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Stimulation of Lipolysis and Hormone-sensitive Lipase via the Extracellular Signal-regulated Kinase Pathway*

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Hormonally stimulated lipolysis occurs by activation of cyclic AMP-dependent protein kinase (PKA) which phosphorylates hormone-sensitive lipase (HSL) and increases adipocyte lipolysis. Evidence suggests that catecholamines not only can activate PKA, but also the mitogen-activated protein kinase pathway and extracellular signal-regulated kinase (ERK). We now demonstrate that two different inhibitors of MEK, the upstream activator of ERK, block catecholamine- and β_3-stimulated lipolysis by ~30%. Furthermore, treatment of adipocytes with dioctanoylglycerol, which activates ERK, increases lipolysis, although MEK inhibitors decrease dioctanoylglycerol-stimulated activation of lipolysis. Using a tamoxifen regulatable Raf system expressed in 3T3-L1 preadipocytes, exposure to tamoxifen causes a 14-fold activation of ERK within 15–30 min and results in ~2-fold increase in HSL activity. In addition, when differentiated 3T3-L1 cells expressing the regulatable Raf were exposed to tamoxifen, a 2-fold increase in lipolysis is observed. HSL is a substrate of activated ERK and site-directed mutagenesis of putative ERK consensus phosphorylation sites in HSL identified Ser^{600} as the site phosphorylated by active ERK. When S600A HSL was expressed in 3T3-L1 cells expressing the regulatable Raf, tamoxifen treatment fails to increase its activity. Thus, activation of the ERK pathway appears to be able to regulate adipocyte lipolysis by phosphorylating HSL on Ser^{600} and increasing the activity of HSL.

Free fatty acids are an essential source of energy for many tissues. The flux of free fatty acids is primarily dependent on the lipolysis of stored triacylglycerols in adipose tissue (1). The control of lipolysis is complex and involves multiple mechanisms (2–4). These include lipolytic (β-adrenergic agonists, ACTH, etc.) and anti-lipolytic (insulin, adenosine, etc.) hormones, their cognate receptors and signaling pathways, lipid droplet-associated proteins, such as perilipins (5, 6), and hormone-sensitive lipase (HSL),[1] the enzyme responsible for mediating the hydrolysis of triacylglycerol (7). Lipolytic stimuli increase lipolysis by activating adenylyl cyclase and raising intracellular concentrations of cyclic AMP, with resultant activation of cyclic AMP-dependent protein kinase (PKA) (4, 8), which phosphorylates both perilipins (5, 9) and HSL (10). The phosphorylation of HSL is associated with an increase in hydrolytic activity of the enzyme (7) and the translocation of HSL from the cytosol to the lipid droplet in some physiological settings (11–13). PKA has been shown to phosphorylate HSL at residues Ser^{563}, Ser^{659}, and Ser^{660}, all of which reside in a 150-amino acid stretch, termed the regulatory module (14, 15). The regulatory module is found within the C-terminal domain of HSL, which also contains the catalytic triad (16).

Although it is generally accepted that lipolysis is stimulated by PKA activation, as a consequence of GTP-binding protein (G protein)-coupled receptors acting through adenylyl cyclase and cyclic AMP, there is accumulating evidence to suggest that, in addition to PKA, G protein-coupled receptors and cyclic AMP can also activate mitogen-activated protein kinase (MAPK) pathways (17–19). Members of the MAPK family enable cells to transduce extracellular signals into an intracellular response (20). In mammalian cells, three parallel MAPK pathways have been identified which include the extracellular signal-regulated kinases (ERKs), p44 MAPK (ERK1), and p42 MAPK (ERK2), stress-activated protein kinases, also referred to as c-Jun-NH_2-terminal kinases (JNK), and the p38 MAPKs. ERKs are activated by growth factors acting via MAPK kinase kinases (such as Raf) and MAPK kinases (MEKs) and are involved in both cell proliferation and differentiation (20). Whereas, stress-activated protein kinases/JNKs and p38 MAPK are thought to be activated primarily in response to proinflammatory cytokines and environmental stress (20). Of particular interest, recent studies have shown that β-adrenergic agonists are capable of activating ERK in adipocytes (21–23). In the current paper, we have addressed whether the MAPK pathway, and specifically ERK, is involved in the regulation of lipolysis.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—All chemicals were from Sigma unless otherwise indicated. Bovine serum albumin (fraction V) was from In-

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[1] The abbreviations used are: HSL, hormone-sensitive lipase; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; JNK, c-Jun-NH_2-terminal kinases; MEK, mitogen-activated protein kinase kinases; PAGE, polyacrylamide gel electrophoresis.

