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Phosphorylation of extracellular signal-regulated kinases 1 and 2 in 3T3-L1 adipocytes by stimulation of β_3-adrenoceptor

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Abstract

Recent studies have revealed that activated extracellular signal-regulated kinases (ERKs) 1 and 2 by the stimulation of β_3-adrenoceptors played a critical role in cell survival in brown adipocytes. On the other hand, phosphorylation of ERK1/2 via β_3-adrenoceptors and its physiological and pathological significance in white adipocyte has remained uncertain despite the increasing significance of functioning white adipocytes. Accordingly, we here studied phosphorylation of ERK1/2 caused by the stimulation of β_3-adrenoceptors in 3T3-L1 adipocytes, and the roles of phosphorylated ERK1/2 in lipolysis. Phosphorylation of ERK1/2 was induced by a selective β_3-adrenoceptor agonist, DL-4-[2′-{2-hydroxy-2-(3-chlorophenyl)ethylamino}propyl] phenoxyacetic acid sodium salt sesquihydrate (BRL37344), in 3T3-L1 adipocytes in a time- and dose-dependent manner. The phosphorylation of ERK1/2 by BRL37344 was sensitive to the cyclic AMP (cAMP)-dependent protein kinase inhibitor, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89). To elucidate the roles of phosphorylated ERK1/2 in lipolysis, the effect of a selective inhibitor of ERK1/2 phosphorylation, 2′-amino-3′-methoxyflavone (PD98059), was examined. This inhibitor did not alter the lipolytic action caused by BRL37344, even at concentrations sufficient to block phosphorylation of ERK1/2, suggesting that ERK1/2 play no role in the lipolysis caused by BRL37344 in 3T3-L1 adipocytes.

Keywords: β_3-Adrenoceptor; Extracellular signal-regulated kinase; 3T3-L1; Adipocyte

1. Introduction

Mammals have two different types of adipose tissues, brown and white (for review, see Ricquier and Cassard-Doulcier, 1993). Brown adipocytes express uncoupling proteins (Garlid et al., 1996), and thus are thought to play a role in thermogenesis and energy expenditure (Rehnmark et al., 1990). White adipocytes are thought to be specialized cells for the synthesis and storage of triacylglycerols, with each adipocyte storing a single large droplet of fat. However, recent studies have also reported that white adipocytes secrete leptin and other bioactive substances (Leroy et al., 1996; Rentsch and Chiesi, 1996). With this discovery, the physiological significance of white adipocytes has increased dramatically.

β_3-Adrenoceptors are predominantly expressed in white and brown adipocytes (Emorine et al., 1989; Granneman et al., 1991; Muzzin et al., 1991; Nahmias et al., 1991). Treatment with β_3-adrenoceptor selective agonists causes lipolysis (Van Liefde et al., 1992) and a number of diverse metabolic effects including increase of energy expenditure (Weyer et al., 1998), reduction of food intake (Mantzoros et al., 1996), and dramatic increase of insulin level in blood (Susulic et al., 1995). These effects have been shown to disappear in β_3-adrenoceptor knockout mice (Susulic et al., 1995). Re-expression of the receptors in both white and brown adipocytes recovered these effects, whereas re-expression of the receptors only in brown adipocytes did not (Grujic et al., 1997). This interesting phenomenon strongly suggests the significance of β_3-adrenoceptor-mediated responses in the functioning white adipocytes.

Extracellular signal-regulated kinases (ERKs) 1 and 2, which belong to a subfamily of mitogen-activated protein kinases (MAPKs), and their signaling cascade play key roles in many physiological events such as proliferation (Pages et al., 1993), differentiation (Pang et al., 1995), gene expression (Vanhoute et al., 1998) and cell homeostasis (Kanda et al., 1998). ERK1/2 are phosphorylated by MAPK kinase at threonine 202 and tyrosine 204 and

