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Inhibition of p38 Mitogen-Activated Protein Kinase Enhances Adrenergic-Stimulated Arylalkylamine *N*-Acetyltransferase Activity in Rat Pinealocytes

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We have previously shown that inhibition of $p38^{MAPK}$ increases adrenergic-stimulated $p42/44^{MAPK}$ activation in rat pinealocytes. In this study we investigated whether $p38^{MAPK}$ played a role in the adrenergic regulation of arylalkylamine-*N*-acetyltransferase (AA-NAT) induction and melatonin (MT) synthesis. Treatment of pinealocytes with norepinephrine (NE) caused a time-dependent increase in the levels of *AA-NAT* mRNA, AA-NAT protein, and enzymatic activity as well as MT production. Cotreatment with SB202190, a selective $p38^{MAPK}$ inhibitor, although having no effect on AA-NAT activity or protein level 3 h after NE treatment, caused a sustained increase in AA-NAT activity and protein level after 6 h of NE treatment. The increases in NE-stimulated AA-NAT activity and protein level by SB202190 occurred in the absence of an increase in *AA-NAT* mRNA. Similar results were obtained when AA-NAT was induced by $(Bu)_2$cAMP or when SB203580 was used to inhibit $p38^{MAPK}$. In comparison, SB202474, the inactive analog, had no effect on NE or $(Bu)_2$cAMP-stimulated AA-NAT activity or protein level. SB202190 also increased cumulative NE-stimulated MT production, provided that the medium was supplemented with 5-methoxytryptamine. $p38^{MAPK}$ inhibitors had no effect on hydroxyindole-*O*-methyltransferase activity. These results show that inhibition of $p38^{MAPK}$, although having no effect on cAMP-mediated *AA-NAT* transcription, appears to increase AA-NAT activity either by increasing translation or by reducing degradation of the AA-NAT protein. The lack of effect on NE-stimulated MT accumulation by $p38^{MAPK}$ inhibitors in the absence of 5-methoxytryptamine could be secondary to a lack of substrate, or alternatively, hydroxyindole-*O*-methyltransferase may become limiting. (*Endocrinology* 145: 1167–1174, 2004)

THE RAT PINEAL gland is stimulated by the nightly release of norepinephrine (NE) from the sympathetic nerve terminals (1). NE, by activating both α- and β-adrenergic receptors, causes 100-fold increases in cAMP and cGMP accumulation (1). The primary function of cAMP in the rat pineal gland is to initiate a series of cellular processes that lead to the synthesis of arylalkylamine-*N*-acetyltransferase (AA-NAT), the rate-limiting enzyme in synthesis of the pineal hormone, melatonin (MT) (2). These cellular events include activation of cAMP-dependent protein kinase and phosphorylation of the transcription factor cAMP response element-binding protein (CREB), followed by an up to 150-fold increase in transcription of *AA-NAT* mRNA (2). At the posttranslational level, cAMP also plays an important role in maintaining AA-NAT activity by switching the fate of AA-NAT protein from destruction by proteasomal proteolysis (3) to protection and activation (4). Increased AA-NAT activity results in an elevation of the intracellular concentration of *N*-acetylserotonin, which is converted to MT by hydroxyindole-*O*-methyltransferase (HIOMT) (5). In contrast to cAMP, the physiological importance of NE-stimulated cGMP accumulation in the pineal gland is not as well established. However, we have shown that the NE→cGMP pathway is the main signaling mechanism involved in activation of $p42/44^{MAPK}$ (6–8), a member of the MAPK family of kinases.

MAPKs, a large family of serine/threonine protein kinases, have important functions as mediators of signal transduction and are activated by diverse stimuli, such as cytokines, growth factors, neurotransmitters, hormones, and cellular stresses (9, 10). Three main groups of MAPKs have been identified: $p42/44^{MAPK}$ (11, 12), $p38^{MAPK}$ (13, 14), and c-Jun N-terminal kinase (15, 16). In rat pinealocytes, we have demonstrated activation of $p42/44^{MAPK}$ (6, 7) and its downstream kinase, the 90-kDa ribosomal S6 kinase by NE (8). Interestingly, we also found that NE activation of $p42/44^{MAPK}$ can be modulated by the state of $p38^{MAPK}$ activation (17). Using specific $p38^{MAPK}$ inhibitors, we showed that inhibition of $p38^{MAPK}$ amplifies adrenergic-stimulated $p42/44^{MAPK}$ phosphorylation (17). This finding is in agreement with the mounting evidence that cross-talk between the $p38^{MAPK}$ pathway and other members of the MAPK family represents an important mechanism of biological response regulation (18–20).

The observation that inhibition of $p38^{MAPK}$ can significantly affect the adrenergic-stimulated phosphorylation of $p42/44^{MAPK}$ suggests that it may also modulate other adrenergic-mediated events, such as AA-NAT induction. Moreover, because $p42/44^{MAPK}$ mediates its cellular responses through phosphorylation of nuclear transcription factors such as CREB (21–23), and CREB phosphorylation is important in the circadian activation of AA-NAT (24), $p38^{MAPK}$ may affect MT biosynthesis in the pineal gland. To

Abbreviations: AA-NAT, Arylalkylamine-*N*-acetyltransferase; CREB, cAMP response element-binding protein; HIOMT, hydroxyindole-*O*-methyltransferase; MT, melatonin; NE, norepinephrine.

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explore this possibility, we investigated the effect of p38^{MAPK} inhibition on basal and stimulated AA-NAT induction and MT production.

Materials and Methods

Materials

$(Bu)_2$cAMP, NE, tryptamine hydrochloride, and antibodies against p38^{MAPK} and phosphorylated p38^{MAPK} were obtained from Sigma-Aldrich Corp. (St. Louis, MO). SB202190, SB 203580, and SB202474 were obtained from Calbiochem (San Diego, CA). [^{3}H]Acetyl-coenzyme A was purchased from NEN Life Science Products (Boston, MA). [^{3}H]MT (specific activity, 81.1 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Polyclonal antibodies for the RIA of MT were obtained from CIDTech Co. (Mississauga, Canada). Antibody against AA-NAT (AB3314) was a gift from Dr. D. C. Klein (NICHHD, NIH, Bethesda, MD). All other chemicals were of the purest grade available commercially.

