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Signaling Pathways Implicated in α-Melanocyte Stimulating Hormone-Induced Lipolysis in 3T3-L1 Adipocytes

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Abstract Melanocortins, besides their central roles, have also recently been reported to regulate adipocyte metabolism. In this study, we attempted to characterize the mechanism underlying α-melanocyte-stimulating hormone (MSH)-induced lipolysis, and compared it with that of the adrenocorticotrophin hormone (ACTH) in 3T3-L1 adipocytes. Similar to ACTH, MSH treatment resulted in the release of glycerol into the cell supernatant. The activity of hormone-sensitive lipase, a rate-limiting enzyme, which is involved in lipolysis, was significantly increased by MSH treatment. In addition, a variety of kinases, including protein kinase A (PKA) and extracellular signal-regulated kinase (ERK) were also phosphorylated as the result of MSH treatment, and their specific inhibitors caused a reduction in MSH-induced glycerol release and HSL activity, indicating that MSH-induced lipolysis was mediated by these kinases. These results suggest that PKA and ERK constitute the principal signaling pathways implicated in the MSH-induced lipolytic process via the regulation of HSL in 3T3-L1 adipocytes. J. Cell. Biochem. 96: 869–878, 2005.

Key words: melanocortins; adipocyte; MSH; ACTH; lipolysis; hormone-sensitive lipase

Lipolysis in white adipose tissue is one of the primary mechanisms, which is involved in the control of fuel partitioning. Under complex hormonal control, the hormone-sensitive lipase (HSL) is the target both of lipolytic and anti-lipolytic hormones [Holm et al., 2000]. The traditional epinephrine-mediated lipolysis pathway involves the cAMP-protein kinase A (PKA) cascade [Egan et al., 1992]. Activated PKA phosphorylates HSL, which is subsequently translocated to the surfaces of lipid droplets, catalyzing lipid hydrolysis [Clifford et al., 2000]. PKA phosphorylates HSL at Ser-563, Ser-659, and Ser-660, although Ser-563 has been believed to be responsible for HSL activation [Stralfors and Belfrage, 1983]. HSL can also be activated via extracellular signal-regulated kinase (ERK), which results in the lipolysis of adipocytes. ERK pathway activation appears to be able to regulate adipocyte lipolysis via the phosphorylation of HSL on Ser-600, and increases in the activity of HSL [Greenberg et al., 2001]. Garton et al. [1989] reported that adenosine 5′-monophosphate-activated protein kinase (AMPK) phosphorylates HSL at Ser-565 in vitro, and results in the abolition of further

Abbreviations used: ACTH, adrenocorticotrophin hormone; AMPK, adenosine 5′-monophosphate-activated protein kinase; DNP, diethyl *p*-nitrophenyl phosphate; ERK, extracellular signal-regulated kinase; HSL, hormone-sensitive lipase; IMX, 3-isobutyl-1-methylxanthine; MCR, melanocortin receptor; MSH, α-melanocyte stimulating hormone; PKA, protein kinase A; POMC, proopiomelanocortin; *p*NPB, *p*-nitrophenyl butyrate; TNF-α tumor necrosis factor-α.

Kyung-Joo Cho and Jung-Hyun Shim equally contributed to this study.

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HSL phosphorylation by PKA at Ser-563. However, a recent report demonstrated that AMPK activation is required for the maximal increase in lipolysis induced by isoproterenol in the 3T3-L1 adipocytes [Yin et al., 2003]. In fact, Su et al. [2003] have reported that mutations of the Ser-565 residue inhibit the ability of HSL to translocate to lipid droplets.

Multiple hormones and receptors are involved in the integration of the cellular response of adipocytes and the determination of the balance between lipogenesis and lipolysis. Many of the peptides derived from the proteolytic cleavage of proopiomelanocortin (POMC) have been classified as melanocortins, and they have been implicated in the regulation of feeding and grooming behaviors, learning and memory, thermogenesis, neural regeneration, metabolism, and inflammation [Boston, 1999]. Adrenocorticotrophin hormone (ACTH) has previously been demonstrated to bind with a high degree of affinity to rat adipocytes, and also exerts a potent lipolytic effect on adipocytes. Other POMC-derived peptides, MSH and α-lipotropin, have also been demonstrated to stimulate lipolysis in adipocytes. These peptides do not bind to ACTH receptors, however, thereby suggesting the presence of a second melanocortin receptor in the adipocytes [Boston and Cone, 1996].

