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Corticosterone-induced rapid phosphorylation of p38 and JNK mitogen-activated protein kinases in PC12 cells

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Abstract The present study showed that corticosterone (B) could induce a rapid activation of p38 and c-Jun NH_2-terminal protein kinase (JNK) in PC12 cells. The dose–response and time–response curves were bell-shaped with maximal activation at 10^{-9} M and at 15 min. RU38486 had no effect, and bovine serum albumin-coupled B could induce the activation. Genistein failed to block the phosphorylation, suggesting the pathway was not involved in tyrosine kinase activity. Phorbol 12-myristate 13-acetate could mimic, while Gö6976 could abolish the actions. These results demonstrated that B might act via a putative membrane receptor to activate p38 and JNK rapidly through a protein kinase C-dependent pathway.

Key words: Corticosterone; Non-genomic; p38; c-Jun NH_2-terminal protein kinase; Protein kinase C; PC12 cell

1. Introduction

Steroid hormones exert important effects through their intracellular receptors, which act as ligand-dependent transcription factors to influence gene expression. These genomic effects have been well known for decades, but fail to explain the rapid effects occurring in minutes or seconds. The non-genomic mechanism(s) underlying the rapid effects are poorly understood, but may play a pivotal role in physiological processes as they regulate ion channels, second messengers and protein kinases, such as protein kinase A (PKA) and/or protein kinase C (PKC) [19]. Mitogen-activated protein kinases (MAPKs) are known as Ser/Thr protein kinases involved in many important cellular processes, such as cell growth, differentiation, survival, apoptosis and cytokine production, etc. They are expressed in all eukaryotic cells and activated by diverse stimuli. Three well-characterized MAPK subfamilies in mammalian cells are the extracellular signal-regulated kinases (Erks), the p38 MAPK and the c-Jun NH_2-terminal protein kinase (JNK). While growth factors elicit the activation of Erks, stress responses and inflammatory mediators lead to the activation of p38 and JNK. Recently, differential effects of 17β-estradiol on MAPK pathways in rat cardiomyocytes were reported [1]. To elucidate the signaling pathways involved in the rapid effect of glucocorticoid we have also investigated the influence of corticosterone (B) on Erk1/2 in PC12 cells [21]. Here we examined the effect of B on rapid phosphorylation of p38 and JNK MAPKs and its underlying mechanism. The results showed that B could induce a rapid activation of p38 and JNK MAPKs through a PKC-dependent pathway.

2. Materials and methods

2.1. Materials

Corticosterone 21-sulfate, bovine serum albumin (BSA; fatty acid-free), corticosterone 21-hemisuccinate, nerve growth factor (NGF), genistein and RU38486 were purchased from Sigma (St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA) and Gö6976 were from Calbiochem (La Jolla, CA, USA). Phospho-p38 MAPK (Thr180/Tyr182) antibody (#9210) that detects Thr180- and Tyr182-phosphorylated p38 MAPK (dual-phosphorylated), p38 MAPK antibody (#9210) that recognizes total p38 MAPK (phosphorylation state-independent) protein levels, phospho-SAPK/JNK (Thr183/Tyr185) antibody (#9251S) that detects SAPK/JNK only when activated by phosphorylation at Thr183 and Tyr185 and SAPK/JNK MAPK antibody (#9252) that recognizes total SAPK/JNK (phosphorylation state-independent) protein levels were purchased from New England Biolabs (Beverly, MA, USA). All other chemicals used were of analytical grade.

2.2. Cell culture

PC12 cells were the kind gift of Dr. K. Kuba (Department of Physiology, Saga Medical School, Saga, Japan). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco BRL, Grand Island, NY, USA) supplied with 10% fetal bovine serum and 5% heat-inactivated horse serum and incubated in a humidified atmosphere containing 5% CO_2 in the air at 37°C. Flasks used to culture PC12 cells were pre-coated with collagen to facilitate cell adherence to the surface of the substrate.