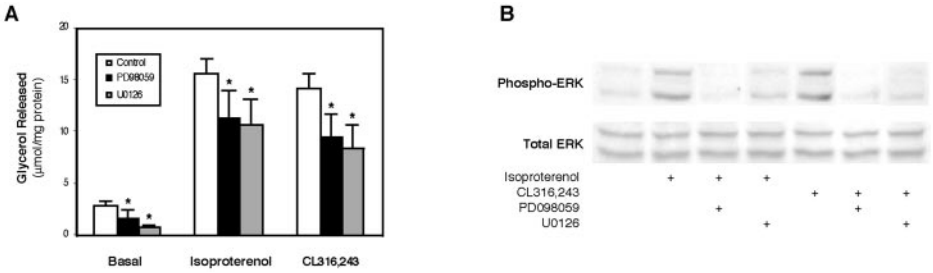

FIG. 1. **Effects of MEK inhibitors on isoproterenol- and β_3-agonist-stimulated lipolysis.** Differentiated 3T3-L1 adipocytes were serum starved overnight, preincubated in the absence or presence of either the MEK inhibitor PD098059 (100 μM for 3 h) or U0126 (10 μM for 1 h), and then incubated in the absence (*basal*) or presence of isoproterenol (10 μM) or CL316,243 (5 μM). *Panel A,* after 30 min incubation, glycerol content in the media was determined. Results are the mean ± S.E. of triplicate measurements and are representative of seven independent experiments. *Panel B,* cell extracts were immunoblotted for phospho-ERK and total ERK as described under "Experimental Procedures." *, $p < 0.01$ compared with no MEK inhibitor.

tergen Co., Purchase, NY; fetal bovine serum from Gemini Bio-Products, Inc., Calabasas, CA; Coon's F12/Dulbecco's modified Eagle's media, Dulbecco's modified Eagle's media, and Lipofectin reagent from Life Technologies, Inc., Gaithersburg, MD; ECL Western blotting detection reagents, horseradish peroxidase-linked whole antibody anti-rabbit IgG, cholesteryl [1-^{14}C]oleate from Amersham Pharmacia Biotech; nitrocellulose paper from Schleicher and Schuell, Keene, NH; Quick-Change Site-directed mutagenesis kit from Stratagene, La Jolla, CA; anti-ACTIVE MAPK and anti-ERK1/2 antibodies from Promega, Madison, WI; anti-phospho-JNK, anti-phospho-p38 MAPK, and anti-total p38 MAPK antibodies from Santa Cruz Biotechnology, Santa Cruz, CA; anti-total JNK antibodies from New England BioLabs, Beverly, MA. The MEK inhibitors U1026 (1,4-diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene) and PD098059 (2′-amino-3′-methoxyflavone) were from Calbiochem, San Diego, CA. Disodium (*R*, *R*)-5-(2[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl] - 1,3-benzodioxole-2,2-dicarboxylate (CL316,243) was a kind gift from Wyeth-Ayerst, Philadelphia, PA. Organic solvents were from J. T. Baker (Phillipsburg, NJ).

Plasmid Construction and Site-directed Mutagenesis—Mutagenesis of putative MAPK recognition sites in HSL was carried out using the Quick-Change Site-directed Mutagensis Kit. To facilitate the selection of positive mutants, an additional, neutral base pair alteration, which either introduced or eliminated a restriction enzyme site, was also incorporated into each set of primers. Primers used for the mutagenesis were: T540A, 5′-CCCCATAAGGCCCCAGTGGCCTGCAAC; S600A, 5′-CCATCAGACGCCCCCGAGATGTCA; T614A, 5′-GGCCCCTCCGCACCTCGGATGTGAACTTT; S680A, 5′-AAGAACCCCTTTATGGCTCCTCTGCTG. All constructs were sequenced to confirm the desired mutations and to ensure that no additional mutation had been introduced.