The recent cloning of a family of melanocortin receptors (MCRs) has identified five distinct G protein-coupled receptors, and all of these receptors are coupled to adenylyl cyclase, via Gs. The MC1 receptor was the first receptor to be cloned, and has been demonstrated to be the classic α-melanocyte stimulating hormone (MSH) receptor responsible for cAMP-induced eumelanin formation in the melanocytes. A second receptor, the MC2 receptor (MC2R), has been identified to correspond to the adrenal cortex adrenocorticotrophin hormone (ACTH) receptor, and is known to bind only to ACTH. Both the cloned MC3 and MC4 receptor genes have been shown to code for neural melanocortin receptors. Finally, the MC5 receptor (MC5R) gene has been determined to encode a melanocortin receptor, exhibiting a very wide tissue distribution. Northern hybridization and RNase protection analyses have been utilized in order to detect MC5R mRNA at high levels in skin and muscle, as well as in lung, liver, spleen, and adrenal tissue. Unlike the MC1 and MC2 receptors, which exhibit specificity for MSH and ACTH, respectively, the MC3, MC4, and MC5 receptors are stimulated by a variety of melanocortin peptides with equal potency [Boston and Cone, 1996]. MC5R has also been shown to be expressed in numerous human peripheral tissues, including the adrenal gland, adipocytes, leukocytes, and many others [Chhajlani, 1996], and MC2R is expressed by adipose tissue in humans [Wikberg, 1999], suggesting that the regulation of adipocyte metabolism by MSH or ACTH occurs primarily via these two receptors. Recently, rodent adipocytes, including differentiated 3T3-L1 cells, have also been reported to express functional MC2R and MC5R, which are receptors which primarily exhibit specificity for ACTH and MSH, respectively [Gantz et al., 1994; Boston and Cone, 1996; Norman et al., 2003].

Although some reports have suggested that MSH, as well as ACTH, are involved in the lipolytic process [Boston, 1999], the mechanism underlying the regulation of lipolysis by MSH has yet to be well elucidated. Moreover, with the exception of the PKA pathway, little remains known with regard to the signaling pathways involved in MSH- or ACTH-induced lipolysis. In this study, our aim was to elucidate the primary signaling pathways involved in the regulation of lipolysis by MSH.

MATERIALS AND METHODS

Chemicals

Cell culture reagents, including Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS), were purchased from Invitrogen (San Diego, CA). [γ-^{32}P]-dATP was obtained from NEN Life Science Products (Boston, MA). Phospho-AMPK and AMPK antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA), and the other antibodies used were obtained from Santa Cruz Biotech, Inc. (Santa Cruz, CA). When not specifically indicated, all the other reagents, including MSH and ACTH (1–24), were obtained from the Sigma-Aldrich Chemical Co. (St. Louis, MO).

Cell Culture

3T3-L1 pre-adipocytes (American Type Culture Collection) were allowed to grow to confluence in DMEM containing 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, as described previously [Moini et al., 2002]. Two

days after attaining confluence (at a post-confluent stage), adipogenesis was induced by treating the cells with a solution containing 5 μg/ml of insulin, 0.25 μM of dexamethasone, and 0.5 mM of 3-isobutyl-1-methylxanthine (IMX), referred to as a "hormonal cocktail." The MSH and ACTH (1–24) were dissolved in 5% acetic acid.

Glycerol Release Assay

The fully differentiated 3T3-L1 adipocytes were then subjected to serum-deprived conditions for 12 h, and treated with either MSH or ACTH for the indicated times. Although the addition of adenosine deaminase was known to increase the lipolytic rate by removing adenosine [Mills, 1999], adenosine deaminase was not added upon lipolysis induction by MSH or ACTH. After incubation, the cell supernatants were collected, and the levels of glycerol release were measured spectrophotometrically using Free Glycerol Reagent (Sigma), according to the methods described in the manufacturer's manual. Glycerol standards (0–1 μM) were run in parallel with test samples, and the concentrations of released glycerol were calculated based on the standard calibration curve. Glycerol release was then represented as fold increase, as compared to control levels.

Preparation of RNA and RT-PCR

Total cellular RNA was purified from cultured cells using the easy-BLUE Total RNA Extraction Kit (Intron, Seoul, Korea), and 1 μg of total RNA was reverse-transcribed (RT) into the cDNA with reverse transcriptase (Promega, Madison, WI). Twenty milliliters of the RT mixture was incubated at 42°C for 90 min, then heated at 90°C for 10 min. PCR was performed in a 20 μl mixture containing 3 μl of RT product, 1× reaction buffer, 250 μM dNTP, 1 μM of each primer, and 1 U Taq DNA polymerase (TaKaRa), with the following oligonucleotide primers, MC5R (product length, 337 bp), 5′-CAT GTT CCT CCT GGC CCG GAA-3′, and 5′-TTA ATA CCC GCC AAG GAG CC-3′; MC2R (product length, 814 bp), 5′-AAC TCC GAT TGT CCT GAT GTA G-3′; GAPDH (product length, 400 bp), 5′-ACC CAG AAG ACT GTG GAT GG-3′, and 5′-TGA GCT TGA CAA AGT GGT CG-3′; under the following conditions: 1 cycle of 95°C for 15 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 2 min, primer extension at 72°C for 2 min, and a final cycle of 72°C for 10 min. A 10 μl aliquot from each PCR reaction was then electrophoresed on 2% agarose gel, and visualized via an ethidium bromide staining method.