Preparation of pinealocytes

All procedures related to animal usage were reviewed and approved by the health sciences animal and welfare committee of University of Alberta. Sprague Dawley rats (male; weighing 150 g) were obtained from the University of Alberta animal unit. Pinealocytes were prepared by trypsinization from freshly dissected rat pineal glands as described previously (25). Cells were suspended in DMEM containing 10% fetal calf serum and were 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 that had been prepared in concentrated solutions in water or dimethylsulfoxide 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, Valencia, CA) according to the manufacturer's instruction. Samples for Western blot analysis were solubilized in 1× sample buffer by boiling for 5 min and were stored until electrophoresis. The homogenization buffer contained 20 mM Tris-HCl; 2 mM EDTA; 0.5 mM EGTA; 2 mM phenylmethylsulfonylfluoride; 1 μg/ml each of aprotinin, leupeptin, and pepstatin; 1 mM sodium orthovanadate; and 1 mM sodium fluoride (pH 7.5). Samples for determination of NAT and HIOMT activities were immediately frozen in dry ice and stored at −75 C. Media were collected for MT determination.

RT-PCR analysis

First strand cDNA was synthesized using 1 μg total RNA, which had been treated with ribonucleaase-free deoxyribonuclease, by using the Superscript preamplification system (Life Technologies, Inc., Grand Island, NY) and oligo(deoxythymidine) primer according to the manufacturer's instructions. After first strand cDNA synthesis, PCR was performed in a 50-μl reaction mixture containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each deoxy-NTP, 1.25 U *Taq* polymerase (PerkinElmer Cetus, Emeryville, CA), and 50 pmol of a previously characterized primer pair for AA-NAT (26) (forward, TGTCTTGTGGCCTTCATCATCGG; reverse, TGCGCAGCTTGGGTCAGCAGCC). After denaturing for 3 min at 94 C, PCR was performed for 30 cycles (1 min at 94 C, 1 min at 63 C, and 1 min at 72 C). A final extension was carried out at 72 C for 5 min. Reference cDNA was generated from extracts of NE-treated pinealocytes incubated along with the experimental samples. Initial cDNA concentrations were confirmed by PCR reactions amplifying cytoplasmic β-actin as an internal standard (not shown). All reaction sets included water blanks as negative controls. The amplified products were separated on 1.5% agarose gels, and images were analyzed using a Kodak 2000R imaging station with Kodak 1-D software (Eastman Kodak Co., Rochester, NY).

Western blot

SDS-PAGE was performed according to the procedure of Laemmli (27) using 10% acrylamide (Mini-Protein II gel system, Bio-Rad Laboratories, Hercules, CA). After electrophoresis, gels were equilibrated for 20 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 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 1.5 h. The blots were then incubated overnight at 4 C with diluted specific antisera as indicated. After washing three times with blocking solution, blots were incubated with diluted with horseradish peroxidase-conjugated second antibodies (Bio-Rad Laboratories) for 1 h at room temperature. They were then washed extensively and developed using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Enzymatic assays

AA-NAT activity was determined as described previously (28). Briefly, treated pinealocytes were stored frozen in dry ice until homogenization in a reaction mixture of 0.1 M phosphate buffer (pH 6.8) containing 30 nmol [^{3}H]acetyl coenzyme A (specific activity, 1 mCi/mmol) and 1 μmol tryptamine hydrochloride in a final volume of 60 μl. The reaction mixture was incubated at 37 C for 1 h. At the end of the incubation period, the reaction was stopped by the addition of 1 ml methylene chloride. After vortexing, the aqueous phase was removed, and the organic phase was washed three times with 0.1 M phosphate buffer (pH 6.8). The organic phase was transferred to a scintillation vial and evaporated to dryness, and radioactive acetylated product was determined by scintillation counting. AA-NAT activity was expressed as nanomoles per hour per 10^5 cells. HIOMT activity was determined using a previously established method (29) similar to the AA-NAT assay. The reaction mixture used for the HIOMT assay was a 0.1 M phosphate buffer (pH 7.9) that contained 1 mM [^{3}H]*S*-adenosyl-L-methionine (specific activity, 5 mCi/mmol) and 1 mM *N*-acetylserotonin. The incubation conditions and product extraction are described above.

MT assay

Briefly, medium MT was extracted from 300 μl medium by vortexing with 1 ml methylene chloride. As 5-methoxytryptamine (0.1 mM) was added to the medium in selected experiments, washing the methylene chloride extracted medium five times with 0.25 ml 0.1 M HCl was included in the procedure. This washing effectively removed any interference of 5-methoxytryptamine in the MT RIA. After centrifugation, the organic phase was collected and evaporated to dryness. The residue was reconstituted in 500 μl assay buffer (0.01 M phosphate buffer, pH 6.5, containing 0.1% gelatin). The recovery of medium MT was greater than 98%. The extracted MT was assayed by RIA as described previously (29).

Statistical analysis

For the Western blots and RT-PCR results, a typical image from at least three similar experiments was presented. Results from the enzymatic assays and MT measurements were analyzed by paired *t* test or ANOVA with Duncan's multiple range test.

Results

Effect of p38^{MAPK} inhibitors on NE-stimulated AA-NAT activity

The effect of SB202190 (10 μM), a specific p38^{MAPK} inhibitor (30, 31), on the time profile of NE-stimulated AA-NAT activity is shown in Fig. 1A. Treatment with NE (1 μM) caused a significant increase in AA-NAT activity at 3 h ($P < 0.05$) that peaked at 6 h. The increase in AA-NAT activity was sustained for at least another 6 h and was followed by a gradual decline. SB202190 (10 μM) alone had no effect on AA-NAT activity. However, whereas the presence of SB202190 (10 μM) had no effect on NE-stimulated AA-NAT activity when measured 3 h after NE stimulation, it significantly enhanced (up to 70%) the NE-stimulated responses at later time points ($P <$ 0.05 between 6 and 21 h after NE stimulation). A concentration-response study indicated that treatment with SB202190

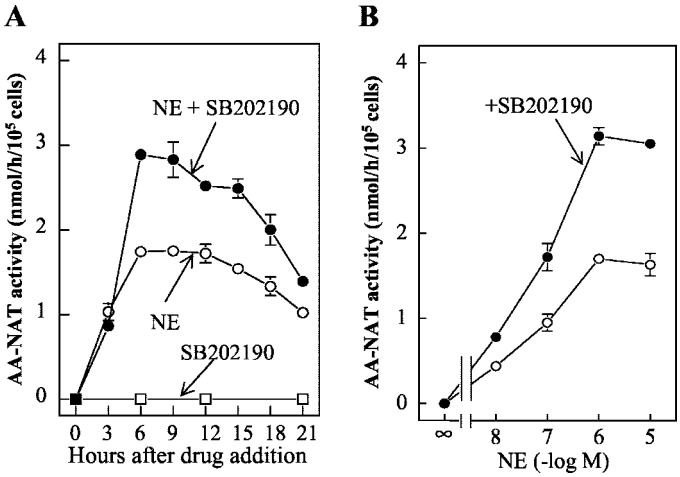

FIG. 1. Effect of a p38^{MAPK} inhibitor on NE-stimulated AA-NAT activity. Pinealocytes (5×10^4 cells/0.5 ml) were cultured for 24 h and treated with NE (1 μM) for different time periods (A) or different concentrations of NE for 12 h (B) in the presence or absence of SB202190 (10 μM) as indicated. The cells were then collected by centrifugation and assayed for AA-NAT activity as described in *Materials and Methods*. Each value represents the mean ± SEM of determinations from three independent experiments.