Cell Culture and Transfection—Chinese hamster ovary cells were grown in Coon's F12/Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2. 3T3-L1 preadipocytes were from the American Type Culture Collection (Manassas, VA). ΔRaf:ER cells were generated as described previously (24) by stably expressing pBABE-ΔRaf:ER/puro (a kind gift of Dr. Martin McMahon, DNAX) in 3T3-L1 cells by retroviral infection. 3T3-L1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum at 37 °C under 5% CO_2. For differentiation into adipocytes, cells were allowed to reach confluence and media changed to Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum along with 2 μM insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine for 48 h. Fully differentiated adipocytes were used 7–10 days later for study of lipolysis. For transient transfection experiments, cells were subcultured at a density of 2×10^5 cells/well in six-well plates the day prior to incubation with 0.75 μg of pCDNA3-HSL or HSL mutants, and 0.25 μg of pCMV β-galactosidase in 10 μl of Lipofectin reagent. Cells were transfected following the procedure from Life Technologies, Inc. Forty h after transfection, cells were washed and changed to serum-free media. Following serum starvation for 16 h, cells were treated with 1 μM tamoxifen to stimulate the activity of Raf. After 20 min treatment, cells were harvested in TES buffer containing protease and phosphatase inhibitors (50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 unit/ml leupeptin, 0.2 mg/ml aprotinin, and 5 mM NaF).

Immunoblotting—Cells were harvested and briefly sonicated in TES buffer with protease and phosphatase inhibitors. Homogenates were centrifuged at 10,000 × *g* for 15 min, and the supernatants were taken for electrophoresis. Samples were electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose, incubated with anti-rat HSL fusion protein IgG (25), anti-ACTIVE MAPK, and anti-ERK1/2 antibodies, and visualized by chemiluminescence as described previously (26).

Measurement of Lipolysis and HSL Activity—For studies with MEK inhibitors, differentiated 3T3-L1 cells were preincubated with PD098059 for 3 h or with U1026 for 1 h prior to initiation of measurement of lipolysis. Lipolysis was measured as glycerol content in the incubation media using a colorimetric assay as described (27, 28). HSL activity was determined on supernatants of cell homogenates after centrifugation at 10,000 × *g* for 15 min, using cholesteryl ester as substrate, as described previously (25).

In Vitro Phosphorylation—Chinese hamster ovary cells that were transfected with different HSL mutants were grown in Coon's F12/Dulbecco's modified Eagle's medium with 10% fetal bovine serum until confluent, and then harvested in lysis buffer (0.15 M NaCl, 3% Triton X-100, 0.1% lauryl Sarcosyl, 1 mM phenylmethylsulfonyl fluoride, 1 unit/ml leupeptin, 0.2 mg/ml aprotinin, and 5 mM NaF). After a brief sonication, the cells were centrifuged at 14,000 × *g* for 10 min at 4 °C. For *in vitro* phosphorylation, 20 μg of cell lysate was mixed with reaction buffer (25 mM Hepes, pH 7.5, 10 mM Mg acetate, 50 μM ATP), 2.0 μCi of [γ-^{32}P]ATP, and 0.01 μg of activated MAP kinase. The mixture was incubated at 30 °C for 30 min; the reaction was stopped, and the volume was adjusted to 250 μl with phosphate-buffered saline (pH 7.4) containing 0.5% bovine serum albumin. The sample was then incubated with rabbit anti-rat HSL/fusion protein IgG (0.5 μg/ml) and 10 μl of protein A beads overnight at 4 °C. The immune complexes were precipitated and washed three times in phosphate-buffered saline containing 0.5% bovine serum albumin. The final pellet were redissolved in 0.63 M Tris (pH 6.8) containing 8 M urea, 1% β-mercaptoethanol, 1% SDS, and 13% glycerol, boiled for 5 min, and electrophoresed on 10% SDS-PAGE. Autoradiographs were visualized in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis—Data are expressed as the mean ± S.E. Statistical analyses were performed by analysis of variance and comparisons among groups by Tukey or Bonferroni/Dunn using either SYSTAT (SPSS, Inc., Chicago, IL) or GB-STAT (Dynamics Microsystems, Inc., Silver Springs, MD).