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catalytically activated by the phosphorylation (Anderson et al., 1990). Recently, activation of ERK1/2 in brown adipocytes by adrenergic stimulation was reported to be mediated through β_3-adrenoceptors (Shimizu et al., 1997; Lindquist and Rehnmark, 1998), and it was also suggested that the activated ERK1/2 play a critical role in cell survival (Lindquist and Rehnmark, 1998). However, there has been no report on phosphorylation and activation of ERK1/2 in white adipocytes by the β_3-adrenoceptor stimulation. In spite of the significance of β_3-adrenergic responses in white adipocytes, the physiological and pathological significance of ERK1/2 in white adipocytes remains uncertain. Therefore, we investigated weather ERK1/2 phosphorylation was caused by, the selective β_3-adrenoceptor agonist, DL-4-[2′-{2-hydroxy-2-(3-chlorophenyl)ethylamino}propyl] phenoxyacetic acid sodium salt sesquihydrate (BRL37344), in 3T3-L1 adipocytes, which have frequently been used as a white adipocyte model (Alvarez, 1991; Camirand et al., 1998). We also examined the role of phosphorylated ERK1/2 in glycerol release, which is well known as a β_3-adrenoceptor-mediated response.

2. Materials and methods

2.1. Cell culture and differentiation

3T3-L1 cells (Green and Kehinde, 1976) were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin and 50 units/ml streptomycin at 37°C in an atmosphere of 5% CO_2/95% air. The culture medium was renewed every other day, except where otherwise described. For adipogenesis, the cells were grown to confluence and differentiation was initiated at 2 days after the confluence by treating with the medium containing 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone and 10 μg/ml insulin (Nishio et al., 1996). After 3-day cultivation, the culture medium was changed (designated day 0). At appropriate culture periods, the cells were washed and cultured in the absence of fetal bovine serum for 1 h before the experiments to avoid interference of serum factors with β-adrenoceptor agonist-mediated responses, and then treated by β-adrenergic reagents.

2.2. Immunoblot analysis

Phosphorylation of ERK1/2 at both sites, threonine 202 and tyrosine 204, was estimated by Western blotting. Whole cell extracts of 3T3-L1 fibroblasts and adipocytes were prepared as follows. After aspiration of the culture medium, cells on the dishes were rinsed with ice-cold phosphate-buffered saline containing 1 mM sodium vanadate, and then gently scraped into 200 μl of cell lysis buffer consisting of 20 mM Tris–HCl (pH 7.5), 10 mM EDTA, 60 mM β-glycerophosphate, 10 mM $MgCl_2$, 1% Triton X-100, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin and 2 μg/ml aprotinin. The cell suspensions were sonicited five times, for 1 s each time, in an ice bath. The homogenates were centrifuged at $12{,}000 \times g$ for 20 min, and the obtained infranatants were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% T gel). Approximately 10 μg of crude cellular protein was loaded per lane, and the separated proteins were transferred to a polyvinilidene difluoride membrane (IPVH000 10; Millipore) by electroblotting (150 mA, 80 min). After blocking in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T), the resulting membranes were incubated with anti-phospho ERK antibody (New England BioLabs) for 1 h, washed with TBS-T three times (10 min for each) and incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IGG; Calbiochem-Novabiochem) for 1 h. After washing with TBS-T, bound antibodies were detected by enhanced chemiluminescence (ECL Western blotting detection reagents; Amersham) according to the manufacturer's instructions. The intensity of each band was quantitatively analyzed by means of NIH image (National Institute of Health).

To elucidate the existence of β_3-adrenoceptors, the antibodies were changed to anti-β_3-adrenoceptor antibody (SC-1473; Santa Cruz Biotechnology) and peroxidase-conjugated anti-Goat IGG (Calbiochem-Novabiochem).

2.3. Lipolytic reaction and glycerol determination

Differentiated 3T3-L1 adipocytes in 24-well plates were washed twice with modified Krebs–Ringer phosphate buffer containing 20 mM HEPES (pH 7.4), 25 mM glucose, 1 mM ascorbate, 50 μg/ml sodium disulfite and 2% bovine serum albumin (fatty acid free). The washed cells were preincubated for 1 h at 37°C in 1 ml of the same buffer, and then stimulated with β-adrenergic reagents. The amount of released glycerol in the buffer was determined by the reaction with glycerol dehydrogenase (EC 1.1.1.6). From each well, 100 μl of aliquot was sampled and added to a reaction mixture consisting of 100 mM carbonate–bicarbonate buffer (pH 10.5), 1 mM NAD^+, 33 mM ammonium sulfate and glycerol dehydrogenase from *Cellulomonas* sp. (Toyobo) and then incubated at 30°C. The amount of released glycerol was estimated from the amount of formed NADH measured at 340 nm by spectrophotometry.

