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ORIGINAL PAPER

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Magnolol induces the distributional changes of p160 and adipose differentiation-related protein in adrenal cells

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Abstract Magnolol stimulates adrenal steroidogenesis and induces the distributional changes of p160 and adipose differentiation-related protein (ADRP) in rat adrenal cells. This study investigated the underlying signaling mechanisms involved in these processes. Magnolol (30 μM) caused a time-dependent increase in the phosphorylation of extracellular signal-related kinase (ERK) in cultured adrenal cells. The following evidence supports a link between ERK activation and p160 translocation. First, the magnolol-induced redistribution of p160 from the lipid droplet surface to the cytosol, resulting in the decrease in the percentages of p160-positive cells, and this decrease in p160-positive cells was completely blocked by pretreatment with either of the MAPK-ERK kinase (MEK) inhibitors PD98059 or U0126. Second, magnolol did not significantly decrease total p160 protein levels but caused an increase in threonine phosphorylation of p160, which reached a maximum after 5 min of magnolol treatment, and this magnolol-induced phosphorylation of p160 was prevented by pretreatment with U0126, suggesting the involvement of ERK. In addition, magnolol decreased both ADRP immunostaining intensity at the lipid droplet surface and the percentage of ADRP-positive cells. This was further confirmed biochemically by the decrease in ADRP levels in total cell homogenates and in lipid droplet fractions. Magnolol-induced decrease in ADRP staining at the lipid droplet surface was not affected by pretreatment with PD98059 or U0126, indicating that ERK signaling was not involved in this event. Furthermore, treatment with 30 μM magnolol for 6 h resulted in about 50% decrease in ADRP protein level. Therefore, decreased protein levels of p160 and ADRP at the lipid droplet surface induced by magnolol were mediated via two different mechanisms: phosphorylation of p160 and downregulation of ADRP expression, respectively.

Keywords Magnolol · Adipose differentiation-related protein · p160 · Adrenal cells

Abbreviations ADRP: Adipose differentiation-related protein · CEH: Cholesterol ester hydrolase · DMEM: Dulbecco's modified Eagle medium · ERK: Extracellular signal-related kinase · HSL: Hormone-sensitive lipase · MEK: MAPK-ERK kinase · PBS: Phosphate-buffered saline · PKA: Protein kinase A · PKC: Protein kinase C · PLC: Phospholipase C

Introduction

Steroidogenic adrenal cells are characterized by the presence in the cytoplasm of many lipid droplets of varying sizes. The surface of the lipid droplets in adrenal cells is coated by at least three different proteins: perilipin, adipose differentiation-related protein (ADRP), and p160 (Fong et al. 2002; Servetinick et al. 1995; Wang and Fong 1995). Perilipin A and C are the major perilipin isoforms in adrenal cells (Londos et al. 1995, 1996; Servetinick et al. 1995), but only perilipin A can be directly phosphorylated by protein kinase A (PKA) (Greenberg et al. 1991). Nonphosphorylated perilipin plays a role in the inhibition of lipolysis (Londos et al. 1999). PKA-mediated phosphorylation of perilipin triggered by isoproterenol is presumably required for the redistribution of perilipin from the lipid droplet surface to the cytosol (Souza et al. 1998). However, transloca-

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tion of perilipin in adipocytes appears to be related to the ages of the rats (Clifford et al. 2000). Nevertheless, reduced expression of perilipin at the lipid droplet surface is associated with lipolysis stimulated by tumor necrosis factor-α and isoproterenol (Souza et al. 1998), and ectopic expression of perilipin A in 3T3-L1 preadipocytes induces the accumulation of lipid droplets (Brasaemle et al. 2000).

ADRP, a 52-kDa protein closely involved in the early differentiation of adipocytes, is found in several types of lipid-droplet-containing cells (Brasaemle et al. 1997; Heid et al. 1996; Jiang and Serrero 1992; Londos et al. 1999). Its major function is the formation of nascent and growing lipid droplets (Imamura et al. 2002; Nakamura and Fujimoto 2003). ADRP is a fatty-acid-binding protein and therefore may regulate fatty acid targeting to lipid droplets (Frolov et al. 2000; Serrero et al. 2000). The presence of ADRP aggregates prior to its association with lipids supports the theory that ADRP plays a role in nascent lipid droplet formation (Wang et al. 2003). In 3T3 fibroblasts, overexpression of ADRP induces the accumulation of intracellular lipid droplets without the induction of other lipogenic genes (Imamura et al. 2002). In adrenal cells, ADRP associates stably with the lipid droplet surface and does not show any redistribution under lipolytic conditions (Fong et al. 2002). In response to dibutyryl cAMP-induced PKA activation, perilipin, and p160, but not ADRP, redistribute from the surface of lipid droplets to the cytoplasm (Fong et al. 2002). Therefore, whether ADRP plays a role in lipolysis remains to be determined.

A 160-kDa protein (p160) was proven to be associated with lipid droplets in adrenal cells by using a monoclonal antibody A2 (mAbA2) (Wang and Fong 1995). Upon activation of PKA, staining of p160 at the lipid droplet surface is decreased while that in the cytosol increased (Fong and Wang 1997). A time-course study showed that the decrease in droplet size is simultaneously associated with loss of p160 staining (Fong and Wang 1997). Thus, in adrenal cells, the PKA-signaling pathway controls both steroidogenesis and the distributional changes of perilipin and p160 from the lipid droplet surface (Fong et al. 2002). These observations indicate that, in adrenal cells, p160 and perilipin may form a barrier against lipolysis (Fong et al. 2002). Although the mechanism for perilipin translocation is well known, how p160 translocation is regulated remains to be answered.