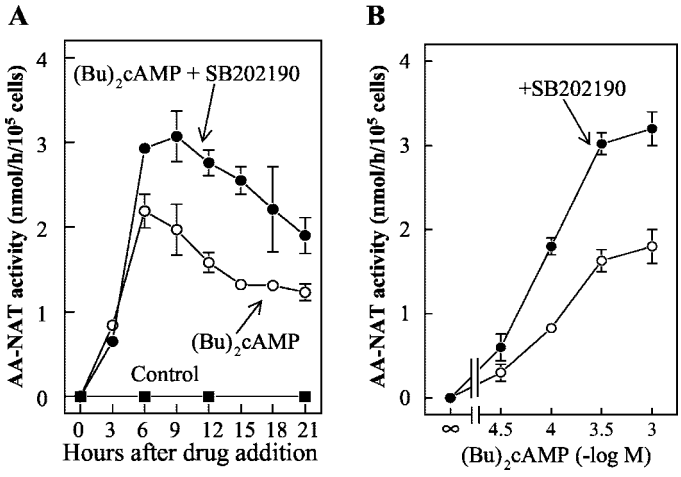

FIG. 2. Effect of a p38^{MAPK} inhibitor on (Bu)$_2$cAMP-stimulated AA-NAT activity. Pinealocytes (5×10^4 cells/0.5 ml) were cultured for 24 h and treated with (Bu)$_2$cAMP (1 mM) for different time periods (A) or different concentrations of (Bu)$_2$cAMP for 12 h (B) in the presence or absence of SB202190 (10 μM) as indicated. The cells were then collected by centrifugation and assayed for AA-NAT activity as described in *Materials and Methods*. Each value represents the mean ± SEM of determinations from three independent experiments.

TABLE 1. Effect of p38^{MAPK} inhibitors on NE- and (Bu)$_2$cAMP-stimulated NAT activity

Treatment	AA-NAT activity (nmol/h/10^5 cells)		
	0.5% DMSO	+ SB202474	+ SB203580
Control	ND	ND	ND
NE (1 μM)	1.81 ± 0.05	1.77 ± 0.11	2.75 ± 0.08[a]
(Bu)$_2$cAMP (1 mM)	1.65 ± 0.10	1.70 ± 0.12	2.65 ± 0.13[a]

Pinealocytes (1.5×10^4 cells/0.4 ml) were incubated in DMEM with 10% fetal bovine serum and treated with NE or (Bu)$_2$cAMP in the presence or absence of 10 μM SB202474 or 203580 for 12 h. ND, Not detectable; DMSO, dimethylsulfoxide. Each value represents the mean ± SEM of determinations from three independent experiments.

[a] Significantly different from corresponding group without SB203580 ($P < 0.05$).

caused an increase in NE-induced maximal AA-NAT activity (Fig. 1B). SB203580 (10 μM), another active p38^{MAPK} inhibitor, had a similar enhancing effect on NE-stimulated AA-NAT activity 12 h after drug addition (Table 1). In comparison, SB202474 (10 μM), the negative control, had no effect on NE-stimulated AA-NAT activity 12 h after drug addition (Table 1).

Effect of p38^{MAPK} inhibitors on (Bu)$_2$cAMP-stimulated AA-NAT activity

We have previously shown that SB202190 enhances NE-stimulated cAMP accumulation through inhibition of phosphodiesterase activity (32). To determine whether the effect of SB202190 on NE-stimulated AA-NAT activity was due to its reported enhancing effect on cAMP synthesis (32), pinealocytes were stimulated with (Bu)$_2$cAMP. Treatment with (Bu)$_2$cAMP (1 mM) caused a maximal increase in AA-NAT activity at 6 h, followed by a gradual decline (Fig. 2A). Cotreatment with SB202190 (10 μM), although having no effect 3 h after drug treatment, significantly increased (Bu)$_2$cAMP-stimulated AA-NAT by 30–90% between 6 and 21 h after addition of (Bu)$_2$cAMP (Fig. 2A). A concentration-response study showed that SB202190 caused a 75% increase in the (Bu)$_2$cAMP-induced maximal AA-NAT response (Fig. 2B). In comparison, SB202474 (10 μM), the negative control, did not have a significant effect on (Bu)$_2$cAMP-stimulated AA-NAT activity 12 h after drug addition (Table 1).

Effect of p38^{MAPK} inhibitors on NE-stimulated AA-NAT mRNA

To investigate the mechanism by which p38^{MAPK} inhibition increased NE-stimulated AA-NAT activity, pinealocytes were treated with NE (1 μM) in the presence or absence of SB202190 (10 μM) or SB202474 (10 μM) for 3, 6, 12, or 16 h. Cells were collected for *AA-NAT* mRNA determination by RT-PCR. Reference cDNA samples for each time point were prepared from pooled NE-treated pinealocyte extracts. Standard curves were plotted from in-gel densitometric analysis of reaction products from a graded series of reference cDNA concentrations. In the linear range of reference cDNA amplification, control cDNA samples generated from untreated pinealocyte extracts showed no detectable *AA-NAT* mRNA expression (Fig. 3). In comparison, treatment with NE (1 μM) caused a significant induction of *AA-NAT* mRNAs at 3, 6, 12, or 16 h (Fig. 3). Neither SB202190 (10 μM) nor SB202474 (10 μM) had an effect on *AA-NAT* mRNAs at any of the time points tested (Fig. 3). These results indicate that p38^{MAPK} does not appear to have an effect on NE-stimulated *AA-NAT* mRNA transcription.