2.3. Western blot analysis

PC12 cells were grown to sub-confluence in tissue culture flasks, serum-starved for 12 h in phenol red-free DMEM, and stimulated with drugs at 37°C for the indicated time. After drug treatment, cells were washed twice with ice-cold phosphate-buffered saline, pH 7.4, suspended in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM dithiothreitol,

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Abbreviations: B, corticosterone; B-BSA, bovine serum albumin-coupled corticosterone; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal protein kinase; PKC, protein kinase C; PKA, protein kinase A; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; NGF, nerve growth factor; RTK, receptor tyrosine kinase

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0.1% (w/v) bromophenol blue), sonicated for 15 s on ice, and heated at 100°C for 5 min. Then a 10% SDS–polyacrylamide gel electrophoresis was performed and proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked in Tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween 20. Immunoblotting was performed with anti-phospho-p38 (1:1000) or anti-phospho-JNK (1:1000) MAPK antibody overnight and with a secondary antibody conjugated to horseradish peroxidase (1:1000) for 1 h. The enhanced chemiluminescence light-based detection system was used to detect the peroxidase activity. Results were quantified by densitometric scan of X-ray films using a densitometer.

2.4. Statistical analysis

At least three independent experiments were carried out and all data are expressed as the mean ± S.E.M. of all determinations. The statistical significance of the results was assessed by Student's *t*-test. Differences were considered significant at $P < 0.05$ levels.

3. Results

To investigate the signal transduction pathway involved in the rapid, non-genomic effects of glucocorticoid in PC12 cells we examined the influence of B on p38 and JNK MAPKs. Immunoblotting with phospho-specific antibodies selective against the activated, phosphorylated MAPKs was performed. In the absence of B, the levels of phosphorylated p38 and JNK were low. Stimulation of PC12 cells with B (10^{-11}–10^{-7} M) for 15 min induced an increase of phosphorylated p38 and JNK with the maximal activation at the 10^{-9} M level (Fig. 1). Treatment of B (10^{-9} M) for 5–120 min also led to a rapid and transient activation of p38 and JNK (Fig. 2). The significant effect appeared at 5 min and with a maximum of 3.72 ± 0.7-fold and 2.98 ± 0.8-fold, respectively, at 15 min. The dose–response and time–response curves were both bell-shaped. Pretreatment of specific p38 antagonist SB203580 (10^{-5} M) for 15 min blocked p38 activation induced by B and the agonist, anisomycin, could significantly activate p38 MAPK in 15 min (Fig. 3).

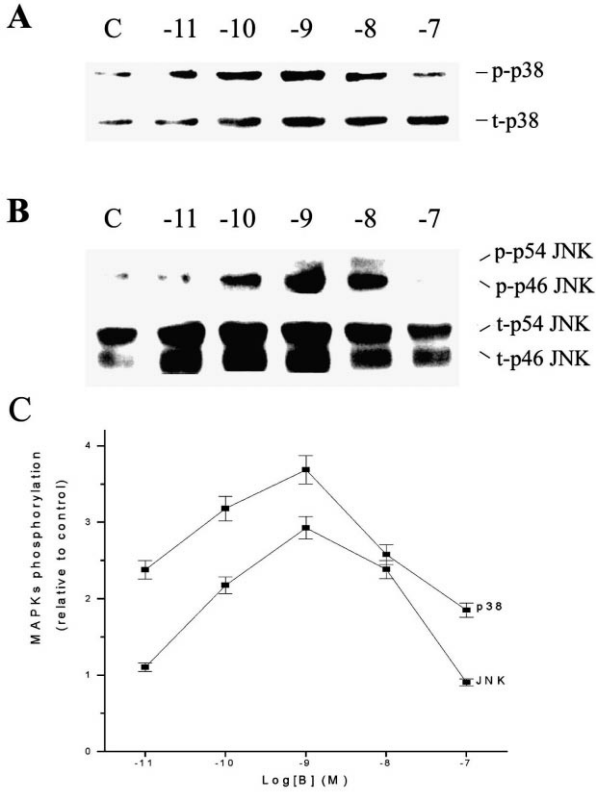

Fig. 1. Dose–response of B-stimulated p38 and JNK activation in PC12 cells. Cells were stimulated with B (10^{-11}–10^{-7} M) for 15 min and the phosphorylation of p38 (parallel A upper) and JNK (parallel B upper) was determined by immunoblot. Parallel A lower and parallel B lower represent total p38 and total JNK using phospho-independent MAPK antibody, respectively. C: Concentration curve of B-stimulated p38 and JNK activation in PC12 cells. Quantification of p38 and JNK phosphorylation was determined by densitometric analysis of immunoblot. Data are the means ± S.E.M. from three independent experiments.

