BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **235,** 99–102 (1997)
ARTICLE NO. RC976746

Rapid Activation of MAP Kinase by Estrogen in the Bone Cell Line

Hideki Endoh,*[,1] Haruna Sasaki,* Kazunori Maruyama,* Ken-ichi Takeyama,† Iwao Waga,‡ Takao Shimizu,‡ Shigeaki Kato,† and Hiroyuki Kawashima*[,§]

**Molecular Medicine Lab, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., Tsukuba, Ibaraki 305, Japan; †Institute for Molecular and Cellular Biosciences and ‡Department of Biochemistry, Faculty of Medicine, The University of Tokyo, Bunkyo, Tokyo 113, Japan; and §Department of Pharmacology, Niigata University School of Dentistry, Gakkomachi-dori, Niigata 951, Japan*

Received May 1, 1997

We examined the effect of estrogen on mitogen-activated protein kinase (MAPK) in osteoblastic cells. Rat ROS 17/2.8 cells were exposed to 17β-estradiol (E_2) and MAPK activity in the cells was measured by an *in vitro* phosphorylation assay. E_2 treatment caused a rapid and transient MAPK activation within 5 min. Insulin-like growth factor-I, which acts via their membrane receptors, caused a similar effect, but it required 10 min to reach the maximum level. Western blot analyses with anti-MAPK and anti-phosphotyrosine antibodies demonstrated that the E_2 activation of MAPK was accompanied by phosphorylation of the enzyme. The concentration range (10 nM-1 pM) of E_2 needed for this MAPK activation was less than that (1 μM-0.1 nM) needed for the transcriptional activation via the nuclear estrogen receptor (ER). These data provide the first evidence of MAPK activation by E_2 through phosphorylation, which may be mediated through a putative plasma membrane receptor in the cultured bone cells.

Steroid hormones exert most of their effects by direct binding to specific nuclear receptors, which act as transcriptional activators (1,2). In contrast, most growth factors act through plasma-membrane receptors to stimulate inherent intracellular signal pathways. On this basis, the above two signalings have to date been considered independent. However, recent studies have indicated the presence of cross-talk between the actions of steroid hormones and those of growth factors. For example, estrogen enhances the signaling of insulin-like growth factor-I (IGF-I) (3); likewise, the transcriptional activity of estrogen is enhanced in the presence of epidermal growth factor (EGF) and or IGF-I (4,5). These facts support the idea that both estrogen and some growth factors may share a common pathway in their signaling cascades. Evidence has accumulated for the presence of estrogen actions mediated not by estrogen receptor (ER), but by unknown plasma-membrane receptors. For instance, estrogen caused a rapid elevation of cAMP or and a rapid release of intracellular calcium in a cell-type-dependent manner (6,7,8). Furthermore, estrogen may act as a ligand for ErbB2, a member of the EGF receptor family (9). If these effects of estrogen are transduced through membrane receptors, it is highly possible that estrogen directly affects the intracellular kinase cascades, which are also located downstream of growth factor signaling and are responsible for cross-talk with growth factors. One possible candidate for cross-talk is mitogen-activated protein kinase (MAPK). MAPK, a serine/threonine kinase, is located in the common downstream of many growth factor signal cascades and is essential for the triggering of cell proliferation and or differentiation (10,11,12).

We report here that 17β-estradiol (E_2) causes rapid MAPK activation in osteoblastic cells. Time course and concentration-response studies with E_2 demonstrated that this E_2 effect on MAPK may be caused through not ER but a cell membrane receptor.

MATERIAL AND METHODS

Cells and materials. A rat osteoblast-like cell line, ROS17/2.8, obtained from Riken Cell Bank (Wako, Saitama, Japan) was maintained in Ham's F-12 from Gibco (Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) at 37°C under 5% CO_2. IGF-I was obtained from Mallinckrodt Chemical (Paris, France). 17β-Estradiol and fatty-acid-free bovine serum albumin (BSA) were obtained from Sigma. The MAP kinase assay system and

[1] To whom correspondence should be addressed. Fax: +81-298-52-5444. E-mail: endoh@yamanouchi.co.jp.

Abbreviations used: MAPK, mitogen-activated protein kinase; E_2, 17β-estradiol; ER, estrogen receptor; IGF-I, insulin-like growth factor-I; EGF, epidermal growth factor; PT, pertussis toxin.