Effect of p38^{MAPK} inhibitors on NE-stimulated AA-NAT protein

To determine whether the enhancing effect of SB202190 on agonist-stimulated AA-NAT activity was related to an increase in AA-NAT protein, pinealocytes were treated with NE (1 μM) for 3, 8, 12, and 16 h, and AA-NAT protein levels

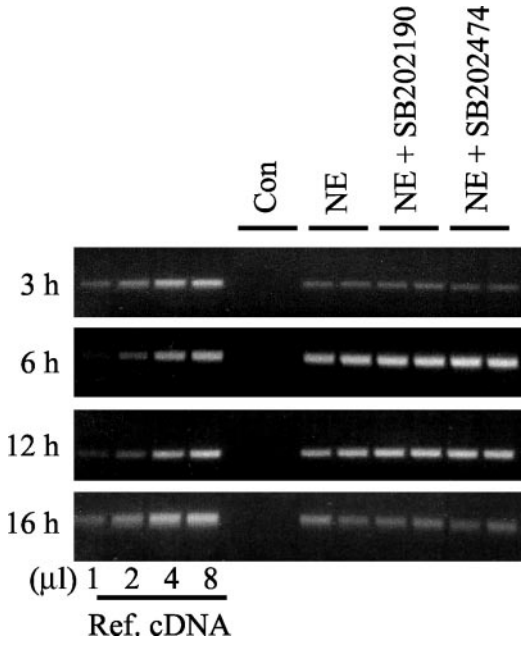

FIG. 3. Effect of p38^{MAPK} inhibitors on NE-stimulated *AA-NAT* mRNA. Pinealocytes (5×10^4 cells/0.5 ml) were cultured for 24 h and treated with NE (1 μM) for different time periods in the presence or absence of 10 μM SB 202190 or SB202474 as indicated. The cells were then collected by centrifugation, total RNA was isolated, and cDNA was prepared. RT-PCR was performed using *AA-NAT* primers. The gel presented is representative of three separate experiments. Reference cDNA samples for each time point were prepared from pooled NE-treated pinealocyte extracts. See *Materials and Methods* for details.

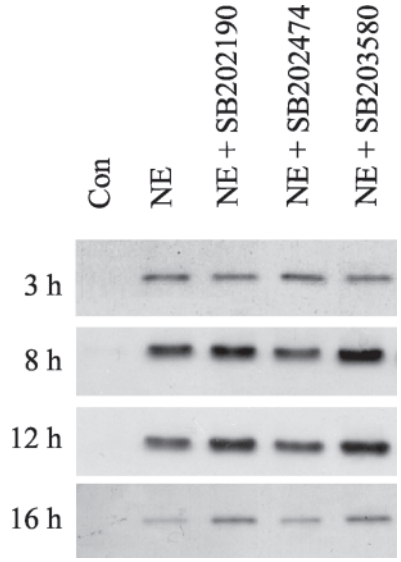

FIG. 4. Effects of p38^{MAPK} inhibitors on NE-stimulated AA-NAT protein. Pinealocytes (5×10^4 cells/0.5 ml) were cultured for 24 h and treated with NE (1 μM) for different time periods in the presence or absence of 10 μM SB202190, SB202474, or SB203580 as indicated. The cells were then collected by centrifugation, dissolved in 1× sample buffer, and analyzed by Western blotting using a specific antibody against AA-NAT protein as described in *Materials and Methods*. The blots presented are representative of three separate experiments.

were determined by a specific antibody (AB3314) using Western blot analysis. There was no detectable AA-NAT protein in control cells (Fig. 4). Treatment with NE (1 μM) caused an increase in AA-NAT protein at 3, 8, 12, and 16 h after drug treatment (Fig. 4). Cotreatment with SB202190 (10 μM) or SB203580 (10 μM) further increased NE-stimulated AA-NAT protein at 8, 12, and 16 h, but not at 3 h. In contrast, SB 202474 (10 μM) had no effect on NE-stimulated AA-NAT protein levels (Fig. 4). These results suggest that p38^{MAPK} inhibition leads to either increased translation of AA-NAT or decreased AA-NAT enzyme degradation.

Effect of a p38^{MAPK} inhibitor on NE-stimulated MT accumulation

The effect of SB202190 (10 μM) on the NE-stimulated MT accumulation is shown in Fig. 5. Treatment with NE (0.1–10 μM) caused a gradual concentration-dependent increase in MT accumulation when measured 12 h after drug addition. In contrast to its effect on NE-stimulated AA-NAT activity (Fig. 1), SB202190 (10 μM) did not have a significant effect on NE-stimulated MT production (Fig. 5A). These results suggest that either the increase in AA-NAT activity can only be observed in broken cell preparations *in vitro* or that other factors may have become rate limiting in the production of MT *in vivo*. To address this issue, the cell culture was supplemented with 5-methoxytrytamine. 5-Methoxytryptamine was selected as an alternate substrate because it can penetrate

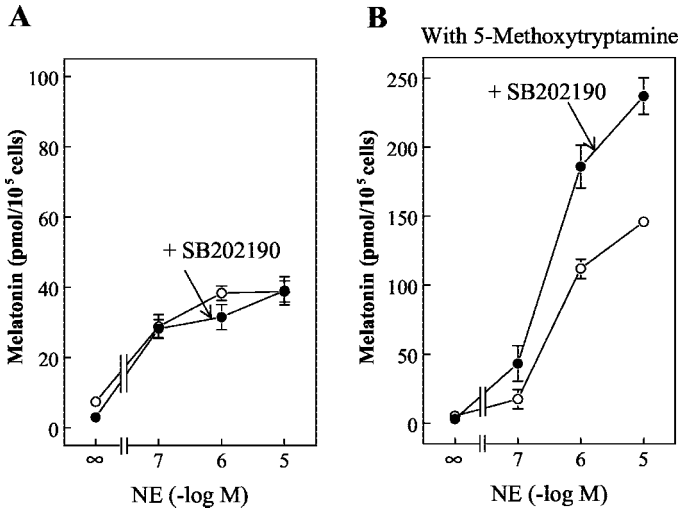

FIG. 5. Effect of a p38^{MAPK} inhibitor on the dose response of NE-stimulated MT accumulation. Pinealocytes (5×10^4 cells/0.5 ml) were cultured for 24 h and treated with different concentrations of NE for 12 h in the presence or absence of SB202190 (10 μM) as indicated in control medium (A) or medium supplemented with 0.1 mM 5-methoxytryptamine (B). After centrifugation, the medium was extracted with methylene chloride before MT determination by RIA as described in *Materials and Methods*. Each value represents the mean ± SEM of determinations from three independent experiments.

the pinealocyte easily and is converted to MT by AA-NAT, thus demonstrating AA-NAT activity *in vivo*.