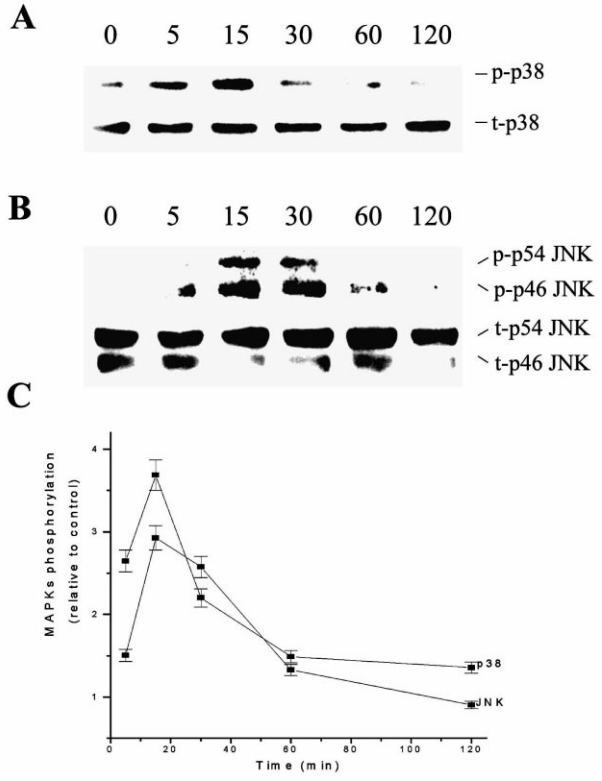

Fig. 2. Time-dependent activation of p38 and JNK by B in PC12 cells. Cells were incubated with B (10^{-9} M) for the indicated times (5–120 min) and the phosphorylation of p38 (parallel A upper) and JNK (parallel B upper) was determined by immunoblot. Parallel A lower and parallel B lower represent total p38 and total JNK using phospho-independent MAPK antibody, respectively. C: Time course of B-stimulated p38 and JNK activation in PC12 cells. Quantification of p38 and JNK phosphorylation was determined by densitometric analysis of immunoblot. Data are the means ± S.E.M. from three independent experiments.

To further elucidate the rapid effect of B in PC12 cells we studied the effect of BSA-coupled B (B-BSA), which is membrane impermeable, and the glucocorticoid nuclear receptor antagonist RU38486 on PC12 cells. Incubation with B-BSA (10^{-7} M) for 15 min could significantly increase the phosphorylation of p38 and JNK (Fig. 4). Pretreatment with RU38486 (10^{-6} M, 30 min) had no effect on the rapid activation induced by B. These results demonstrated that the rapid action might be mediated by the B binding to its putative membrane receptor.

Many kinds of cell surface receptor possess the ability to phosphorylate tyrosine residue, which may lead to the phosphorylation of MAPKs via activation of several adapter proteins. We used receptor tyrosine kinase (RTK) inhibitor, genistein, to examine the involvement of RTK in the process. The