RESULTS

MEK Inhibitors Decrease β-Adrenergic-stimulated Lipolysis—To determine whether the ERK pathway contributes to catecholamine-stimulated lipolysis, two specific MEK (MAP kinase kinase) inhibitors PD098059 (29) and U0126 (30) were used to block activation of the MEK/ERK pathway. PD098059 suppresses MEK activation by binding inactive MEK and preventing its phosphorylation by upstream kinases such as Raf-1, while U0126 binds to MEK and inhibits the catalytic activity of the active enzyme. As shown in Fig. 1, incubation of differentiated 3T3-L1 adipocytes with either PD098059 or U0126 resulted in a decrease in the low levels of glycerol released from cells in the absence of added lipolytic stimuli, suggesting that a portion of basal lipolysis might depend on signals via MAPK. When adipocytes were exposed to isoproterenol, a nonselective β-adrenergic agonist, or to CL316,243, a specific β_3-adrenergic agonist, there was an ~6-fold increase in lipolysis. Co-incubation with PD098059 or U0126 caused a 27 and 32% inhibition

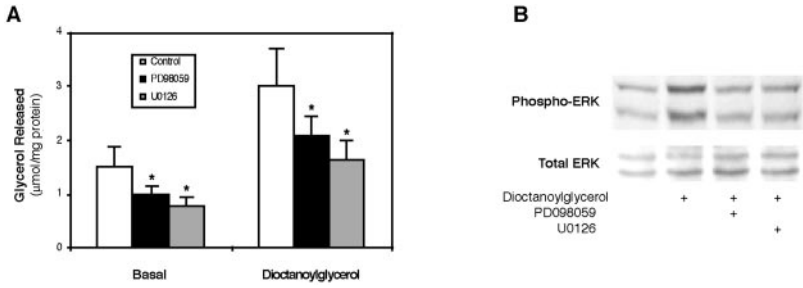

FIG. 2. **Effect of dioctanoylglycerol and MEK inhibitors on lipolysis.** Differentiated 3T3-L1 adipocytes were serum starved overnight, preincubated in the absence or presence of either the MEK inhibitor PD098059 (100 μM for 3 h) or U0126 (10 μM for 1 h), and then incubated in the absence (*basal*) or presence of dioctanoylglycerol (50 μM). *Panel A,* after 30 min incubation, glycerol content in the media was determined. Results are the mean ± S.E. of triplicate measurements and are representative of six independent experiments. *Panel B,* cell extracts were immunoblotted for phospho-ERK and total ERK as described under "Experimental Procedures." *, $p < 0.01$ compared with no MEK inhibitor.

of isoproterenol-stimulated lipolysis ($p < 0.01$), respectively, and a 33 and 41% inhibition of β_3-stimulated lipolysis ($p < 0.01$), respectively. Fig. 1*B* shows that isoproterenol and CL316,243 increased ERK activation, as detected by phospho-ERK, and that this activation was prevented by preincubation with either PD098059 or U0126, while the total amount of ERK was unchanged. Thus, based on data using inhibitors, it appears that ~1/3 of β-adrenergic-stimulated lipolysis could be attributed to activation of the MAPK pathway and ERK.

PKC Stimulates Lipolysis through MAPK/ERK—As another approach to assess whether the MAPK pathway is involved in mediating lipolysis, differentiated 3T3-L1 adipocytes were treated with dioctanoylglycerol, a cell-permeable diacylglycerol that activates protein kinase C (31). The protein kinase C family consists of at least 11 members that are involved in signal transduction (32), mediating a portion of their effects via activation of the MAPK/ERK pathway (33, 34). When adipocytes were exposed to dioctanoylglycerol (Fig. 2), there was a statistically significant ~2-fold increase in glycerol release ($p < 0.01$), although the magnitude of the increase was only ~30% of that seen with maximal β-adrenergic stimulation. Co-incubation with PD098059 or U0126 caused a 57 and 88% inhibition of dioctanoylglycerol-stimulated lipolysis ($p < 0.01$), respectively. Fig. 2*B* shows that dioctanoylglycerol increased ERK activation, as detected by phospho-ERK, and that this activation was prevented by preincubation with either PD098059 or U0126, while the total amount of ERK was unchanged. These data are again consistent with the MAPK/ERK pathway participating in the regulation of lipolysis.