In the presence of 5-methoxytryptamine (0.1 mM), not only was the MT response to NE stimulation alone substantially higher, but cotreatment with SB202190 was also effective in further increasing NE-stimulated MT accumulation (Fig. 5B). By comparing Fig. 6, A and B, it was evident that the presence

of 5-methoxytryptamine by itself had little effect on AA-NAT activity. A similar result was obtained when the effect of SB202190 (10 μM) on the time profile of NE-stimulated MT accumulation was investigated. As shown in Fig. 7, treatment with NE (1 μM) caused a gradual increase in MT accumulation up to 21 h after drug treatment. SB202190 (10 μM) further increased the NE-stimulated MT accumulation provided that 5-methoxytryptamine was present in the culture medium. These results suggest that in the absence of 5-methoxytryptamine supplementation, the discordance between the NE-stimulated increase in AA-NAT activity and MT output after SB202190 treatment (Figs. 5A and 7A) could be related to reduced HIOMT activity or substrate availability.

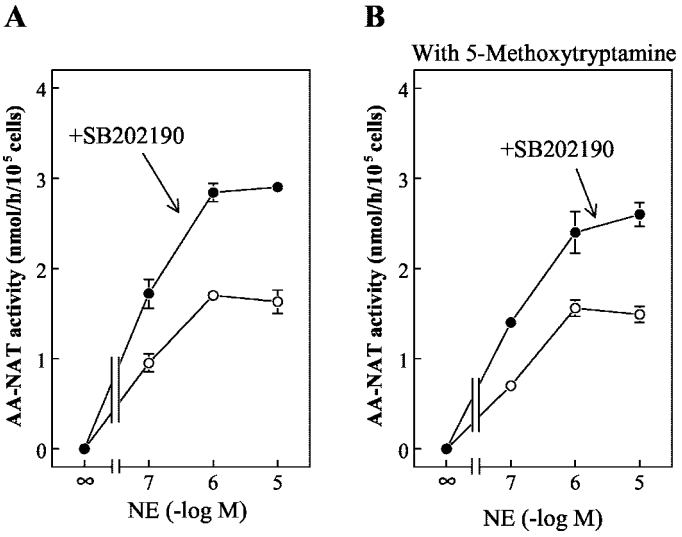

FIG. 6. Effect of a p38^{MAPK} inhibitor on the dose response of NE-stimulated AA-NAT activity. Pinealocytes (5×10^4 cells/0.5 ml) were cultured for 24 h and treated with different concentrations of NE for 12 h in the presence or absence of SB202190 (10 μM) as indicated in control medium (A) or medium supplemented with 0.1 mM 5-methoxytryptamine (B). The cells were then collected by centrifugation and assayed for AA-NAT activity as described in *Materials and Methods*. Each value represents the mean ± SEM of determinations from three independent experiments.

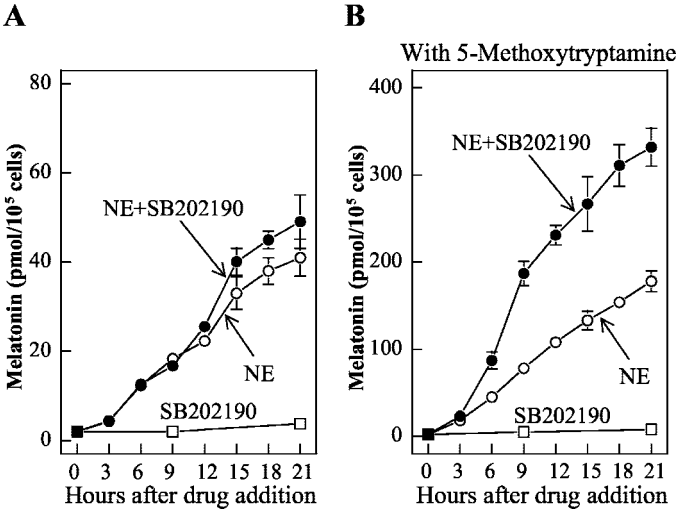

FIG. 7. Effect of a p38^{MAPK} inhibitor on the time-course of NE-stimulated MT accumulation. Pinealocytes (5×10^4 cells/0.5 ml) were cultured for 24 h and treated with NE (1 μM) for different time periods in the presence or absence of SB202190 (10 μM) as indicated in control medium (A) and medium supplemented with 0.1 mM 5-methoxytryptamine (B). After centrifugation, the medium was extracted with methylene chloride before MT determination by RIA as described in *Materials and Methods*. Each value represents the mean ± SEM of determinations from three independent experiments.

Effect of p38^{MAPK} inhibitors on HIOMT activity

Because the discrepancy between the agonist-stimulated AA-NAT activity and MT accumulation (Figs. 5A and 7A) could be related to inhibition of HIOMT activity, the *in vitro* and *in vivo* effects of p38^{MAPK} inhibitors on HIOMT activity were determined. Addition of 10 μM SB202190, SB203580, or SB202474 directly to the reaction mixture had no effect on pinealocyte HIOMT activity (Fig. 8). Moreover, when pinealocytes were treated with SB202190, SB203580, or SB202474 (10 μM) for 12 h, none of the treatments had a significant effect on HIOMT activity (Fig. 8). These results suggest that p38^{MAPK} inhibitors do not have a direct effect on HIOMT activity *in vitro* or *in vivo*.

Effect of NE on p38^{MAPK} activation in rat pinealocytes

To provide support for a role of p38^{MAPK} activation in the regulation of AA-NAT induction, the effect of NE on p38^{MAPK} activation in rat pinealocytes was determined. Western blot analysis showed that treatment with NE (3 μM) caused a time-dependent increase in phosphorylation of p38^{MAPK} without a change in p38^{MAPK} protein levels (Fig. 9). The increase in phosphorylated p38^{MAPK} was observed after 1 h of NE stimulation and was sustained for more than 4 h.