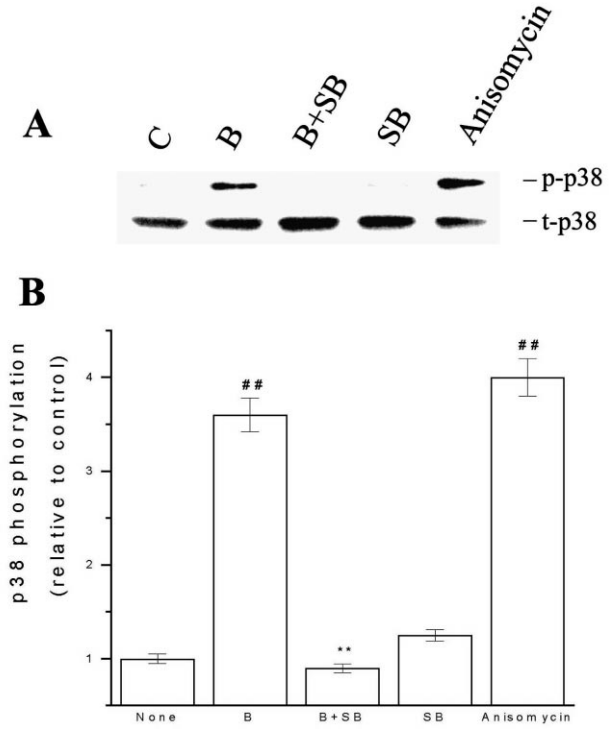

Fig. 3. The effect of SB203580 on B-induced p38 phosphorylation in PC12 cells. Cells were incubated with or without B (10^{-9} M) in the presence or absence of the specific inhibitor of p38, SB203580 (10^{-5} M), and the agonist anisomycin (10^{-5} M) for 15 min. The phosphorylation of p38 (parallel A upper) was determined by immunoblot. Parallel A lower represents total p38 using phospho-independent p38 MAPK. B: Quantification of p38 phosphorylation by densitometry. Data are the means ± S.E.M. from three independent experiments. $^{**}P < 0.01$ compared with cells incubated with B alone. $^{\#\#}P < 0.01$ compared with cells left untreated.

result showed that co-treatment with genistein (10^{-4} M, 15 min) could completely block NGF-induced activation of p38 and JNK (Fig. 5). However, it had no effect on the B-induced rapid phosphorylation. These findings implied that the rapid, non-genomic effect of B did not involve tyrosine kinase activity.

Some recent studies showed that PKC might regulate the MAPK pathways [12–15]. To extend our previous study [21] we investigated the involvement of PKC in p38 and JNK rapid activation. Western blot showed that co-treatment of PC12 cells with the specific PKC inhibitor, Gö6976 (10^{-7} M, 15 min), completely inhibited the rapid phosphorylation of MAPKs (Fig. 6) and PMA, a PKC activator, could mimic the actions of B. These findings indicated that the PKC is involved in the rapid activation of MAPKs by B.

4. Discussion

Recent evidence showed the involvement of the G protein–PKC pathway and/or the G protein–PKA pathway in the putative steroid membrane receptor-mediated glucocorticoid's rapid actions [7,19]. However, the signaling pathways in the steroid rapid, non-genomic actions need more investigation. Differential effects of 17β-estradiol on MAPK pathways in rat cardiomyocytes were reported [1]. It is known that MAPKs are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAPK kinase [20]. Our previous work found, for the first time, that B could rapidly activate Erk1/2 MAPK in PC12 cells [21].

The present study showed that treatment with B in PC12 cells also resulted in a rapid activation of p38 and JNK in a dose- and time-dependent manner. The concentration-dependent curve and the time-dependent curve were both bell-shaped. However, the maximal activation of p38 and JNK induced by B were both 10^{-9} M within 15 min. This result was similar to the one of Erk1/2 [21]. Though differential activation of MAPK families were reported in recent studies [1–6], similar time-dependent and concentration-dependent activation of three MAPK subfamilies were revealed in our results. These differences may be due to the different cell lines and different extracellular stimuli used in each experiment, as well as different upstream processes involved, such as RTK, and PKC or PKA, in different reactions.

One characteristic of a non-genomic effect is the short time-lag of action. Our findings indicated that B could induce the phosphorylation of p38 and JNK even in 5 min. Furthermore; the effect was not affected by RU38486, the antagonist of classical nuclear glucocorticoid receptor. In addition, B-BSA could also lead to the activation of p38 and JNK. Since this compound is practically membrane impermeable, these results supported the notion that B might act via putative glucocorticoid membrane receptors. In mammalian cells, a single receptor type can activate different MAPK pathways. Phosphorylation of the RTKs, such as NGF receptor [16,17], leads to the activation of small G proteins of the Ras and Rho family. While Ras can activate the Raf/MEK/Erk pathway, cdc42 and Rac, which belong to the Rho family, can mediate the activation of p38 and JNK [9–11]. We examined the involvement of RTK in the rapid steroid action. While genistein, a RTK inhibitor, successfully blocked the NGF-induced MAPK phosphorylation, it had no effect on B-induced MAPK activation. The findings implied that RTK activity is