2.4. Materials

3T3-L1 fibroblast cells (JCRB 9014) were obtained from the Health Science Research Resources Bank of Japan Health Sciences Foundation. [(7*S*)7-{(2*R*)2-(3-chlorophenyl)-2-hydroxyethyl-amino} -5,6,7,8-tetrahydronapht-2-yl]ethyl oxyacetate, hydrochloride (SR58611) was a kind gift from Dr. Luciano Manara (Research Centre Sanofi

Midy) and Dr. Martine Combes (Sanofi Recherche). The following materials were purchased from the sources indicated: DMEM from Nissui; fetal bovine serum from JRH Biosciences; penicillin and streptomycin from Gibco BRL; DL-4-[2′-{2-hydroxy-2-(3-chlorophenyl)ethylamino}propyl phenoxyacetic acid sodium salt sesquihydrate (BRL37344), DL-4-[3-(1,1-dimethylethyl)amino]-(2-hydroxylpropoxy)]-1,3-dihydro-2*H*-benzimidazol-2-one hydrochloride (CGP12177) and L-propranolol from Research Biochemical International; {*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCl} (H89) and 2′-amino-3′-methoxyflavone (PD98059) from Calbiochem-Novabiochem. Other reagents used in the study were of the highest grade commercially available.

3. Results

3.1. Phosphorylation of ERK1/2 in 3T3-L1 adipocyte by the stimulation of β_3-adrenoceptors

β_3-Adrenoceptors were not found in 3T3-L1 fibroblasts, whereas these were found in 3T3-L1 adipocytes at 8 days after the initiation of differentiation (Fig. 1a).

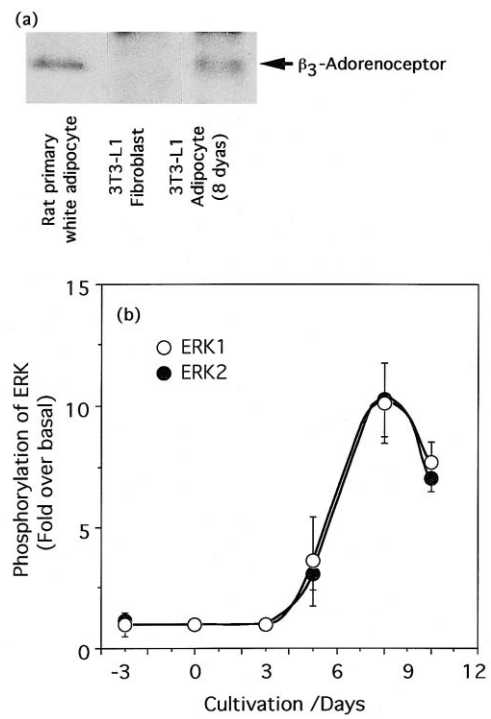

Fig. 1. Effect of BRL37344 on phosphorylation of ERK1/2 in 3T3-L1 cells during differentiation. (a) Whole cell extracts of rat white adipocytes, 3T3-L1 fibroblasts, and 3T3-L1 adipocytes were subjected to SDS-PAGE and immunobloted by anti-β_3-adrenoceptor antibody. (b) The 3T3-L1 cells cultured in 35-mm dishes were stimulated by 1 μM BRL37344 for 30 min at 37°C at the indicated cultivation periods. Phosphorylation extents of ERK1/2 in the cells were expressed in fold of phosphorylation level over each basal. Values represents mean ± S.D. ($n = 3$). Open and closed circles represent the results of ERK1 and ERK2, respectively.