Activation of MAPK/ERK Increases Lipolysis—To study the effects of ERK activation on lipolysis in the absence of confounding effects of other signaling cascades that can be simultaneously activated by hormone treatment, we used a regulatable Raf system in which ERK activation is controlled. Although Raf directly activates MEK and, consequently, ERKs, Raf does not activate any other MAP kinase pathways (35). For this purpose we used 3T3-L1 cells into which a tamoxifen-activable Raf:ER fusion protein (ΔRaf-1:ER) containing the hormone-binding domain of a mutant estrogen receptor that binds tamoxifen, but not estrogen, and the kinase domain of B-Raf was introduced (24, 36). Binding of an estrogen analog to the expressed ΔRaf-1:ER causes a conformational change in the fusion protein, resulting in the activation of Raf-1 and the subsequent rapid activation of ERK1 and ERK2. Fig. 3 shows that there was an 8–14-fold increase in ERK1 and -2 activation, as detected by phospho-ERK, within 20–30 min following exposure of cells to tamoxifen, while the total amount of ERK was unchanged. In contrast, there was no evidence for the activation of either JNK (Fig. 3*C*) or p38 MAPK (Fig. 3*D*), as detected by phospho-JNK and phospho-p38 MAPK, respectively, by tamoxifen, although both JNK and p38 MAPK proteins were detected by immunoblotting. Thus, ERK appears to be the only MAPK pathway regulated by tamoxifen-activable Raf in these cells. The ΔRaf:ER cells were allowed to differentiate into adipocytes, serum starved overnight, and then exposed to 1 μM tamoxifen to examine the effects of ERK activation on lipolysis (Fig. 4). The specific activation of ERK resulted in an ~2-fold increase in glycerol release ($p < 0.01$) over 90 min, further demonstrating a role for the MAPK/ERK pathway in regulating lipolysis. In comparison, isoproterenol (10^{-7} M) stimulated glycerol release ~5-fold in differentiated ΔRaf:ER cells (data not shown).

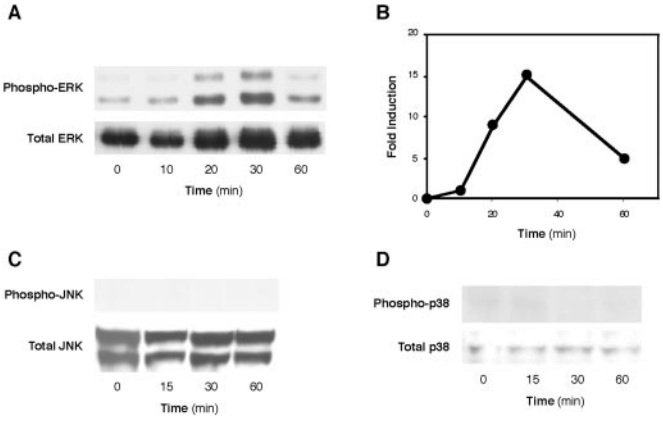

FIG. 3. **Time course of MAPK activation by tamoxifen in ΔRaf-1:ER cells.** ΔRaf-1:ER cells were generated as described under "Experimental Procedures." Cells were exposed to tamoxifen (1 μM) for the indicated times and cell extracts immunoblotted for phospho-ERK and total ERK (*Panel A*) (*Panel B* is a densitometric scan of *Panel A*), for phospho-JNK and total JNK (*Panel C*), and for phospho-p38 and total p38 MAPK (*Panel D*), as described under "Experimental Procedures." Results are representative of three independent experiments.

Activation of MAPK/ERK Increases HSL Activity—To explore the mechanism whereby MAPK/ERK activation leads to an increase in lipolysis, ΔRaf:ER preadipocytes were transfected with normal rat HSL cDNA. Forty-eight h after transfection, the cells were washed, treated with 1 μM tamoxifen for 20 min, and cell extracts assayed for HSL activity. As shown in Fig. 5, activation of ERK by exposure to tamoxifen increased HSL activity, as measured as neutral cholesteryl ester hydrolase, ~2-fold ($p < 0.01$). Thus, ERK activation appears to result in the rapid stimulation of HSL activity, which is followed by a parallel increase in lipolysis.

Identification of HSL Site Phosphorylated and Activated by MAPK/ERK—HSL activity is known to be regulated by phosphorylation (3). PKA increases HSL activity via phosphorylation at Ser^{563}, Ser^{659}, and Ser^{660} (14, 15), although phosphorylation at Ser^{565} by glycogen synthase kinase, AMP-dependent protein kinase, or Ca^{2+}/calmodulin-dependent protein kinase II has been

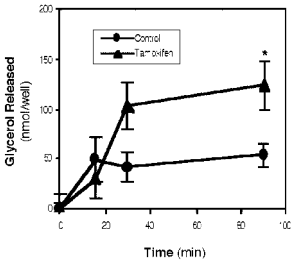

FIG. 4. **Effect of ERK activation on lipolysis in ΔRaf:ER cells.** ΔRaf:ER cells were allowed to differentiate into adipocytes, serum starved overnight, and then exposed to 1 μM tamoxifen. Glycerol content in the media was determined at the indicated times. Results are the mean ± S.E. of sextuplicate measurements and are representative of four independent experiments. *, $p < 0.01$ compared with no tamoxifen.