pNPB Hydrolytic Activity

HSL activity was measured based on its esterase property, using *p*-nitrophenyl butyrate (*p*NPB) (Sigma) as a substrate. In 15 ml tubes, the assay buffer (0.9% NaCl, 0.1 M sodium phosphate, pH 7.25) was pre-incubated at 37°C. Enzyme (20 μg/20 μl of 3T3-L1 cell lysates) and 10 μl of substrate (50 mM in acetonitrile) were added to each tube. The total reaction volume was 1 ml, and the final concentration of the substrate was 0.5 mM. After 10 min of incubation, the reactions were terminated by the addition of 3.25 ml of methanol-chloroform-heptane (10:9:7, v/v/v). After vortex mixing (10 s per tube) and centrifugation (20 min at 800*g*), the tubes were heated at 42°C for 3 min. The absorbance (optical density) of 1 ml of the upper phase was then measured at 400 nm.

PKA Activity

PKA activity was determined by measuring the transfer of [^{32}P]-labeled phosphates to a phosphocellulose filter-bound peptide substrate, using the SignaTECT PKA assay kit (Promega). In brief, the kinase reaction was initiated by the addition of 25 μg of proteins with 100 μM biotinylated Kemptide (LRRASLG) to 25 μl of reaction mixture. After 5 min of incubation at 30°C, the reaction was terminated by the addition of 12.5 μl of termination buffer. An aliquot of the reaction mixture was spotted onto a phosphocellulose filter, and PKA activity was measured with an LS 6000 TA liquid scintillation counter (Beckman, Fullerton, CA).

Immunoblotting

Cells in 100-mm dishes were washed in ice-cold PBS containing 1 mM of Na_3VO_4, and lysed using a lysis buffer (pH 7.2) consisting of 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P 40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 25 mM NaF, 1 mM sodium orthovanadate, and 0.25% protease inhibitor cocktail solution (Sigma). Cytosolic proteins were separated on 10% SDS–polyacrylamide gel, electro-transferred to a PVDF membrane, and immunoblotted with appropriate antibodies. The immunoreactive bands were then

visualized with Enhanced Chemiluminescence Reagent (Amersham).

Data Presentation

Data were obtained from at least three independent experiments performed in triplicate, and the results of one of those experiments was represented as mean ± SD. Differences between means were evaluated by one-way analysis of variance (ANOVA). The minimum level of significance was set at $P < 0.05$.

RESULTS

Lipolysis Induction by MSH

Over 90% of the 3T3-L1 pre-adipocytes were fully differentiated into 3T3-L1 adipocytes 6 days after the 3-day treatment with hormonal cocktail (Fig. 1A). As reported previously [Boston and Cone, 1996], our RT-PCR analysis indicated that MC2R and MC5R mRNAs were expressed in the 3T3-L1 adipocytes but not in the 3T3-L1 pre-adipocytes, thereby suggesting that peripherally secreted MSH or ACTH can act directly on the 3T3-L1 adipocytes, probably through these receptors (Fig. 1B).

Lipolysis induction was determined by measurements of the levels of glycerol release from differentiated 3T3-L1 cells, as previously described [Arner, 1995].

In the unstimulated adipocytes, about 100 pmol of glycerol per milligram protein per hour was detectable in the cell supernatants, while 6 h of MSH treatment of the cells resulted in a significant increase in the amount of released glycerol in its nM range (50–1,000 nM),