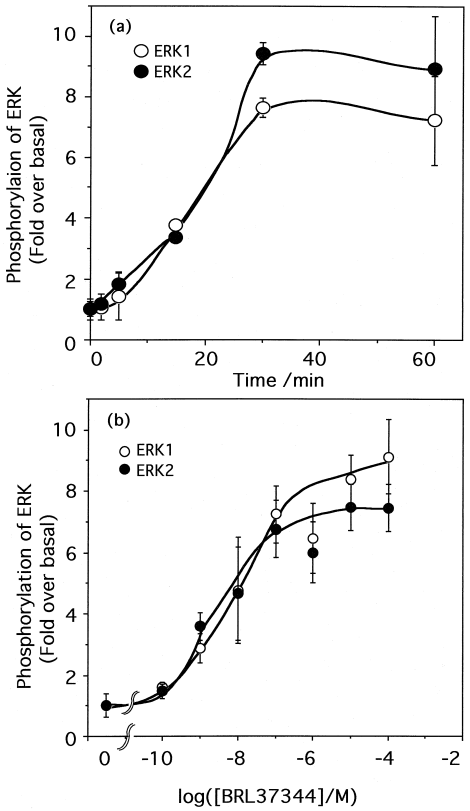

Fig. 2. Time- (a) and dose-dependency (b) of ERK1/2 phosphorylation by BRL37344 in 3T3-L1 adipocytes. 3T3-L1 adipocytes (35-mm dish, 8-day culture) were treated for the indicated periods with 1 μM BRL37344 at 37°C (a), or for 30 min at the indicated concentrations (b). The increase of phosphorylation levels over the basal level was measured as described previously. Open and closed circles represent the results for ERK1 and ERK2, respectively (mean ± S.D., $n = 3$).

ERK1/2 were not phosphorylated by the stimulation with the selective β_3-adrenoceptor agonist BRL37344 in

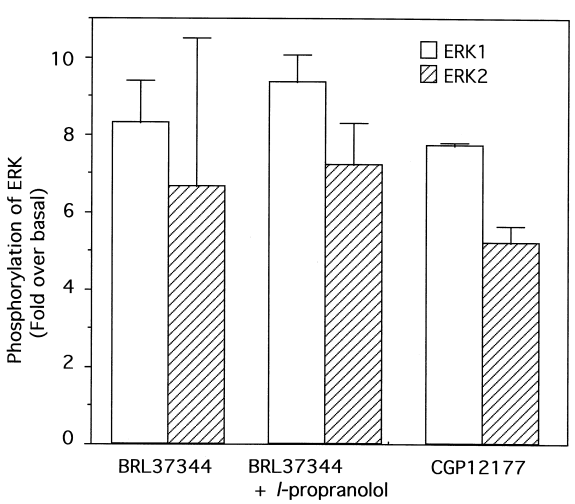

Fig. 3. Effect of β-adrenergic reagents on phosphorylation of ERK1/2. 3T3-L1 adipocytes (35-mm dish, 8-day culture) were stimulated by 1 μM BRL37344 in the absence or presence of 0.1 μM L-propranolol, or stimulated by 1 μM CGP12177 for 30 min at 37°C. Open and hatched bars represent the results for ERK1 and ERK2, respectively (mean ± S.D., $n = 3$).

either 3T3-L1 fibroblasts or cells within 0 to 3 days after the initiation of differentiation. On the other hand, 5 days after the initiation, threonine and tyrosine phosphorylation levels of both isoforms of ERK1/2 in the cells were slightly increased by the stimulation. The phosphorylation levels of ERK1/2 were increased to a similar extent, and reached a level about 7–10 times higher than that of the basal level over longer cultivation periods (Fig. 1b).

Phosphorylation of ERK1/2 by BRL37344 occurred in a time- and dose-dependent manner. The stimulation increased phosphorylation levels of ERK1/2 with a maximal level at 30 min, and the phosphorylation was sustained for at least 60 min after the stimulation (Fig. 2a and b).

As shown in Fig. 3, the β-adrenoceptor antagonist L-propranolol did not affect ERK1/2 phosphorylation by BRL37344 at 0.1 μM, despite the fact that this concentration was reported to be sufficient to block β_1- and β_2-adrenoceptors (Arch et al., 1984; Murphy et al., 1993). Another selective β_3-adrenoceptor agonist, CGP12177, known as an antagonist for β_1- and β_2-adrenoceptors, also caused ERK1/2 phosphorylation.