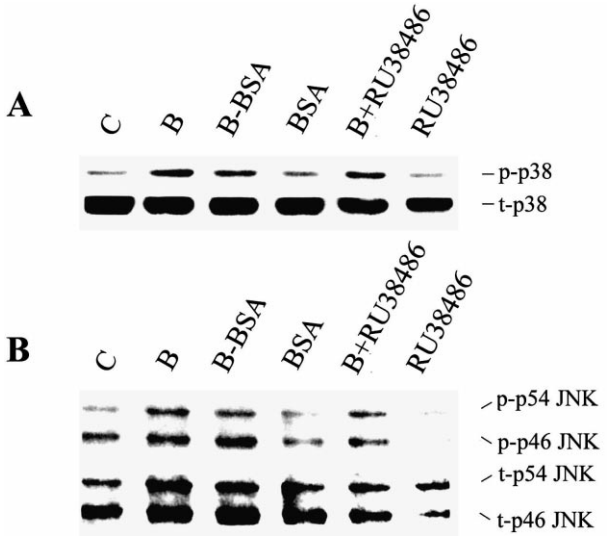

Fig. 4. The effect of RU38486 on B-induced p38 and JNK phosphorylation in PC12 cells. Cells were incubated with B (10^{-9} M), B-BSA (10^{-7} M) and BSA (10^{-9} M) in the presence or absence of glucocorticoid nuclear receptor antagonist, RU38486 (10^{-6} M, 30 min) for 15 min. The phosphorylation of p38 (parallel A upper) and JNK (parallel B upper) was determined by immunoblot. Parallel A lower and parallel B lower represent total p38 and total JNK using phospho-independent MAPK antibody, respectively.

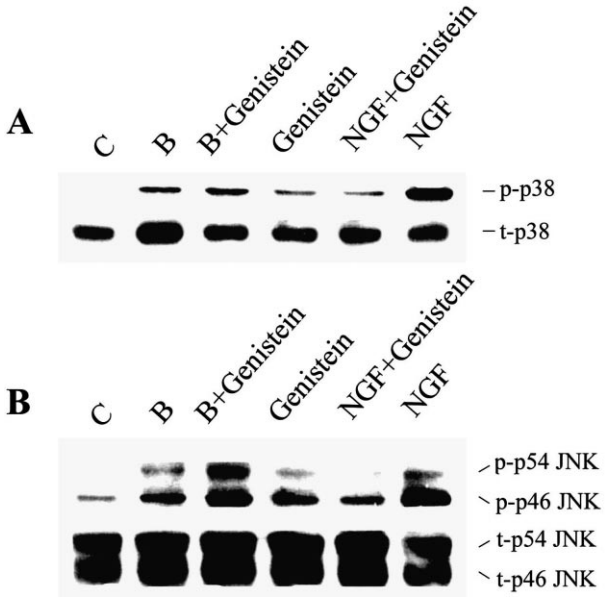

Fig. 5. The effect of genistein on B-stimulated p38 and JNK activation in PC12 cells. Cells were stimulated with B (10^{-9} M) and NGF (10^{-7} g/ml) in the absence or presence of genistein (10^{-4} M) for 15 min and the phosphorylation of p38 (parallel A upper) and JNK (parallel B upper) was determined by immunoblot. Parallel A lower and parallel B lower represent total p38 and total JNK using phospho-independent MAPK antibody, respectively.

not involved in the responses via the putative glucocorticoid membrane receptor.