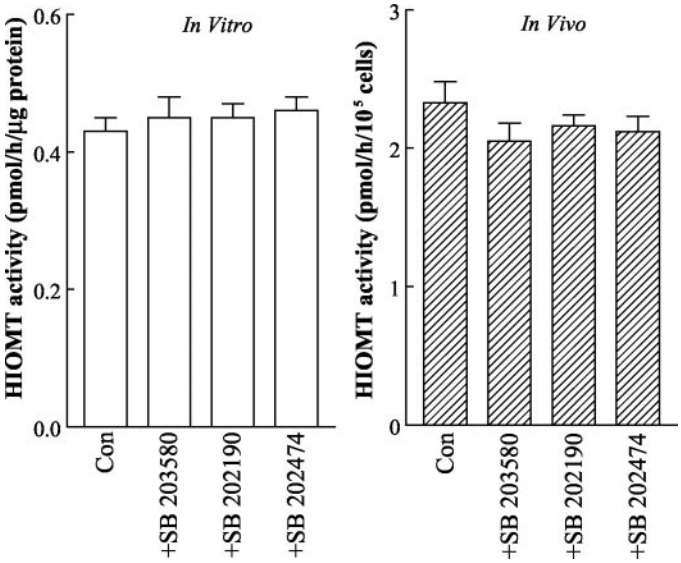

FIG. 8. Effect of p38^{MAPK} inhibitors on HIOMT activity. In the *in vitro* experiment, 3×10^6 pinealocytes were collected by centrifugation and homogenized in HIOMT assay buffer. An aliquot of the homogenate was used for HIOMT activity determination in the absence or presence of 10 μM SB202190, SB203580, or SB202474 in the reaction mixture as indicated. In the *in vivo* experiment, pinealocytes were cultured for 24 h and treated with 10 μM p38^{MAPK} inhibitor for 12 h as indicated. The cells were then collected by centrifugation and assayed for HIOMT activity as described in *Materials and Methods*. Each value represents the mean ± SEM of determinations from three independent experiments.

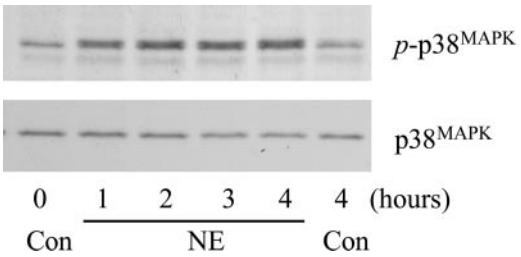

FIG. 9. Effect of NE on phosphorylated p38^{MAPK} in rat pinealocytes. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with NE (10 μM) for different time periods as indicated. The cells were collected by centrifugation, dissolved in 1× sample buffer and analyzed by Western blotting using an antibody against phosphorylated p38^{MAPK} (*p*-p38^{MAPK}) as described in *Materials and Methods*. The immunoblot was stripped and reprobed with specific antibodies against total p38^{MAPK}. The blot presented is representative of three separate experiments.

These results suggest that activation of p38^{MAPK} is a natural event that occurs after adrenergic stimulation in the rat pineal gland.

Discussion

In this study we provided experimental evidence that activation of p38^{MAPK} can modulate NE-stimulated AA-NAT activity in rat pinealocytes. Treatment with SB202190 or SB203580, two specific inhibitors of p38^{MAPK}, but not with their inactive analog, SB202474, has an enhancing effect on NE-stimulated AA-NAT activity. Together with the observation that NE activates p38^{MAPK} in a sustained manner, these results suggest that p38^{MAPK} is part of the mechanism by which NE regulates AA-NAT activity in rat pinealocytes.

Previously we have shown that p38^{MAPK} inhibitors can inhibit phosphodiesterase activity in rat pinealocytes, resulting in enhanced cyclic nucleotide accumulation (32). The mechanism involved appears to be independent of p38^{MAPK} inhibition, because SB202474, the compound that is inactive against p38^{MAPK}, is equally effective in this capacity as SB202190 and SB203580, the two active compounds (32). In contrast, the effect of p38^{MAPK} inhibitors on AA-NAT appears to be dependent on p38^{MAPK} inhibition and independent of phosphodiesterase inhibition because the enhancing effect on agonist-stimulated AA-NAT activity is only observed with SB202190 and SB203580, the two active compounds. Moreover, SB202190 remains effective in enhancing AA-NAT activity when cells are maximally stimulated by a membrane-permeable cAMP analog, $(Bu)_2$cAMP. Taken together, these results suggest that p38^{MAPK} inhibitors increase agonist-stimulated AA-NAT activity at a post-cAMP step that is involved in the synthesis of AA-NAT.

Signaling pathways activated by members of the MAPK family represent important intracellular mechanisms that transmit extracellular signals into target gene expression (for review, see Refs. 33 and 34). Activated p38^{MAPK} is known to phosphorylate transcription factors and kinases involved in the regulation of mRNA synthesis (35; for review, see Ref. 33). In addition, p38^{MAPK} has been shown to cause stabilization of mRNAs (33, 36). However, our results indicate that the effect of p38^{MAPK} inhibition on AA-NAT is probably not due to changes in the transcription of *AA-NAT*, because neither p38^{MAPK} inhibitor has an effect on *AA-NAT* mRNA levels up to 16 h after NE stimulation. In comparison, the two p38^{MAPK} inhibitors produce a sustained increase in the amount of AA-NAT protein from 8–16 h after NE stimulation. Moreover, p38^{MAPK} inhibitors increase the maximal fold AA-NAT response that can be elicited by NE or $(Bu)_2$cAMP. These results point toward a posttranscriptional site of action of p38^{MAPK} inhibitors on AA-NAT regulation. This is also supported by the observation of an enhancing effect of SB202190 on AA-NAT activity at 6 h, but not at 3 h, after NE or $(Bu)_2$cAMP stimulation. Posttranscriptional regulation of gene expression by p38^{MAPK} has previously been demonstrated in other cell types (for review, see Ref. 33).

Another possible mechanism that can account for our observations of the effect of p38^{MAPK} inhibitors on AA-NAT is that p38^{MAPK} may regulate the degradation of AA-NAT protein. Degradation of AA-NAT protein has been shown to be controlled by proteasomal proteolysis (3). The rapid decline of AA-NAT that follows removal of NE is blocked by treatment with a proteasomal inhibitor (3). Moreover, complex formation of 14-3-3 with AA-NAT has also been shown to prevent degradation of the AA-NAT enzyme (4). Therefore, it is possible that p38^{MAPK} inhibitors may reduce the degradation of the AA-NAT protein either by enhancing its binding with 14-3-3 or by reducing proteosomal-dependent AA-NAT degradation. In this regard, activation of other members of the MAPK family has been shown to promote phosphorylation and proteasome-dependent proteolysis (37). Therefore, it would be of interest to determine whether AA-NAT or peptides containing one of the other cyclic nucleotide kinase sites involved in binding to 14-3-3 are phosphorylated by p38^{MAPK}.