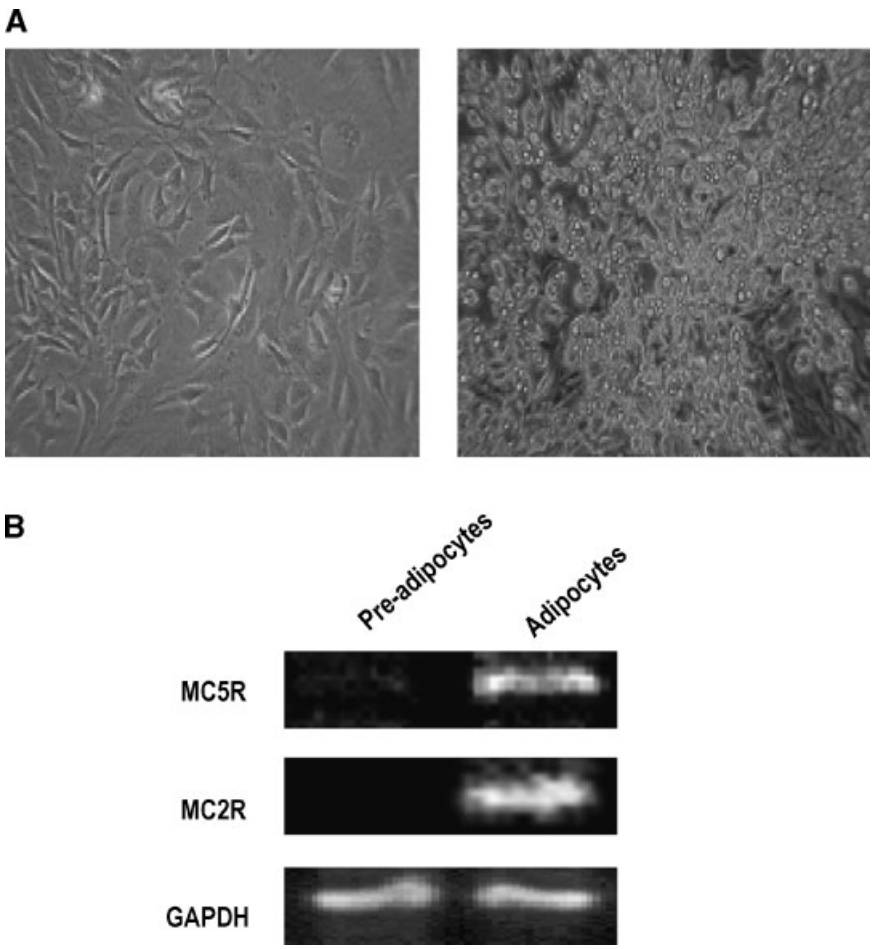

Fig. 1. RT-PCR analysis of MC5R or MC2R expression in 3T3-L1 adipocytes. **A**: Phenotypes of 3T3-L1 pre-adipocytes (**left panel**) and adipocytes (**right panel**). 3T3-L1 pre-adipocytes were differentiated into adipocytes by treatment with hormonal cocktail, as described in the Materials and Methods section. **B**: Total RNA was reverse-transcribed, then amplified using the specific primers for MC5R, MC2R, and GAPDH, as described in Materials and Methods.

which is consistent with ACTH (data not shown). When 1 μM of MSH or ACTH was administered over the course of time, glycerol release increased significantly at 30 min (data not shown), and persisted for up to 6 h (2.4- or 2.1-fold increase by MSH or ACTH, respectively) (data not shown). Interestingly, MSH failed to significantly induce glycerol release at lower concentrations (5 or 10 nM), or within 1 h (Fig. 2A), while ACTH could significantly induce release, even at lower concentrations, within 1 h (Fig. 2B). Moreover, 4 h of treatment with IMX, a representative cAMP activator, also increased glycerol release, up to 1.3 fold at 100 μM, while 10 nM of insulin failed to significantly induce such a release (Fig. 3). When the 3T3-L1 pre-adipocytes were treated with either MSH or ACTH, we detected no increases in the optical density as compared to the basal levels (open bar), suggesting that the increase in the adipocyte optical density was not induced by MSH or ACTH itself, but rather by its induction of glycerol release.

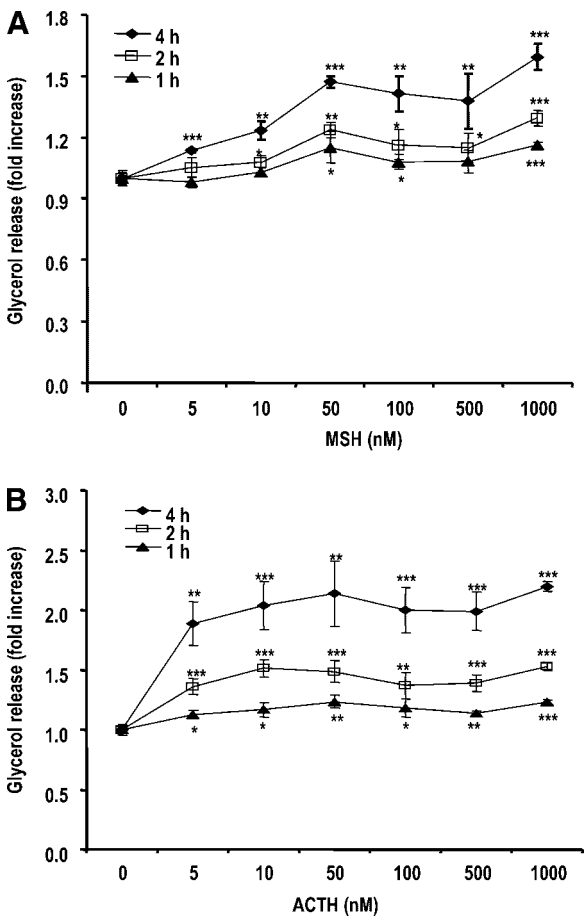

Fig. 2. Lipolysis induction by MSH in 3T3-L1 adipocytes. **A**: Cells were treated with indicated concentrations of MSH (A) or ACTH (**B**) for 1, 2, or 4 h in the absence of FBS, and the glycerol concentrations in the supernatants were measured using Free Glycerol Reagent (Sigma) as described in Materials and Methods. The glycerol release was represented as a fold increase as compared to control values. *, **, or ***; significant at $P < 0.05$, $P < 0.01$, or $P < 0.001$, respectively.

