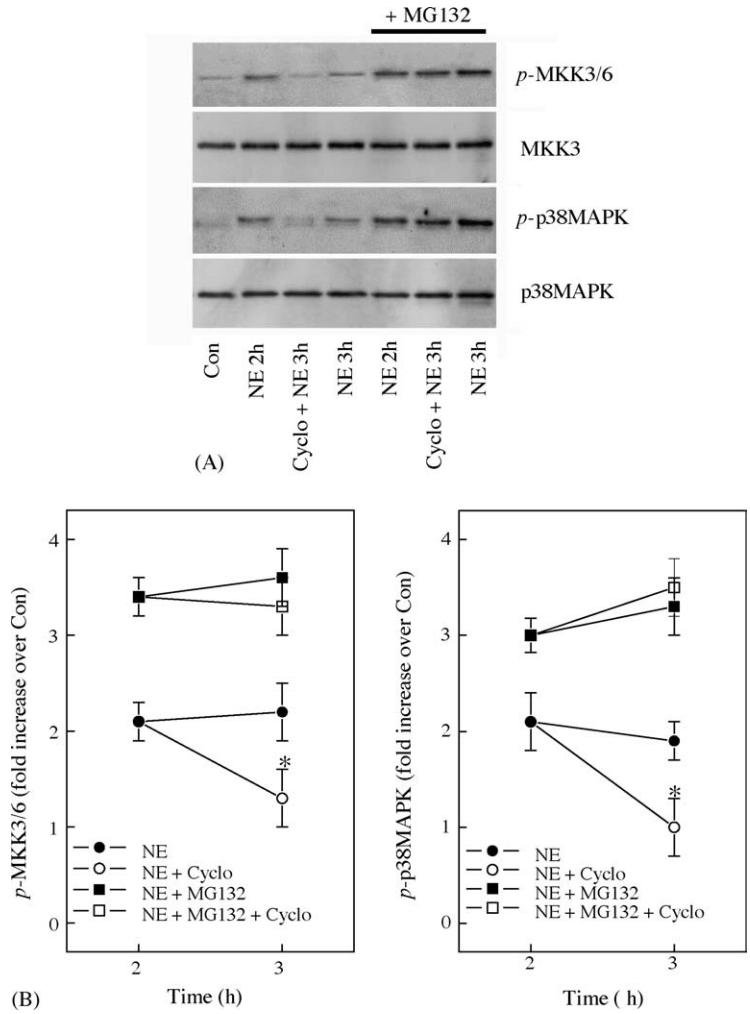

Fig. 8. Effects of MG132 on the inhibitory effect of cycloheximide on p38MAPK activation during NE stimulation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with NE (3 μM) or NE with MG132 (1 μM) for 2 h before addition of cycloheximide (15 μg/ml; Cyclo) and incubated for an additional 1 h. The cells were then collected by centrifugation and prepared for Western blot analysis as described in Section 2. (A) Immunoblots of p-MKK3, MKK3, p-p38MAPK and p38MAPK from pinealocytes treated as indicated. The blots presented are representative of at least three separate experiments. (B) Graphical summary from experimental replicates of densitometric values from Western blots for p-MKK3 and p-p38MAPK. *Significantly different from NE treatment at 2 or 3 h ($p < 0.05$).

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