In the present study, treatment of PMA could induce the rapid activation of p38 and JNK. But Gö6976, a specific inhibitor of PKCα and PKCβ, could block the rapid action induced by B. Since there is no PKCβ in PC12 cells [18], the results indicated that PKCα might mediate the rapid activation of the MAPK family elicited by B. Our previous work found that B or B-BSA had no effect on either the resting or forskolin-stimulated cAMP level and PKA activities [8]. Thus, the B-stimulated phosphorylation of p38 and JNK might involve the PKC pathway, while the PKA pathway played no part. It is interesting to observe whether the non-differential activation of Erk, p38 and JNK is a feature characteristic of PKC activity in MAPK activation [8].

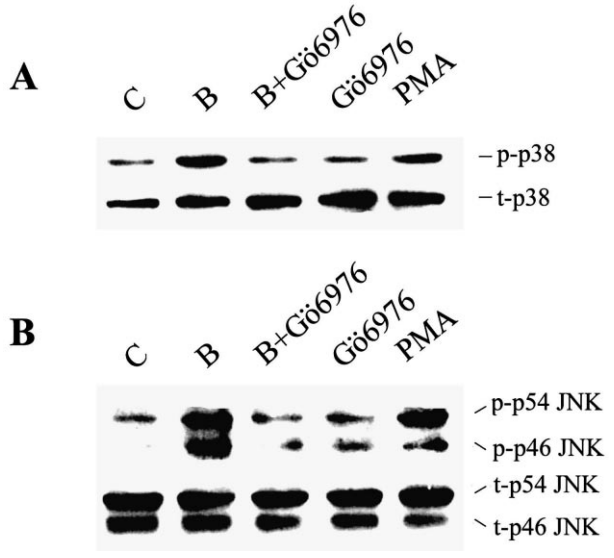

Fig. 6. The effect of Gö6976 on B-stimulated p38 and JNK activation in PC12 cells. Cells were stimulated with B (10^{-9} M) in the absence or presence of Gö6976 (10^{-7} M) for 15 min. Cells treated with PMA (10^{-6} M, 15 min) alone could also lead to the phosphorylation of p38 (parallel A upper) and JNK (parallel B upper). Parallel A lower and parallel B lower represent total p38 and total JNK using phospho-independent MAPK antibody, respectively.

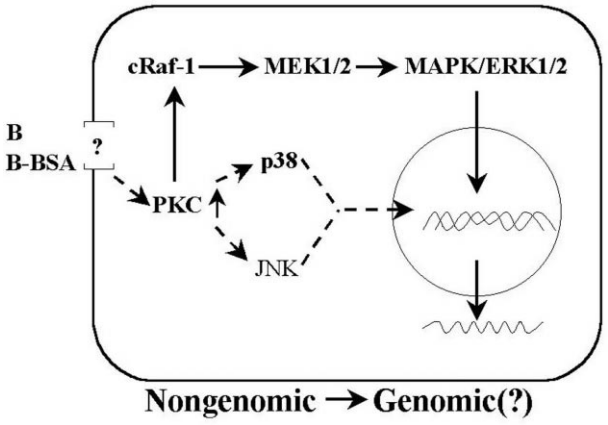

Fig. 7. A new model of the rapid non-genomic action of B in PC12 cells. Arrows with dashed lines represent the steps that are not well defined.

None of the single MAPK pathway is activated in response to stimuli. Some investigation shows the crosstalk between the specific MAPKs; for instance, MEKK1, the Erk1 kinase kinase, is capable of regulating the JNK and p38-induced NFκB pathway. Some downstream substrates of the MAPKs are the upstream of their own or other MAPKs. The relevance of the rapid activation of MAPKs by steroid hormone is poorly understood, but the activated MAPKs may translocate to the nucleus to influence gene expression [21]. This raises the assumption that a delayed effect occurred after the rapid action of steroids (Fig. 7), which may be a supplement to the classical genomic effect of steroid hormone through the classical nuclear receptor.

However, activation patterns of the MAPK family members are complex. Recent evidence showed that transforming growth factor β-activated kinase 1 (TAK1), a member of the MAPK kinase kinase superfamily, acted as a link in the delayed response to mechanical stress to phosphorylate p38 MAPK in vivo which elicits myocardial hypertrophy and fulminant heart failure [22]. TAK1 also participated in the JNK signaling pathway during *Drosophila* development to control the cell shape and regulation of apoptosis [23]. Whether TAK1 is involved in the responses described in the present study needs further investigation.