Our results also suggest that during NE stimulation, activation of p38^{MAPK}, apart from initiation of the transcription and translation of AA-NAT, also sets into motion a series of cellular events that limit the maximal response attainable poststimulation. This is not a new concept, as the inhibitory transcription factor inducible cAMP early repressor has also been shown to be stimulated by NE (38). The degree of enhancement of *AA-NAT* transcription by CREB phosphorylation (24, 39) is inversely linked to inducible cAMP early repressor, which competes with CREB for CRE sites on the *AA-NAT* promoter (38, 40). Our findings of p38^{MAPK} inhibition on AA-NAT add to the importance of this concept by indicating that a negative signal also operates at the translational level and limits the magnitude of the AA-NAT response poststimulation.

Similar to previous studies (41, 42), our results showed that NE treatment causes parallel increases in AA-NAT activity and MT accumulation. However, the increase in NE-stimulated AA-NAT activity produced by p38^{MAPK} inhibition alone is not accompanied by a corresponding increase in MT production. Our results suggest that the lack of effect on NE-stimulated MT accumulation by p38^{MAPK} inhibitors could be secondary to a lack of substrate because of the prolonged incubation time, or alternatively, HIOMT may become limiting. In the presence of 5-methoxytryptamine, which acts as an alternate substrate for conversion to MT by

AA-NAT, SB202190 has an enhancing effect on NE-stimulated MT production that parallels its effect on NE-stimulated AA-NAT activity, confirming that SB202190 enhances NE-stimulated AA-NAT activity in intact cells. However, we could not exclude the possibility that HIOMT may become limiting even though p38^{MAPK} inhibition has no effect on HIOMT activity. Interestingly, HIOMT has been reported to drive the photoperiodic changes in the amplitude of the MT peak in Siberian hamsters (43). However, in rats there is only a small nocturnal increase in HIOMT activity (44).

Recently, p38^{MAPK} has been shown to regulate the oscillation of AA-NAT in the chick pineal gland (45). Our results indicate that p38^{MAPK} also plays an important role in regulating rat pineal function. This is based on the observations that NE can activate p38^{MAPK} and that inhibition of p38^{MAPK} amplifies the NE-stimulated AA-NAT protein and enzymatic activity. Together, these findings suggest that activation of p38^{MAPK} is part of the negative signal used by the pineal gland to control the magnitude of the AA-NAT response to adrenergic stimulation. To better understand the mechanism by which p38^{MAPK} affects AA-NAT activity, it will be of interest to distinguish whether inhibition of p38^{MAPK} has an effect on the translation efficiency or degradation of the AA-NAT protein.