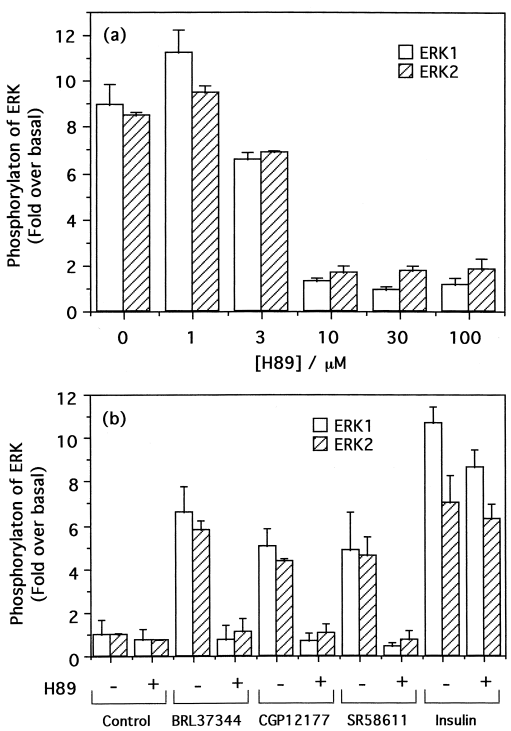

Fig. 4. Effect of the cAMP-dependent protein kinase inhibitor on phosphorylation of ERK1/2 by β_3-adrenoceptor agonists in 3T3-L1 adipocytes. (a) 3T3-L1 adipocytes (35-mm dish, 8-day culture) were treated by H89 at the indicated concentrations for 30 min and then stimulated by 1 μM BRL37344 at 37°C. (b) The adipocytes cultured as described in (a) were incubated for 30 min at 37°C in the absence or presence of 10 μM H89, and then stimulated by 1 μM BRL37344, 1 μM CGP12177 or 1 μM SR58611 for 30 min, or by 0.1 μM insulin for 10 min at 37°C. Open and hatched bars represent the results for ERK1 and ERK2, respectively (mean ± S.D., $n = 3$).

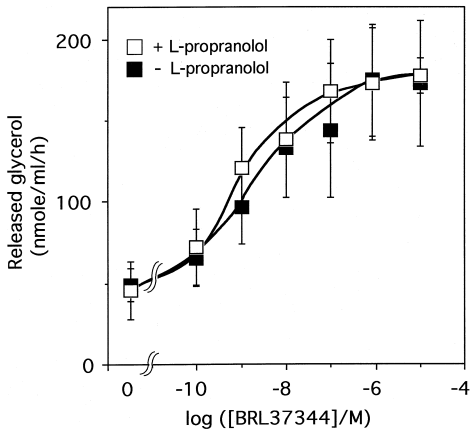

Fig. 5. Effect of L-propranolol on glycerol release by BRL37344. 3T3-L1 adipocytes (24-well plate, 8-day culture) were treated with BRL37344 at the indicated concentrations in the absence or presence of 0.1 μM L-propranolol. After 1 h of incubation at 37°C, the released glycerol in each well was assayed as described in Section 2. Open and closed squares represent the results in the presence or absence of L-propranolol, respectively (mean ± S.D., $n = 3$).

Preincubation of the adipocytes with the cyclic AMP (cAMP)-dependent protein kinase inhibitor H89 decreased the phosphorylation level of ERK1/2 induced by BRL-37344 in a dose-dependent manner and completely abol-