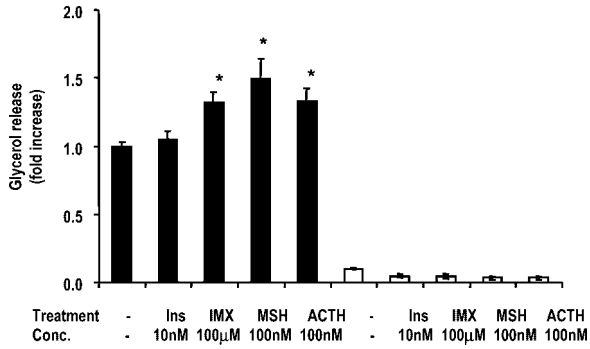

Fig. 3. Lipolysis induction by 3-isobutyl-1-methylxanthine in the 3T3-L1 adipocytes and pre-adipocytes. Cells were treated with indicated concentrations of insulin (Ins), 3-isobutyl-1-methylxanthine (IMX), MSH, and ACTH for 4 h in 3T3-L1 adipocytes (closed bar) and in pre-adipocytes (open bar), and the glycerol concentrations in the supernatants were measured using Free Glycerol Reagent (Sigma), as described in the Materials and Methods section. Glycerol release levels were represented as a fold increase as compared to the control. *; significant at $P < 0.05$.

Regulation of pNPB Hydrolytic Activity by MSH

HSL is presumed to be essential for lipolysis, which can be defined as the mobilization of free fatty acids from the adipocytes [Egan et al., 1992]. According to a previous report [Laurell et al., 2000], the HSL-derived activity was found to be 6–10-fold as high as the endogenous esterase activity at all temperatures, when *p*NPB was used as a substrate. In order to determine whether or not the regulation of lipolysis by MSH occurred via the regulation of HSL activity, the activity was measured after treatment with 1 μM of MSH for 10–120 min. Basal *p*NPB hydrolytic activity was approximately 1.05 ± 0.28 pmol *p*-nitrophenol generated per milligram protein per hour. The activity was highest at 30 min, and tended to decrease to basal levels after 60 min (Fig. 4A). However, treatment with either MSH or ACTH for up to 24 h failed to induce any detectable changes in the levels of HSL mRNA, when measured by RT-PCR analysis (data not shown), suggesting that the induction of HSL activity due to MSH or ACTH treatment was not mediated by its regulation of HSL mRNA expression.

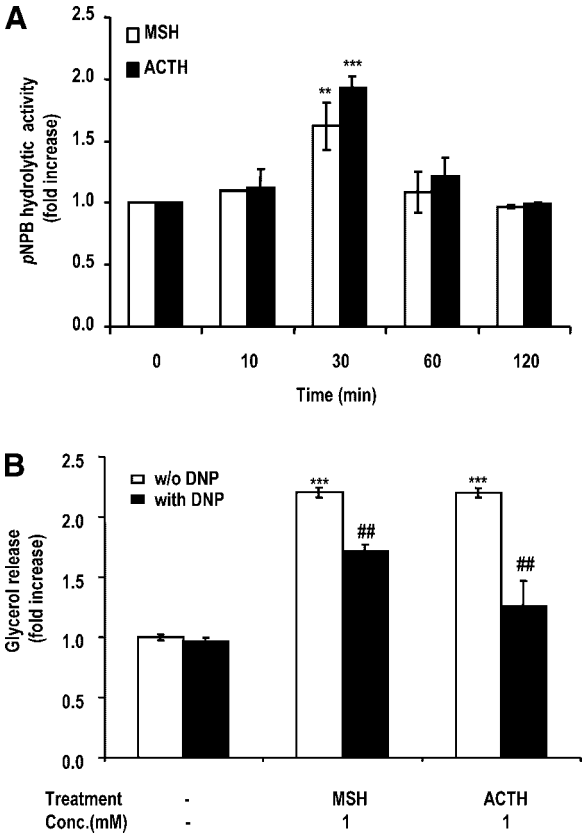

Fig. 4. Effect of MSH on pNPB hydrolytic activity in 3T3-L1 adipocytes. **A**: Cells were treated with 1 μM of MSH or ACTH for the indicated times in the absence of FBS, and *p*NPB hydrolytic activity was measured in the supernatant, using *p*NPB as a substrate, as described in the Materials and Methods section. The activity was represented as fold increase, as compared to control values. ** or ***; significant at $P < 0.01$ or $P < 0.001$, respectively. **B**: Cells were pre-treated with 100 μM of diethyl *p*-nitrophenyl phosphate (DNP) for 30 min, then treated with MSH (A) or ACTH (B) for 2 h in the absence of FBS, and the glycerol concentration in the supernatant was measured using the Free Glycerol Reagent (Sigma) as described in Materials and Methods. The glycerol release was represented as a fold increase compared to the control values. *** or ##; significant at $P < 0.001$ or $P < 0.05$ compared to control- or MSH (or ACTH)-treated groups, respectively.