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References

1. **Klein DC** 1985 Photoneural regulation of the mammalian pineal gland. In: Evered D, Clark S, eds. Photoperiodism, melatonin and the pineal. Ciba Foundation Symposium. London: Pitman; vol 117:38–56
2. **Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone PM, Rodriguez IR, Begay V, Falcon J, Cahill GM, Cassone VM, Baler R** 1997 The melatonin rhythm-generating enzyme: molecular regulation of serotonin *N*-acetyltransferase in the pineal gland. Recent Prog Horm Res 52:307–357
3. **Gastel JA, Roseboom PH, Rinaldi PA, Weller JL, Klein DC** 1998 Melatonin production: proteasomal proteolysis in serotonin *N*-acetyltransferase regulation. Science 279:1358–1360
4. **Ganguly S, Gastel JA, Weller JL, Schwartz C, Jaffe H, Namboodiri MA, Coon SL, Hickman AB, Rollag M, Obsil T, Beauverger P, Ferry G, Boutin JA, Klein DC** 2001 Role of a pineal cAMP-operated arylalkylamine *N*-acetyltransferase/14–3-3-binding switch in melatonin synthesis. Proc Natl Acad Sci USA 98:8083–8088
5. **Klein DC** 1974 Circadian rhythms in indole metabolism in the rat pineal gland. In: Schmitt FO, Worden FG, eds. The neurosciences: third study program. Cambridge: MIT Press; 509–516
6. **Ho AK, Hashimoto K, Chik CL** 1999 3′,5′-Cyclic guanosine monophosphate activates mitogen-activated protein kinase in rat pinealoctyes. J Neurochem 73:598–604
7. **Ho AK, Chik CL** 2000 Adrenergic regulation of mitogen-activated protein kinase in rat pinealocytes: opposing effects of protein kinase A and protein kinase G. Endocrinology 141:4496–4502
8. **Ho AK, Mackova M, Cho C, Chik CL** 2003 Regulation of 90-kilodalton ribosomal S6 kinase phosphorylation in the rat pineal gland. Endocrinology 144:3344–3350
9. **Seger R, Krebs EG** 1995 The MAPK signaling cascade. FASEB J 9:726–735
10. **Cobb MH** 1999 MAP kinase pathways. Prog Biophys Mol Biol 71:479–500
11. **Ray LB, Sturgill TW** 1987 Rapid stimulation by insulin of a serine/threonine kinase in 3T3–L1 adipocytes that phosphorylates microtubule-associated protein 2 in vitro. Proc Natl Acad Sci USA 84:1502–1506
12. **Boulton TG, Yancopoulos GD, Gregory JS, Slaughter C, Moomaw C, Hsu J, Cobb MH** 1990 An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science 249:64–67
13. **Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, Han J** 1996 Characterization of the structure and function of a new mitogen-activated protein kinase (p38β). J Biol Chem 271:17920–17926
14. **Stein B, Yang MX, Young DB, Janknecht R, Hunter T, Murray BW, Barbosa MS** 1997 p38–2, a novel mitogen-activated protein kinase with distinct properties. J Biol Chem 272:19509–19517
15. **Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR** 1991 Phosphorylation of c-jun mediated by MAP kinases. Nature 353:670–674
16. **Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, Derijard B, Davis RJ** 1996 Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J 15:2760–2770
17. **Mackova M, Man JR, Chik CL, Ho AK** 2000 p38^{MAPK} inhibition enhances basal and norepinephrine-stimulated p42/p44^{MAPK} phosphorylation in rat pinealocytes. Endocrinology 141:4202–4208
18. **Alexandrov A, Keffel S, Goepel M, Michel MC** 1998 Stimulation of α_{1A}-adrenoceptors in Rat-1 cells inhibits extracellular signal-regulated kinase by activating p38 mitogen-activated protein kinase. Mol Pharmacol 54:755–760
19. **Singh RP, Dhawan P, Golden C, Kapoor GS, Mehta KD** 1999 One-way cross-talk between p38^{MAPK} and p42/44^{MAPK}. Inhibition of p38^{MAPK} induces low density lipoprotein receptor expression through activation of the p42/44^{MAPK} cascade. J Biol Chem 274:19593–19600
20. **Rolli-Derkinderen M, Machavoine F, Baraban JM, Grolleau A, Beretta L, Dy M** 2003 ERK and p38 inhibit the expression of 4E-BP1 repressor of translation through induction of Egr-1. J Biol Chem 278:18859–18867
21. **Rosenberger SF, Finch JS, Gupta A, Bowden GT** 1999 Extracellular signal-regulated kinase 1/2-mediated phosphorylation of JunD and FosB is required for okadaic acid-induced activator protein 1 activation. J Biol Chem 274:1124–1130
22. **Xing J, Kornhauser JM, Xia Z, Thiele EA, Greenberg ME** 1998 Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Mol Cell Biol 18:1946–1955
23. **Zanassi P, Paolillo M, Feliciello A, Avvedimento EV, Gallo V, Schinelli S** 2001 cAMP-dependent protein kinase induces cAMP-response element-binding protein phosphorylation via an intracellular calcium release/ERK-dependent pathway in striatal neurons. J Biol Chem 276:11487–11495
24. **Roseboom PH, Klein DC** 1995 Norepinephrine stimulation of pineal cyclic AMP response element-binding protein phosphorylation: primary role of a β-adrenergic receptor/cyclic AMP mechanism. Mol Pharmacol 47:439–449
25. **Buda M, Klein DC** 1978 A suspension culture of pinealocytes: regulation of *N*-acetyltransferase activity. Endocrinology 103:1483–1493
26. **Pfeffer M, Stehle JH** 1998 Ontogeny of a diurnal rhythm in arylalkylamine-*N*-acetyltransferase mRNA in rat pineal gland. Neurosci Lett 248:163–166
27. **Laemmli UK** 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
28. **Ho AK, Grota LJ, Brown GM** 1984 Relationship between pineal *N*-acetyltransferase activity, pineal melatonin and serum melatonin in rats under different lighting conditions. Neuroendocrinology 39:465–470
29. **Ho AK, Smith JA** 1982 Effect of benserazide on the levels of pineal 5-hydroxytryptamine, melatonin synthesizing enzymes and serum melatonin. Biochem Pharmacol 31:2251–2255
30. **Young PR, McLaughlin MM, Kumar S, Kassis S, Doyle ML, McNulty D, Gallagher TF, Fisher S, McDonnell PC, Carr SA, Huddleston MJ, Seibel G, Porter TG, Livi GP, Adams JL, Lee JC** 1997 Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. J Biol Chem 272:12116–12121
31. **Hazzalin CA, Cano E, Cuenda A, Barratt MJ, Cohen P, Mahadevan LC** 1996 p38/RK is essential for stress-induced nuclear responses: JNK/SAPKs and c-Jun/ATF-2 phosphorylation are insufficient. Curr Biol 6:1028–1031
32. **Ho AK, Price L, Mackova M, Chik CL** 2001 Potentiation of cAMP and cGMP accumulation by p38 mitogen-activated protein kinase inhibitors in rat pinealocytes. Biochem Pharmacol 62:1605–1611
33. **Clark AR, Dean JL, Saklatvala J** 2003 Post-transcriptional regulation of gene expression by mitogen-activated protein kinase p38. FEBS Lett 546:37–44
34. **Johnson GL, Lapadat R** 2002 Mitogen-activated protein kinase pathways mediated by ERK, JNK, p38 protein kinase. Science 298:1911–1912
35. **Pramanik R, Qi X, Borowicz S, Choubey D, Schultz RM, Han J, Chen G** 2003 p38 isoforms have opposite effects on AP-1-dependent transcription through regulation of c-Jun. The determinant role of the isoforms in the p38 MAPK signal specificity. J Biol Chem 278:4831–4839
36. **Reunanen N, Li S-P, Ahonen M, Foschi M, Han J, Kähäri V-M** 2002 Activation of p38α MAPK enhances collagenase-1 (matrix metalloproteinase (MMP)-1) and stromelysin-1 (MMP-3) expression by mRNA stabilization. J Biol Chem 277:32360–32368
37. **Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ** 2003 Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-

dependent degradation of the BH3-only protein, Bim. J Biol Chem 278:18811–18816
38. **Stehle JH, Foulkes NS, Molina CA, Simmoneaux V, Pévet P, Sassone-Corsi P** 1993 Adrenergic signals direct rhythmic expression of transcriptional repressor CREM in the pineal gland. Nature 365:314–320
39. **Baler R, Covington S, Klein DC** 1997 The rat arylalkylamine *N*-acetyltransferase gene promoter. cAMP activation via a cAMP-responsive element-CCAAT complex. J Biol Chem 272:6979–6985
40. **Maronde E, Pfeffer M, Olcese J, Molina CA, Schlotter F, Dehghani F, Korf HW, Stehle JH** 1999 Transcription factors in neuroendocrine regulation: rhythmic changes in pCREB and ICER levels frame melatonin synthesis. J Neurosci 19:3326–3336
41. **Klein D, Weller JL** 1973 Adrenergic-adenosine 3′, 5′-monophosphate regulation of serotonin *N*-acetyltransferase activity and the temporal relationship of serotonin *N*-acetyltransferase activity synthesis of ^{3}H-N-acetylserotonin and ^{3}H-melatonin in the cultured rat pineal gland. J Pharmacol Exp Ther 186:516–527
42. **Ho AK, Chik CL** 1992 Inhibitors of Na^{+}/H^{+} exchange block stimulus-provoked pineal melatonin synthesis. Am J Physiol 263:E481–E488
43. **Ribelayga C, Pévet P, Simonneaux V** 2000 HIOMT drives the photoperiodic changes in the amplitude of the melatonin peak of the Siberian hamster. Am J Physiol 278:R1339–R1345
44. **Ribelayga C, Gauer F, Calgari C, Pévet P, Simonneaux V** 1999 Photoneural regulation of rat pineal hydroxyindole-*O*-methyltransferase (HIOMT) messenger ribonucleic acid expression: an analysis of its complex relationship with HIOMT activity. Endocrinology 140:1375–1384
45. **Hayashi Y, Sanada K, Hirota T, Shimizu F, Fukada Y** 2003 p38 mitogen-activated protein kinase regulates oscillation of chick pineal circadian clock. J Biol Chem 278:25166–25171

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