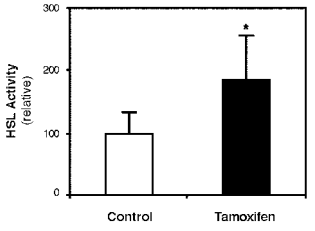

FIG. 5. **Effect of ERK activation on HSL activity.** ΔRaf:ER cells were transfected with normal rat HSL cDNA. Forty-eight h after transfection, the cells were washed, treated with 1 μM tamoxifen for 20 min, and cell extracts assayed for neutral cholesteryl ester hydrolase activity as described under "Experimental Procedures." Results are the mean ± S.E. of sextuplicate measurements and are representative of four separate experiments. *, $p < 0.01$ compared with no tamoxifen.

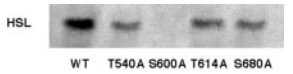

FIG. 6. **Phosphorylation of HSL and HSL mutants by activated MAPK/ERK *in vitro*.** Site-directed mutagenesis was used to generate T540A HSL, S600A HSL, T614A HSL, and S680A HSL as described under "Experimental Procedures." Chinese hamster ovary cells were transiently transfected with the mutants or wild-type HSL. Cellular extracts were then incubated with [^{32}P]ATP and activated MAPK/ERK *in vitro*. HSL was immunoprecipitated with anti-HSL IgG, separated on 10% SDS-PAGE, and visualized as described under "Experimental Procedures."

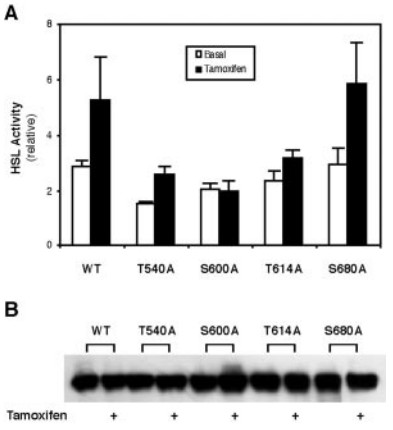

FIG. 7. **Identification of the site required for ERK-mediated stimulation of HSL hydrolytic activity.** ΔRaf:ER cells were transfected with normal HSL (WT), T540A HSL, S600A HSL, T614A HSL, or S680A HSL cDNA. Forty-eight h after transfection, the cells were washed, treated with 1 μM tamoxifen for 20 min, and cell extracts assayed for neutral cholesteryl ester hydrolase activity as described under "Experimental Procedures" (*Panel A*). Results are the mean ± S.E. of quintuplicate measurements and are representative of three independent experiments. *Panel B,* immunoblot of HSL in transfected cells.

reported to prevent activation of the enzyme (37). Computer analysis of the sequence of HSL revealed four putative MAPK phosphorylation sites (Thr^{540}, Ser^{600}, Thr^{614}, and Ser^{680}) located within the regulatory module of the protein. To examine whether any of these sites is recognized and phosphorylated by activated MAPK/ERK, we performed site-directed mutagenesis on each of the putative phosphorylation sites, converting each to Ala. After sequencing to confirm the identity of the clones, Chinese hamster ovary cells were transiently transfected with the mutants or wild-type HSL. Cellular extracts were then incubated with [^{32}P]ATP and activated MAPK/ERK *in vitro* and HSL immunoprecipitated. As shown in Fig. 6, HSL is a substrate for activated MAPK/ERK since wild-type HSL was phosphorylated. Mutation of T540A, T614A, and S680A had no effects on the ability of HSL to be phosphorylated by activated MAPK/ERK, when corrected for the amount of HSL expressed. In contrast, S600A completely eliminated the phosphorylation of HSL by activated MAPK/ERK, suggesting that Ser^{600} is the target of MAPK/ERK.