Correspondingly, the increase in glycerol release resultant from MSH or ACTH treatment was inhibited up to 22% or 43%, respectively, by treatment with diethyl *p*-nitrophenyl phosphate (DNP), an inhibitor of HSL and other lipases/esterases, implying that the regulation of glycerol release by MSH or ACTH was mediated by its regulation of HSL or other *p*NPB-specific lipase/esterase activity (Fig. 4B).

Regulation of Various Kinases by MSH

The activity of HSL is primarily regulated by PKA, while ERK and AMPK have been also

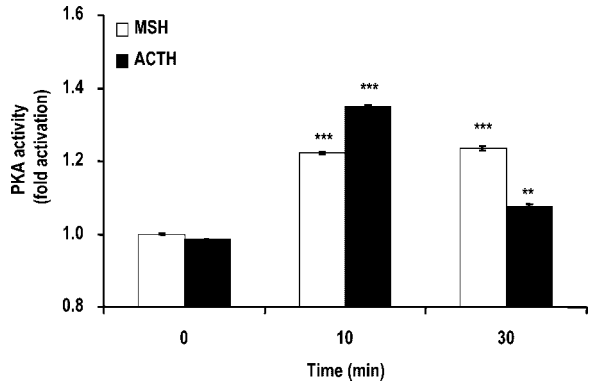

Fig. 5. Effects of MSH on PKA activity in 3T3-L1 adipocytes. Cells were treated with 1 μM of MSH or ACTH for indicated times in the absence of FBS, then lysed. PKA activity was then measured using the SignaTECT PKA assay kit, as described in the Materials and Methods section. The PKA activity was represented as a fold increase as compared to control values. ** or ***; significant at $P < 0.01$ or $P < 0.001$, respectively.

reported to regulate HSL activity [Stralfors and Belfrage, 1983; Greenberg et al., 2001; Birnbaum et al., 2003]. In order to demonstrate the involvement of these kinases in MSH-induced lipolysis, their activities were measured with a PKA assay kit and by Western blotting analysis, using the appropriate antibodies. As shown in Figure 5, significant increases in PKA activity were detectable within 30 min, whereas the total PKA amounts were almost identical at each time point (data not shown). Treatment with 1 μM of either MSH or ACTH resulted in increases in the phosphorylation of AMPK, ERK, respectively, and JNK at 10, 30, and 60 min, while the total amounts of PKA, AMPK, ERK, and JNK were almost identical in all lanes (Fig. 6A,B), suggesting their involvement in the lipolytic process.

In order to determine whether the activation of these kinases resulted in the induction of lipolysis via HSL activation, a variety of inhibitors, including H89 (PKA inhibitor), PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) were administered at their appropriate concentrations, before being treated with MSH or ACTH. As expected, treatment with H89 and PD98059 resulted in a significant reduction of MSH- or ACTH-induced glycerol release at various time points, while SP600125 and SB203580 exerted no notable effects (Fig. 7), suggesting that the PKA and ERK pathways constituted the principal pathways through which MSH or ACTH could induce lipolysis. In addition, MSH- or ACTH-