0006-291X/97 $25.00

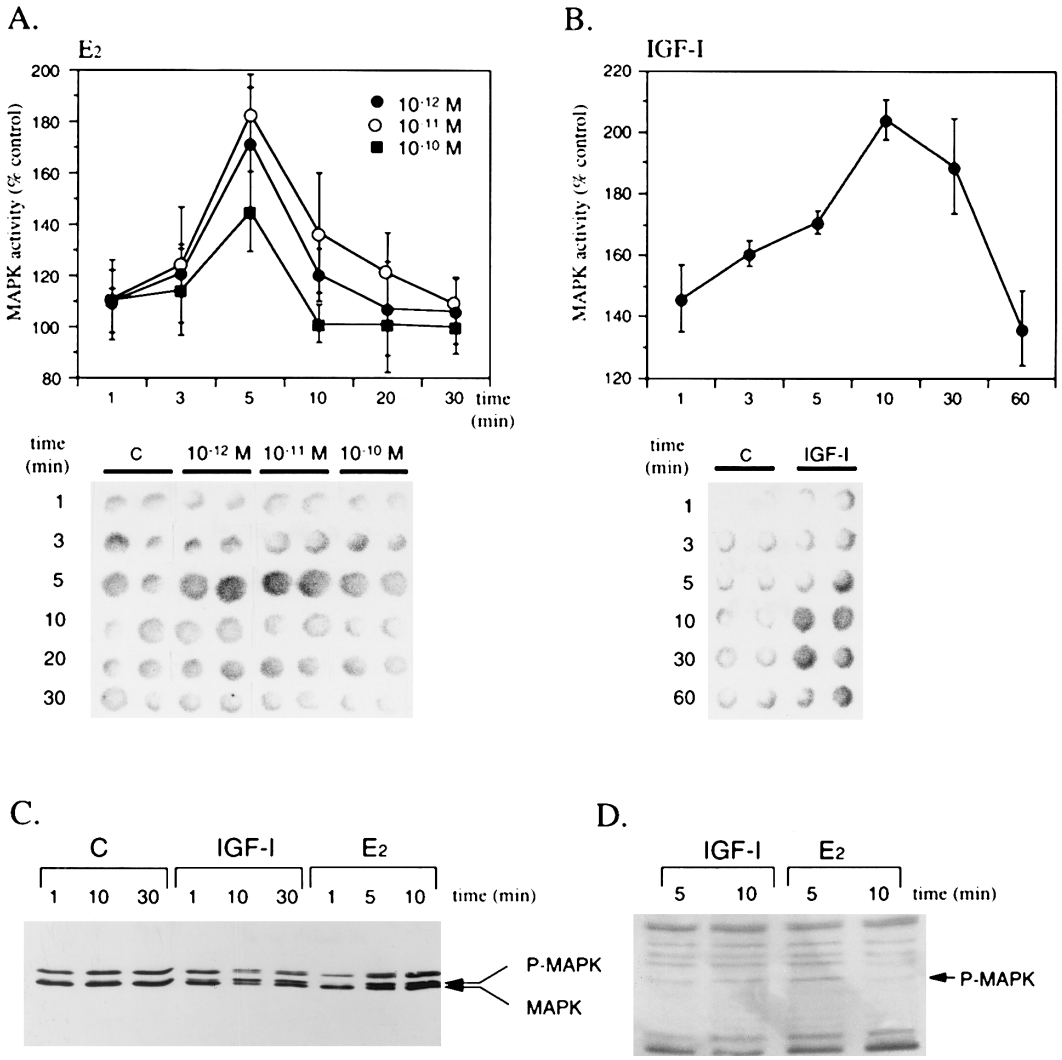

FIG. 1. Effect of E_2 (**A**) and IGF-I (**B**) on the activity of MAPK in ROS17/2.8. Cells (3×10^4) were treated with E_2 (10^{-10}-10^{-12} M) or IGF-I (100 ng/ml) and harvested at the time indicated. Bottom shows a typical pattern of autoradiography of lysates dotted onto P-81 paper. Each point on the graph represents the mean value of three independent experiments. Western blotting of MAPK in ROS17/2.8 treated with E_2 or IGF-I (**C and D**). Ten μl aliquot of cell lysates (6×10^6 cells/ml) from vehicle (C)- or 100 ng/ml IGF-I- or 10^{-11} M 17 β-estradiol (E_2)-treated cells were run on polyacrylamide gel and blotted onto a nitrocellulose membrane. The MAPK were probed with anti-MAPK antibodies (anti-ERK1 and ERK2) to detect the band shift of MAPK (shown in (**C**)) or with anti-phosphotyrosine antibody to detect a tyrosine phosphorylation (shown in (**D**)). MAPK and P-MAPK indicate the signal bands of inactivated (i.e. unphosphorylated) MAPK and activated (i.e. phosphorylated) MAPK, respectively.