To directly determine whether the phosphorylation of HSL at Ser^{600} is required for activation of HSL activity by MAPK/ERK, ΔRaf:ER preadipocytes were transfected with mutant or wild-type HSL cDNA. Forty-eight h after transfection, the cells were washed, treated with 1 μM tamoxifen for 20 min, and cell extracts assayed for HSL activity (Fig. 7*A*). As seen previously, activation of ERK by exposure to tamoxifen increased neutral cholesteryl ester hydrolase activity ~2-fold ($p < 0.01$) in cells transfected with wild-type HSL. Basal (no tamoxifen treatment) HSL activity was not appreciably affected by mutation of T540A, T614A, or S680A, and, consonant with the phosphorylation of these mutants by activated MAPK/ERK, exposure to tamoxifen increased neutral cholesteryl ester hydrolase activity. However, the tamoxifen-induced increases in HSL hydrolytic activity of the T540A and T614A mutants, although statistically significant, were not as robust as seen with wild-type HSL. The tamoxifen-induced increase in the hydrolytic activity of the S680A mutant was indistinguishable from wild-type HSL. Mutation of S600A also had no appreciable effect on basal HSL activity, but eliminated the ability of ERK activation to increase neutral cholesteryl ester hydrolase activity, demonstrating that phosphorylation of Ser^{600} by activated MAPK/ERK is required for stimulating HSL hydrolytic activity. Equivalent amounts of wild type and mutant HSL were expressed in the transfected cells as documented by immunoblot with anti-HSL antibodies (Fig. 7*B*).

DISCUSSION

The MAPK family consists of several kinase cascades that respond to mitogens, cytokines, and environmental stress, leading to changes in gene expression, metabolism, and cellular regulatory events that mediate cell proliferation, differentiation, and survival, and inflammatory and stress responses (20, 38, 39). MAPK specificity is controlled by multiple mechanisms, including sequential physical interactions of members within a particular cascade, interactions with docking proteins that act as scaffolds, and spatial localization (20). The three-tiered MAPK/ERK pathway has classically been viewed to respond to growth factors, with activation of tyrosine kinase receptors acting through small G-proteins, such as Ras, leading to the activation of the proximal MAPK kinase kinase, Raf (20). Raf then phosphorylates and activates the MAPK kinases MEK1 and MEK2, which in turn phosphorylate and activate the MAP kinases ERK1 and ERK2 (20). In addition to growth factor receptors, accumulating evidence supports a role for regulation of MAPK by G protein-coupled receptors (17, 19, 40). A number of different mechanisms appear to link G protein-coupled receptors with MAPK activation. For instance, sub-

units of G proteins have been linked to ERK activation via Ras-dependent and independent pathways (41, 42); recruitment of nonreceptor tyrosine kinases, such as Src, by G proteins have been reported (40); and the participation of scaffolding proteins in the binding of receptor- and nonreceptor-tyrosine kinases, as well as members of the MAPK cascade have been suggested (40, 43). Although regulation of transcription factors and gene expression is an important function of MAPK cascades, they also have a number of cytoplasmic targets (20).

Adipocyte lipolysis has been viewed to be controlled via activation of G protein-coupled receptors acting through PKA (4, 8). In the present studies we provide evidence that components regulating adipocyte lipolysis are cytoplasmic targets of MAPK and that a portion of β-adrenergic-stimulated lipolysis in 3T3-L1 adipocytes is mediated via ERK. First, lipolytic stimulation by agonists acting either nonselectively via β-adrenergic receptors or selectively via β_3-adrenergic receptors was inhibited ~1/3 by two different, highly specific MEK inhibitors, PD098059 and U0126. Both PD098059 and U0126 block ERK activation, but via different mechanisms. PD098059 prevents the activation of MEK by Raf (29) and U0126 directly inhibits MEK from phosphorylating ERK (30). These observations on lipolysis are in contrast to a previous report where PD098059 was found to block ERK activation by a different β_3 agonist (BRL37344) than we used, but to have no effect on BRL37344 stimulated lipolysis (44). This discrepancy might be explained by the β-agonists used, since BRL37344 has been reported to have biological effects even in β_3-adrenoreceptor null mice, although CL316,243 has no activity in these mice (45). Second, avoiding G protein-coupled receptor pathways, lipolysis was stimulated, albeit to only ~30% of the magnitude seen with β-adrenergic stimulation, when ERK activation was achieved by protein kinase C signaling using dioctanoylglycerol (33, 34). Importantly, up to ~90% of dioctanoylglycerol-stimulated lipolysis was blocked by the MEK inhibitors PD098059 and U0126. Third, using a regulatable Raf system in which ERK activation is controlled in the absence of confounding effects of other signaling cascades, we show that activation of ERK is sufficient to stimulate lipolysis, although the magnitude of the stimulation is only a fraction of that achieved by β-adrenergic agonists. Fourth, we show that HSL activity is rapidly increased by activation of ERK. Fifth, HSL is a substrate of activated ERK, with activated ERK directly phosphorylating HSL. And sixth, we identify Ser^{600} as the site phosphorylated by activated ERK and demonstrate that the integrity of this site is required not only for phosphorylation of HSL by activated ERK, but also for activated ERK to increase HSL activity. Taken together, these data strongly support a role for MAPK signaling in the regulation of lipolysis and identify HSL as a cytoplasmic target of ERK.