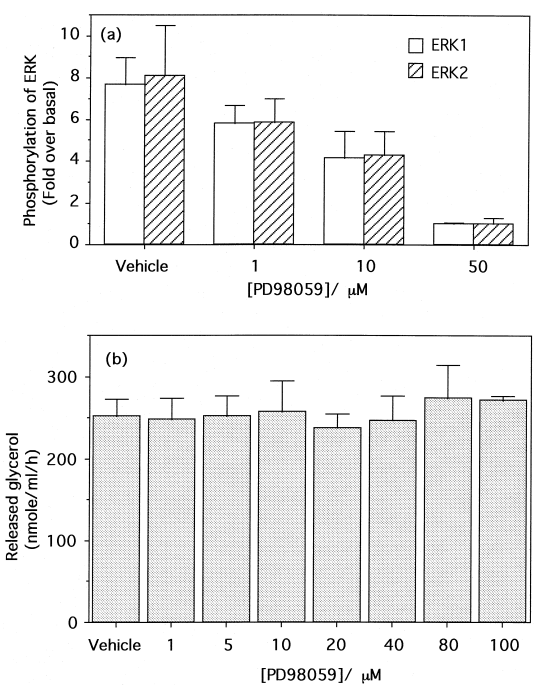

Fig. 6. Effect of PD98059 on phosphorylation of ERK1/2 (a) and glycerol release (b) caused by BRL37344 in 3T3-L1 adipocytes. (a) 3T3-L1 adipocytes (35-mm dish, 8-day culture) were treated with PD98059 at 37°C for 20 min at the indicated concentrations, and then stimulated by 1 μM BRL37344 for 30 min. Open and hatched bars represent the results for ERK1 and ERK2, respectively (mean ± S.D., $n = 3$). (b) 3T3-L1 adipocytes (24-well plate, 8-day culture) were preincubated with PD98059 as described in (a), and then stimulated by 1 μM BRL37344 for 1 h. The shaded bar represents the amount of released glycerol (mean ± S.D., $n = 3$).

ished at 10 μM (Fig. 4a). In addition, the preincubation with 10 μM H89 also completely abolished phosphorylation of ERK1/2 induced by the other β_3-adrenoceptor agonists CGP12177 and SR58611, whereas their phosphorylation by insulin was not altered, suggesting some possible roles of cAMP-dependent protein kinase in ERK1/2 phosphorylation induced by the stimulation of β_3-adrenoceptors (Fig. 4b).

3.2. Effect of the inhibitor of ERK1/2 phosphorylation on lypolysis by the stimulation of β_3-adrenoceptors

Glycerol release by BRL37344 occurred in a dose-dependent manner with a maximal effect at 1 μM. This dose dependency was not altered by 0.1 μM L-propranolol (Fig. 5). This result suggests that glycerol release was also mediated by β_3-adrenergic stimulation in 3T3-L1 adipocytes.

To date, no information about the roles of phosphorylated ERK1/2 in glycerol release have been obtained. To elucidate the role, the effect of a highly selective MAPK kinase inhibitor, PD98059, on the glycerol release was examined, and the results are shown in Fig. 6. PD98059 impaired ERK1/2 phosphorylation in a dose-dependent manner and completely inhibited it at 50 nM (Fig. 6a). On the other hand, this inhibitor did not alter the lipolytic action even at higher concentrations (up to 100 nM) than that sufficient to block ERK1/2 phosphorylation (Fig. 6b).

4. Discussion

3T3 fibroblasts are known to have a unique ability of differentiating to adipocytes, and sublines such as 3T3-L1 and 3T3-F442A, which were obtained by serial selection, show a much higher frequency of differentiation (Wise and Green, 1978), although appropriate treatment is required for efficient differentiation in the case of 3T3-L1 fibroblasts. The differentiated cells express many proteins characteristic for adipocytes, such as hormone-sensitive lipase (Kawamura et al., 1981), lipoprotein lipase (Wise and Green, 1978), glycerophosphate dehydrogenase (Tomiyama et al., 1995; Engelmant et al., 1998), β_3-adrenoceptors (Hadri et al., 1996) and so on (Alvarez, 1991). These sublines have therefore been used in many studies as a useful model of white adipocytes.

The absence of β_3-adrenoceptors in 3T3-L1 fibroblasts and the existence of these receptors in differentiated 3T3-L1 adipocytes (8-day cultivation) were confirmed at the start of the study, and as shown in Fig. 1, the selective β_3-adrenoceptor agonist, BRL37344, did not cause phosphorylation of ERK1/2 in either 3T3-L1 fibroblasts or immature 3T3-L1 adipocytes lacking β_3-adrenoceptors. On the other hand, ERK1/2 phosphorylation by BRL37344 was found in 3T3-L1 adipocytes cultured for 5 or more days after the initiation of differentiation. The cultivation-time dependency of ERK1/2 phosphorylation was similar to the gradual expression of β_3-adrenoceptors in differentiating 3T3-F442A cells (Hadri et al., 1996). Thus, phosphorylation of ERK1/2 might be mediated by β_3-adrenoceptors expressed during differentiation to adipocytes.

The phosphorylation of ERK1/2 in 3T3-L1 adipocytes was relatively delayed compared to those occurring in brown adipocytes (Shimizu et al., 1997; Lindquist and Rehnmark, 1998). Some reports have suggested that stimulation of β_3-adrenoceptors causes opposite responses in the two kinds of adipocytes, at least in terms of lipoprotein lipase mRNA level, which was increased in brown adipocytes (Kuusela et al., 1997), but decreased in white adipocytes (Raynolds et al., 1990; Antras et al., 1991; Ong et al., 1992). The differences in the phosphorylation time course of ERK1/2 might reflect difference in the functions of ERK1/2 and/or their roles in mediating events responding to the β_3-adrenergic stimulation, although the roles of ERK1/2 in such regulation remain uncertain.