[γ-^{32}P] ATP (specific activity, ~6000 Ci/mmol) were purchased from Amersham (Little Chalfont, Buckinghamshire, England). Q-Sepharose Fast Flow was obtained from Pharmacia (Uppsala, Sweden). Anti-MAPK (ERK1 and 2) antibodies were purchased from Zymed Laboratories, Inc. (San Francisco, CA, USA), and anti-phosphotyrosine antibody was obtained from UBI (Lake Placid, NY, USA).

Plasmid. The reporter plasmid was constructed by inserting a synthetic oligonucleotide of a perfect estrogen response elememt (ERE) (5′- AGGTCACTGTGACCT -3′) into pG-CAT (13), containing the minimum promoter of β-globin gene and the gene encoding chloramphenicol acetyltransferase (CAT).

In vitro MAP kinase assay. MAPK activity was measured as described previously (14) using EGF receptor peptide as a substrate instead of myelin basic protein (MBP). ROS17/2.8 maintained in Ham's F-12 without serum or phenol red for 36 h was treated with E_2 (17 β-estradiol; 10 nM-1 pM) or IGF-I (100 ng/ml).

Immunoblot analysis of MAPK. The cell lysates of ROS 17/2.8 treated with E_2 (10 pM) or IGF-I (100 ng/ml) were applied to SDS-PAGE (10% polyacrylamide), and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham). These membranes were probed with anti-MAPK anibodies or anti-phosphotyrosine antibody, and then with the peroxidase-labeled second antibody. Antibody-staining was visualized with an ECL system (Amersham).

CAT assay. ROS 17/2.8 cells were cultured in Ham's F-12 medium without phenol red, supplmented with 10% charcoal-treated FBS. The cells were transfected at 40-50% confluence to 100-mm dishes with 2 μg of ERE-G CAT reporter plasmid, 0.5 μg of rat ER expression vector, 3 μg of pCH110 β-galactosidase reporter