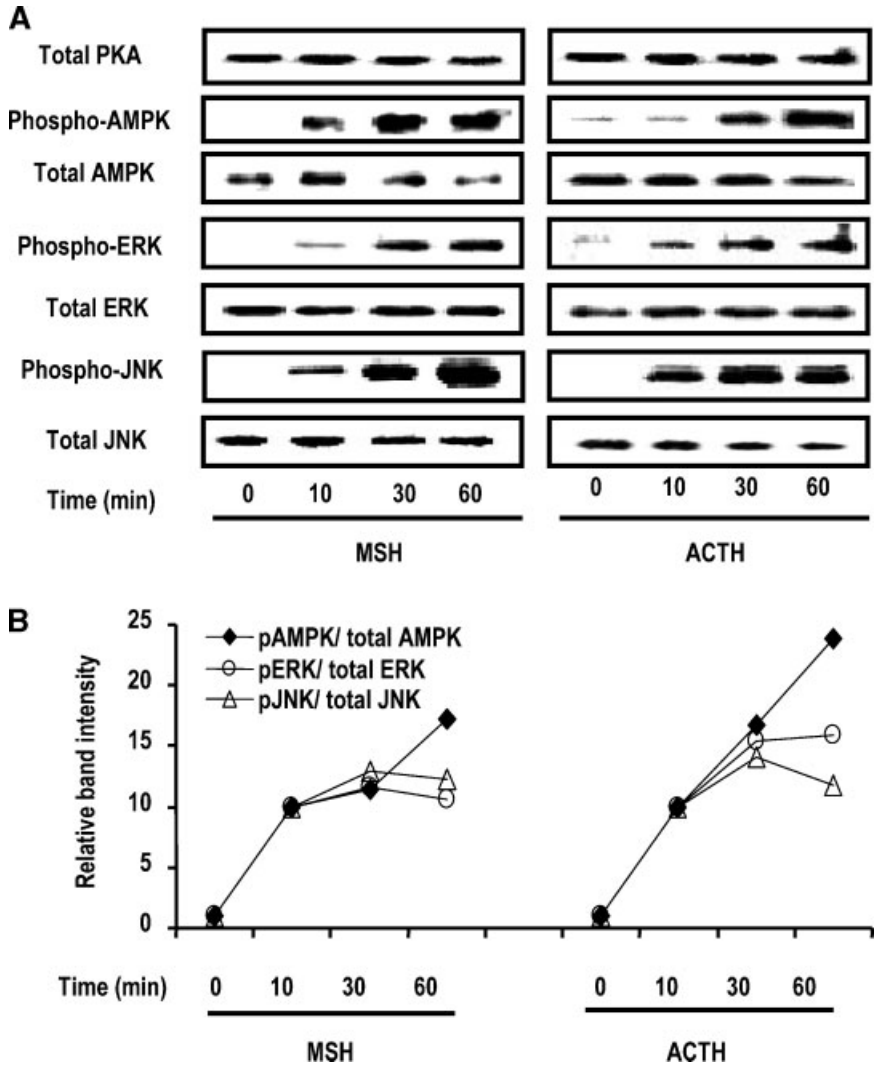

Fig. 6. Regulation of various kinases by MSH in 3T3-L1 adipocytes. Cells were treated with 1 μM of MSH or ACTH for the indicated times in the absence of FBS and lysed, and total- or phospho-AMPK, ERK, and JNK were detected by Western blotting, using their specific antibodies, as described in the Materials and Methods section (**A**). Relative band intensity was shown as a graph, where the Y-axis represents the ratio of phosphorylated kinase to its total amount (**B**).

induced activities of *p*NPB-specific lipases/esterases decreased significantly as the result of treatment with H89 or PD98059, further supporting the notion of PKA or ERK involvement in MSH-induced *p*NPB-specific lipases/esterases and glycerol release (Fig. 8). Pretreatment with diethyl *p*-nitrophenyl phosphate substantially decreased basal HSL activity, as well as MSH or ACTH-induced HSL activity, which might have been caused by its non-specific inhibition of other cellular esterases.

DISCUSSION

Until now, the regulation of lipolysis by melanocortins has remained ill-defined. Also, as compared to ACTH-induced regulation, MSH-induced regulation has been considered not to be important. Boston [1999] previously reported that MSH also stimulates adenylase cyclase in 3T3-L1 cells, thereby suggesting that its regulation of lipolysis occurs by virtue of the cAMP pathway. However, only a few reports have demonstrated the manner in which MSH signals the key lipolytic enzyme HSL, and regulates lipolysis in the 3T3-L1 adipocytes.

Although it was likely that the absence of adenosine deaminase in the incubations attenuated the lipolytic response, MSH, as well as ACTH, significantly increased glycerol release

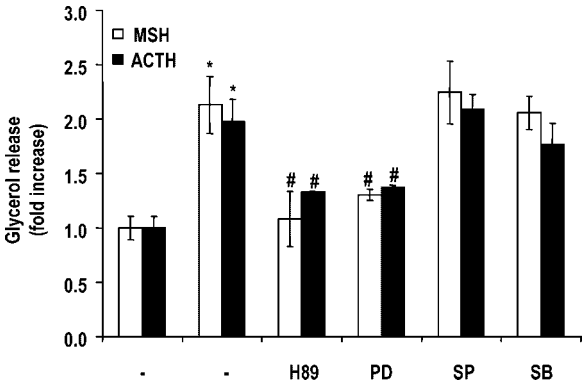

Fig. 7. Effects of various kinase inhibitors on MSH-induced lipolysis in 3T3-L1 adipocytes. Cells were pre-treated with 10 μM H89, 50 μM PD98059 (PD), 100 nM SP600125 (SP), 10 μM SB203580 (SB) for 30 min, and stimulated with 1 μM MSH or ACTH for 6 h. The glycerol concentrations in the supernatants were measured using Free Glycerol Reagent (Sigma) as described in the Materials and Methods section, and represented as a fold increase as compared to the control. * or #; significant at $P < 0.05$ as compared to the control or MSH (or ACTH)-treated groups, respectively.

and HSL activity. Moreover, not only ACTH, but also MSH, could activate a variety of kinases, including PKA, AMPK, and ERK, all of which turned out to be important in the control of lipolysis, probably via HSL regulation.