The phosphorylation and activation of HSL by ERK provides an additional pathway and mechanism for the regulation of HSL and the control of lipolysis. HSL has a primary amino acid sequence that is unrelated to any of the other known mammalian lipases; however, it shares some sequence similarity with liver arylacetamide deacetylase within its catalytic domain (46) and also some homology with several bacterial and fungal lipases and esterases (16, 47–51). The C-terminal portion of HSL displays secondary structural homology with that of acetylcholinesterase and several fungal lipases (16) and bacterial brefeldin A esterase (52), consisting of parallel β-sheets flanked by α-helical connections, that has allowed these proteins to be classified as α/β hydrolases (53). Using limited proteolysis, it has been suggested that HSL is composed of two major structural domains (48, 54). The N-terminal domain in rat HSL constitutes the first 323 amino acids, which display no sequence or structural similarity with any other known proteins (48, 54). The function of this N-terminal region of HSL has been proposed to represent a docking domain for the interaction of HSL with adipocyte lipid-binding protein or other proteins (55). Based on sequence alignment, structural homology with fungal lipases, and mutational analyses (14, 16), the C-terminal domain has been shown to contain the catalytic triad and other residues important in hydrolytic activity, as well as a 150-amino acid insert that has been termed the regulatory module because several serines located within this region have been shown to be phosphorylated (14, 15). The site of phosphorylation and activation of HSL by PKA was originally determined to be Ser^{563} (56); however, others have reported that Ser^{659} and Ser^{660} were phosphorylated by PKA *in vitro* and were required for the phosphorylation-induced increase in hydrolytic activity against triacylglycerol (15). In addition to PKA, other kinases can phosphorylate HSL, such as glycogen synthase kinase, AMP-dependent protein kinase, Ca^{2+}/calmodulin-dependent protein kinase II, at Ser^{565} that does not activate the enzyme (37). Our demonstration of phosphorylation of Ser^{600} by ERK identifies another kinase for which HSL is a substrate. It is interesting that Ser^{600} is located in the middle of the regulatory module and phosphorylation of Ser^{600} by ERK results in a similar magnitude of activation of HSL hydrolytic activity as seen with PKA (7, 15). The fact that ERK stimulation of hydrolytic activity of the T540A and T614A HSL mutants, although statistically significant, was not as robust as seen with wild-type HSL suggests that these mutations might have resulted in some alterations of HSL conformation. The mechanisms whereby phosphorylation of sites within the regulatory module controls HSL hydrolytic activity are not known. However, in view of the comparable changes in hydrolytic activity, phosphorylation of Ser^{600} by ERK might be expected to cause a similar conformational change as phosphorylation of PKA sites.

Although phosphorylation of HSL by PKA increases HSL hydrolytic activity moderately, PKA signaling via G protein-coupled receptors results in a dramatic increase in lipolysis. This discrepancy is due, at least in part, to the translocation of HSL to the lipid droplet and the possible movement of perilipins on the droplet (11–13, 57). The moderate stimulation of lipolysis that occurs with ERK activation and which parallels the increase in HSL hydrolytic activity suggests that ERK activation might not cause HSL translocation; however, this awaits direct experimental evidence. Nonetheless, this raises the possibility that the robust lipolytic stimulation observed with activation of G protein-coupled receptors might be due to a combination of PKA-mediated translocation and activation of HSL and MAPK-mediated regulation of HSL hydrolytic activity. Additional studies will be required to investigate these possibilities. Nevertheless, our current studies document that HSL is a cytoplasmic target of ERK and that a portion of β adrenergic-stimulated lipolysis is mediated via the MAPK pathway.

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