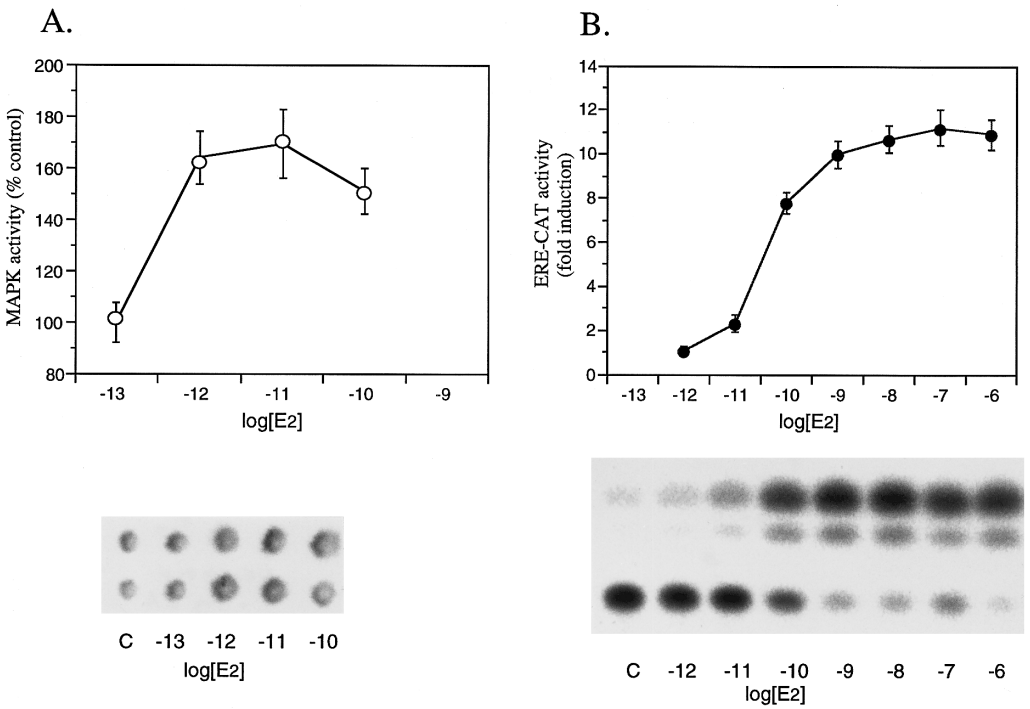

FIG. 2. Dose-response relationship for activation of MAPK (**A**) and for induction of ERE-mediated CAT activity (**B**) by E_2. ROS17/2.8 were treated with various concentrations of E_2 or control vehicle for 5 min (**A**) or 48 h (**B**). Bottom shows a typical view of autoradiography.

plasmid and 14.5 μg of Bluescribe M13+ (Stratagene, La Jolla, CA, USA) carrier DNA. The medium was replaced with to fresh medium after 24 h. E_2 (1 μM-10 pM) was added to the medium 1 h after transfection at each exchange of medium. The cells were harvested after 20 h and the cells lysed by freeze-thawing. CAT assays, normalized for the β-galactosidase activity of each extract, were performed as described (13).

RESULTS AND DISCUSSION

MAPK activation by treatment of osteoblast-like cells with IGF-I or estrogen. Fig.1 A clearly shows that E_2 rapidly activated MAPK in ROS17/2.8 cells. Maximum activation of MAPK by E_2 was achieved at 5 min and activity fell to control values within 30 min. Since IGF-I is a well-known mitogen for bone cells (15), we used it as a positive control for this assay. As shown in Fig.1 B, the addition of IGF-I caused a transient activation of MAPK with a peak at 10 min.

Phosphorylation of MAPK by estrogen. MAPK has been reported to be activated through phosphorylation of both intramolecular tyrosine and threonine residues, giving a slower migration during polyacrylamide electrophoresis (16). As expected, Western blot analysis revealed that E_2 caused a gel shift pattern of MAPK exactly as IGF-I did (Fig.1 C), a finding attributable to the effect of phosphorylation of MAPK at its intramolecular residues, including tyrosine residues (Fig.1 D). These changes showed good correlation with total MAPK activity (Fig.1 A and 1 B). Thus, estrogen activated MAPK within short periods possibly through putative membrane surface receptor(s).

Dose-response relationship of MAPK activation by estrogen. Dose-response studies revealed that low but physiological concentrations of E_2 (10 nM-1 pM) were able to induce the activation of MAPK in ROS17/2.8 cells (Fig.2 A). Full activation of MAPK was achieved with 10 pM E_2. On the other hand, higher concentrations of E_2 (1 μM-0.1 nM) were needed for induction of gene expression via ER (fig. 2 B). The most effective concentration for transcriptional induction via ER was 0.1 μM E_2.

These results show the first evidence that estrogen, one of steroid hormones, causes a transient activation of MAPK in bone cell line (Fig.1). This effect seems to be one of non-genomic actions of estrogen, which is independent of ER-mediated gene expression, since it needed only 5 min to reach maximum activation of MAPK (Fig.1 A) and activation of MAPK could be achieved with a concentration of E_2 significantly lower than that of its genomic effect (Fig.2). We recently found that neither a transcriptional inhibitor (actinomycin D) nor a translational inhibitor (cycloheximide) affected the estrogen-induced activation of MAPK. On the other hand, pretreatment with pertussis toxin (PT), an uncoupler of G-protein and mambrane receptors, abrogated the E_2 activation of MAPK (our unpublished

results). In fact, evidence has been reported indicating the presence of an estrogen-binding protein/receptor located on plasma membrane (17). The effect of estrogen on MAPK might be related to the reported non-genomic action of the hormone in which production of inositol 1,4,5-trisphosphate and diacylglycerol increased, because this was also prevented by pretreatment with PT (8).

MAPK activation has been shown to be essential for mitogenic signaling in many cell types inducing cell proliferation or differentiation (10). MAPK activation by estrogen therefore implies that estrogen shares a part of its signaling pathway with some growth factors, thereby being capable of directly regulating the cell proliferation and differentiation. It may furthermore be possible that this novel effect of estrogen is responsible for its tissue-specific effects, because the time course of MAPK activation by estrogen is different between osteoblast-like cells and cells derived from mammary gland and uterus (our unpublished results).