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References

[1] Nuedling, S., Kahlert, S., Loebbert, K., Meyer, R., Vetter, H. and Grohé, C. (1999) FEBS Lett. 454, 271–276.

[2] Hahn, M.J., Yoon, S.S., Sohn, H.W., Song, H.G., Park, S.H. and Kim, T.J. (2000) FEBS Lett. 470, 350–354.
[3] Ito, T., Kozawa, O., Tanabe, K., Niwa, M., Matsuno, H., Sakai, N., Ito, H., Kato, K. and Uematsu, T. (2000) Hypertension 35, 673–678.
[4] Huang, C., Li, J., Chen, N., Ma, W., Bowden, G.T. and Dong, Z. (2000) Mol. Carcinog. 27, 65–75.
[5] Clerk, A., Harrison, J.G., Long, C.S. and Sugden, P.H. (1999) J. Mol. Cell Cardiol. 31, 2087–2099.
[6] Alexandrov, A., Keffel, S., Goepel, M. and Michel, M.C. (1999) Biochem. Biophys. Res. Commun. 261, 372–376.
[7] Yasumasa, I., Yoshiaki, A., Masahito, K., Yutaka, O. and Hidehiko, S. (1997) Biochem. Biophys. Res. Commun. 235, 295–299.
[8] Qiu, J., Lou, L.G., Huang, X.Y., Lou, S.J., Pei, G. and Chen, Y.Z. (1998) Endocrinology 139, 5103–5108.
[9] Bagrodia, S., Derijard, B., Davis, R.J. and Cerione, R.A. (1995) J. Biol. Chem. 270, 27995–27998.
[10] Whitmarsh, A.J. and Davis, R.J. (1996) J. Mol. Med. 74, 589–607.
[11] Zhang, S., Huan, J., Sells, M.A., Chernoff, J., Knaus, U.G., Ulevitch, R.J. and Bokoch, G.M. (1995) J. Biol. Chem. 270, 23934–23936.
[12] Lee, M. and Yea, S.S. (2000) Immunopharmacology 48, 165–172.
[13] Vuong, H., Patterson, T., Shapiro, P., Kalvakolanu, D.V., Wu, R., Ma, W.Y., Dong, Z., Kleeberger, S.R. and Reddy, S.P. (2000) J. Biol. Chem. 275, 32250–32259.
[14] de Windt, L.J., Lim, H.W., Haq, S., Force, T. and Molkentin, J.D. (2000) J. Biol. Chem. 275, 13571–13579.
[15] Harris, V.K., Coticchia, C.M., Kagan, B.L., Ahmad, S., Wellstein, A. and Riegel, A.T. (2000) J. Biol. Chem. 275, 10802–10811.
[16] Barbacid, M. (1995) Curr. Opin. Cell Biol. 7, 148–155.
[17] Rosalind, A., Segal and Michael, E. (1996) Annu. Rev. Neurosci. 19, 463–489.
[18] Ohmichi, M., Zhu, G. and Saltiel, A.R. (1993) Biochem. J. 295, 767–772.
[19] Chen, Y.Z. and Qiu, J. (1999) Mol. Cell Biol. Res. Commun. 2, 145–149.
[20] Nishida, E. and Gotoh, Y. (1993) Trends Biochem. Sci. 18, 128–131.
[21] Qiu, J., Wang, P., Li, X.Y., Jing, Q., Zhang, W.B., Huang, Y.L., Sun, G., Pei, G. and Chen, Y.Z. (2001) Submitted for publication.
[22] Zhang, D., Gaussin, V., Taffet, G.E., Belaguli, N.S., Yamada, M., Schwartz, R.J., Michael, L.H., Overbeek, P.A. and Schneider, M.D. (2000) Nat. Med. 6, 556–563.
[23] Takatsu, Y., Nakamura, M., Stapleton, M., Danos, M.C., Matsumoto, K., O'Connor, M.B., Shibuya, H. and Ueno, N. (2000) Mol. Cell Biol. 20, 3015–3026.