To elucidate the role of β_3-adrenoceptors in ERK1/2 phosphorylation, the effect of L-propranolol, an antagonist of both β_1- and β_2-adrenoceptors, was examined. The antagonist did not block phosphorylation of ERK1/2 induced by BRL37344 (Fig. 3). At the same time, another selective β_3-adrenoceptor agonist, CGP12177, also caused phosphorylation of ERK1/2 (Fig. 3). These results suggest that phosphorylation of ERK1/2 in 3T3-L1 adipocytes is mediated by β_3-adrenoceptors.

Stimulation of β_3-adrenoceptors is known to increase intracellular cAMP level and cAMP-dependent protein kinase activity. As shown in Fig. 4b, preincubation of the adipocytes with the highly selective cAMP-dependent protein kinase inhibitor, H89 (De Rooij et al., 1998), selectively inhibited ERK1/2 phosphorylation induced by the three β_3-adrenoceptor agonists, BRL37344, CGP12177 and SR58611, but not the phosphorylation by insulin. These results suggest that ERK1/2 phosphorylation induced by the β_3-adrenoceptor agonists occurred via a cAMP-dependent protein kinase-dependent pathway. On the other hand, several groups reported that stimulation of β_3-adrenoceptors activated ERK1/2 via a inhibitory G-protein coupled, but cAMP-dependent protein kinase-independent pathway (Gerhardt et al., 1999; Soeder et al., 1999). In these studies, (*R*,*R*)-5-[2[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL316, 243) was used as a β_3-adrenoceptor agonist instead of BRL37344, CGP12177 and SR58611 used in this study. The difference between our study and the others might be explained by a very recent study with β_3-adrenoceptor knockout mice showing that BRL37344 and CGP12177, but not CL316,243, were still effective in decreasing mRNA level of uncoupling proteins 2 and 3 in β_3-adrenoceptor knockout mice, and suggested that these agonists were also able to act through an as yet undefined receptor, possibly a β_4-adrenoceptors (Boss et al., 1999). Further investigations on the signaling pathway leading to phos-

phorylation of ERK1/2 induced by the three β_3-adrenoceptor agonists used in this study might be indispensable to reveal the properties of β_3- and/or possibly β_4-adrenoceptors and complicated adrenergic controls by the receptors in white adipocytes.

Two main explanations have been proposed to account for the increase of glycerol release from adipocytes. The first is that this increase is related to activation of hormone-sensitive lipase, a rate-limiting enzyme of lipolysis. Activated cAMP-dependent protein kinase by stimulation of the β_3-adrenoceptors is known to phosphorylate hormone-sensitive lipase and this phosphorylation to lead to an increase of catalytic activity (Khoo et al., 1976; Severson et al., 1977; Anthonsen et al., 1998). The other possible explanation is that the increase is caused by re-distribution of hormone-sensitive lipase to membranes (Hirsch and Rosen, 1984) and/or fat droplets (Egan et al., 1992), with the latter thought to play greater role than the former (Egan et al., 1992). The re-distribution of hormone-sensitive lipase is also thought to be achieved by the phosphorylation of hormone-sensitive lipase (Egan et al., 1992), although the mechanism of this re-distribution and the participation of other molecules are not fully understood. In this study, we showed that the selective MAPK kinase inhibitor PD98059 did not alter the lipolytic action induced by BRL37344, even at a concentration that completely inhibited phosphorylation of ERK1/2. This result suggests that ERK1/2 did not contribute to either modulation of hormone-sensitive lipase activity or the re-distribution of the enzyme.

Recent studies have shown that β_3-adrenoceptors in white adipocytes play highly important roles in mediating various physiological events, although the downstream of β_3-adrenoceptors and/or the regulation mechanism of the responses have yet to be fully clarified. Therefore, the present findings of phosphorylation of ERK1/2 via β_3-adrenoceptors and of the possible role of cAMP-dependent protein kinase in the phosphorylation might be valuable for an enhanced understanding and investigation of the diverse responses caused by the receptors.

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