Several genes without ERE on their promoter, such as IGF-I and brain creatine kinase gene, have been reported to be induced by estrogen (18,19). Because MAPK has been shown to activate several transcriptional factors through phosphorylation (20,21), activation of MAPK by estrogen may play an important role in the gene induction via an ER-independent manner. Indeed, our recent studies have shown that phosphorylation of ER enhanced estrogen-mediated transcription and that this phosphorylation can be achieved by MAPK in a cell type-specific manner (22). It is therefore possible that estrogen causes phosphorylation of some other nuclear receptors like ER through MAPK activation, thereby modifying the genomic actions of estrogen.

In conclusion, the steroid hormone estrogen causes activation of MAPK in a manner independent of ER in osteoblast-like cells. This non-genomic effect of estrogen may play a crucial role in the proliferation and differentiation of osteoblast-like cells, and may be, in part, responsible for the mechanism(s) of the cell-specific action of estrogen.

ACKNOWLEDGMENTS

We thank Dr. M Muramatsu, Saitama Medical School, for rat ER cDNA and, Dr. M. Sato, Gifu Pharmaceutical University, for technical comments.

REFERENCES

1. Gronemeyer, H. (1991) *Ann. Rev. Genet.* **25,** 89–123.
2. Gorski, J., Furlow, J. D., Murdoch, F. E., Fritsch, M., Kaneko, K., Ying, C., and Malayer, J. R. (1993) *Biol. Reprod.* **48,** 8–14.
3. van der Burg, B., de Groot, R. P., Isbrucker, L., Kruijer, W., and de Laat, S. W. (1991) *J. Steroid Biochem. Mol. Biol.* **40,** 215–221.
4. Aronica, S. M., and Katzenellenbogen, B. S. (1993) *Mol. Endocrinol.* **7,** 743–752.
5. Ignar-Trowbridge, D. M., Teng, C. T., Ross, K. A., Parker, M. G., Korach, K. S., and McLachlan, J. A. (1993) *Mol. Endocrinol.* **7,** 992–998.
6. Aronica, S. M., Kraus, W. L., and Katzenellenbogen, S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8517–8521.
7. Morley, P., Whitfield, J. F., Vanderhyden, B. C., Tsaang, B. K., and Schwartz, J. L. (1992) *Endocrinology* **131,** 1305–1312.
8. Lieberherr, M., Grosse, B., Kachkache, M., and Balsan, S. (1993) *J. Bone. Miner. Res.* **8,** 1365–1376.
9. Matsuda, S., Kadowaki, Y., Ichino, M., Akiyama, T., Toyoshima, K., and Yamamoto, T. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10803–10807.
10. Pages, G., Lenormand, P., L'Allemain, G., Chambard, J. C., Meloche, S., and Pouyssegur, J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8319–8323.
11. Troppmair, J., Bruder, J. T., Munoz, H., Lloyd, P. A., Kyriakis, J., Banerjee, P., Avruch, J., and Rapp, U. R. (1994) *J. Biol. Chem.* **269,** 7030–7035.
12. Davis, R. J. (1993) *J. Biol. Chem.* **268,** 14553–14556.
13. Kato, S., Tora, L., Yamauchi, J., Masushige, S., Bellard, M., and Chambon, P. (1992) *Cell* **68,** 731–743.
14. Waga, I., Kume, K., Ferby, I., Honda, Z., and Shimizu, T. (1996) *J. Immunol. Methods* **190,** 71–77.
15. Hock, J. M., Centrella, M., and Canalis, E. (1988) *Endocrinology* **122,** 254–260.
16. Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, D. F, Weber, M. J., and Sturgill, T. W. (1991) *EMBO. J.* **10,** 885–892.
17. Brubaker, K. D., and Gay, C. V. (1994) *Biochem. Biophys. Res. Comm.* **200,** 899–907.
18. Umayahara, Y., Kawamori, R., Watada, H., Imano, E., Iwama, N., Morishima, T., Yamasaki, Y., Kajimoto, Y., and Kamada, T. (1994) *J. Biol. Chem.* **269,** 16433–16442.
19. Sukovich, D. A., Mukherjee, R., and Benfield, P. A. (1994) *Mol. Cell. Biol.* **14,** 7134–7143.
20. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991) *Nature* **353,** 670–674.
21. Whitmarsh, A. J., Shore, P., Sharrocks, A. D., and Davis, R. J. (1995) *Science* **269,** 403–406.
22. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) *Science* **270,** 1491–1494.