Using the RT-PCR method, we observed that MC2R and MC5R were expressed in the 3T3-L1 adipocytes, but not in the pre-adipocytes. Moreover, both MSH and ACTH significantly

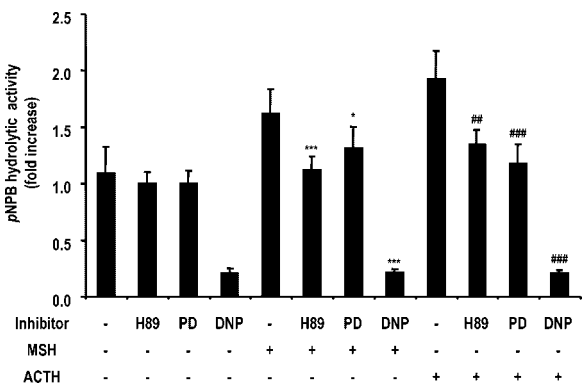

Fig. 8. Effects of various kinase inhibitors on MSH-induced pNPB hydrolytic activity in 3T3-L1 adipocytes. Cells were pre-treated with 10 μM H89, 50 μM PD98059 (PD), or 100 μM diethyl *p*-nitrophenyl phosphate (DNP) for 30 min, then stimulated with 1 μM MSH or ACTH for 30 min. *p*NPB hydrolytic activity was measured in the supernatant, using *p*NPB as a substrate, as described in *Methods*. The activity was represented as fold increase compared to the control. *, **, or *** (or #, ##, or ###); significant at $P < 0.05$, $P < 0.01$, or $P < 0.001$ compared to the MSH (or ACTH)-treated group, respectively.

induced glycerol release in their nM ranges, from 0.5 to 6 h of treatment. HSL is considered to be a major rate-limiting enzyme, which catalyzes the hydrolysis of triglycerides to diglycerides and monoglycerides, ultimately resulting in glycerol release. Although isoproterenol and tumor necrosis factor-α (TNF-α) markedly stimulated lipolysis rates via the PKA-dependent pathway, even in the absence of HSL [Okazaki et al., 2002], a variety of lipolytic hormones, including catecholamines [Large et al., 1998; Morimoto et al., 2001; Palin et al., 2003] have been reported to induce lipolysis via their regulation of HSL activity. According to our results, *p*NPB hydrolytic activity was induced by MSH beginning at 10 min, and was highest at 30 min, suggesting that HSL and other *p*NPB-specific lipases/esterases are involved in the process of MSH-induced lipolysis. Interestingly, PKA and *p*NPB hydrolytic activities occurred only transiently, while the increase in glycerol release persisted for up to 6 h. This discrepancy can be explained as follows. The activation of PKA or HSL is one of the mechanisms by which glycerol release can occur in adipocytes, and its transient activation can contribute to this release. However, many other unidentified mechanisms, other than PKA or HSL, can also induce glycerol release, suggesting that glycerol release can be maintained for a prolonged period. Moreover, the facts that purified HSL showed low specificity to *p*NPB, acting on triolein, cholesteryl oleate, and water-soluble *p*NPB at relative rates of 1:1.8:6.5, respectively [Tsujita et al., 1989], and *p*NPB is not specific to HSL might partly explain the inconsistency between HSL activity and glycerol release.

It has been reported that HSL is principally regulated both by phosphorylation, and by translocation to lipid droplets [Egan et al., 1992; Anthonsen et al., 1998], but not by transcriptional regulation. Our RT-PCR analysis also indicated that MSH-induced HSL regulation did not occurred transcriptionally, indicating that this regulation is probably mediated post-translationally, via phosphorylation. HSL phosphorylation is reported to be primarily controlled by PKA, and several other kinases, including ERK and AMPK, are known to play important roles. According to the results of our study, PKA activity was increased significantly as the result of MSH treatment within 10 min. Moreover, MSH treatment for up to 60 min

induced the phosphorylation of other kinases, such as AMPK, ERK, and JNK, all of which were also reported to regulate HSL, suggesting that the HSL activation due to treatment with MSH or ACTH occurred via multiple signaling pathways. Pre-treatment with H89 and PD98059, but not with SP600125 and SB203580, resulted in reductions in MSH-induced lipolysis and HSL activity, verifying that PKA and ERK are the primary pathways through which MSH can induce lipolysis.

In this study, we attempted to determine the primary signaling pathways that are involved in MSH-induced lipolysis, and determined that a variety of kinases, particularly PKA and ERK, are concomitantly involved in the induction of its lipolysis, probably via HSL activation. However, considering that DNP is not specific to HSL but inhibits other lipases, MSH affects lipolysis via PKA and ERK pathways, perhaps affecting other lipases as well as HSL. Similar patterns were also observed in regard to ACTH-induced lipolysis, suggesting that MSH and ACTH probably share several important signaling pathways, as well as specific receptors. However, further study will be necessary in order to distinguish MSH-induced lipolysis from that of ACTH, thereby providing insight into the precise roles of their specific receptors, MC2R and MC5R, in adipocyte metabolism.

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