Previously, we reported that magnolol, a compound isolated from *Magnolia officialis* (Tseng et al. 1990), stimulates steroidogenesis and results in the decrease in p160 staining at the lipid droplet surface in adrenal cells, and this magnolol-induced translocation of p160 is PKA- and PKC-independent (Wang et al. 2000). In the present study, we examined the signaling pathway and the mechanism involved in magnolol-induced p160 translocation. It is interesting to note that magnolol also induced the redistribution of ADRP in adrenal cells. Efforts were also made to elucidate the possible underlying mechanisms.

Materials and methods

Cell culture

Adult female Wistar rats (8- to 12-weeks-old) were purchased from the Facility for Research Animals of the National Taiwan University. The maintenance and use of the animals were in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985). The animals were anesthetized with 7% chloral hydrate (6 ml/kg) by intraperitoneal injection; then, primary cultures of adrenocortical cells were prepared, as described previously (Wang and Fong 1995). Briefly, adrenal gland fragments in serum-free Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) containing 0.5 mg/ml of type II collagenase (C-6885, Sigma, St Louis, MO, USA) were incubated for 15 min in a 37°C water bath, then the reaction was stopped by addition of ice-cold culture medium (a 1:1 v/v mixture of Ham's F12 medium and DMEM containing 25 mM HEPES, 1.2 g/l of $NaHCO_3$, 2.5% horse serum, 2.5% fetal bovine serum, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin). The dissociated cells were collected by centrifugation at 1,000 *g* for 10 min at room temperature, resuspended, seeded on coverslips for immunostaining or in 35-mm dishes for Western blotting, and maintained for 3–4 days at 37°C in a 95% air/5% CO_2 atmosphere.

Drug treatment

Magnolol was purchased from the Pharmaceutical Industry Technology and Development Center (Taiwan) and was used at a final concentration of 30 μM. The extracellular signal-related kinase (ERK) inhibitors PD98059 or U0126 were purchased from Biolmol Research Laboratories (Plymouth Meeting, PA, USA) and were used at a final concentration of 30 μM or 10 μM, respectively. In general, the effects of these two inhibitors on magnolol-induced changes were tested by preincubating the cells with the inhibitor for 30 min, then incubating the cells with magnolol in the continued presence of the inhibitor for 6 h. Sodium orthovanadate (Sigma), tautomycin (Biomol Research Lab), and calyculin A (Biomol Research Lab) were used at final concentrations of 1 mM, 10 nM, and 5 nM, respectively.

Immunofluorescence

Cultured adrenocortical cells were treated as indicated, fixed for 5 min at room temperature in 0.15% glutaral-

dehyde in phosphate-buffered saline, pH 7.4, (PBS), and permeabilized for 10 min at room temperature with 0.15% Triton X-100 in PBS. To reduce free aldehyde groups, the cells were then treated for 30 min at room temperature with $NaBH_4$ (1 mg/ml). After PBS washes, the cells were incubated overnight at 4°C with mouse mAb A2 (IgM) (1:200 dilution of ascites in PBS), which recognizes p160 protein on the lipid droplet capsule (Wang and Fong 1995), or rabbit anti-ADRP antibodies (a kind gift from Dr. Thomas Keenan; Heid et al. 1996). They were then washed with PBS (3 min × 5 min) and incubated with FITC-conjugated goat antimouse IgG or antirabbit IgG (Sigma). After incubation for 1 h at 37°C, the cells were washed extensively with PBS, mounted using 3% *n*-propyl gallate and 50% glycerol in PBS, and examined using a Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Oberkocheu, Germany) equipped with a Nikon DIX digital camera (Nikon, Tokyo, Japan).

Preparation of lipid droplet fractions for Western blot analysis

The isolation of lipid droplet fraction was prepared according to the method of Clifford et al. (2000). Minced adrenal cortex fragments were incubated with 30 μM DMSO or 30 μM magnolol in the culture medium for 6 h at 37°C in a CO_2 incubator. Then the tissue fragments were lysed and homogenized in buffer A (50 mM Tris-HCl, pH 7.4, containing 225 mM sucrose, 1 mM EDTA, 1 mM benzamidine, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml antipain, and 50 mM NaF) and the lysate centrifuged at 13,000 *g* at 4°C for 15 min. The top fatcake layer was collected for analysis.

Western blot analysis

After various treatments, cell homogenates (50 μg of protein/lane) were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel and the proteins transferred to nitrocellulose paper, as described by Fritz et al. (1989). Membrane strips were blocked for 1 h at room temperature with 5% nonfat milk in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris base, pH 8.2), then incubated overnight at 4°C with mouse antiphosphorylated ERK mAb (Santa Crutz Biotechnology, Santa Crutz, CA, USA), rabbit polyclonal anti-ADRP antibodies or mouse mAb A2 (Wang et al. 1995), mouse anti-β actin mAb (Sigma), or rabbit polyclonal antibodies specific for phosphothreonine, phosphoserine, or phosphotyrosine (Zymed Lab, South San Francisco, CA, USA) diluted in PBS. After washes with TBS-0.1% Tween, for color substrate development, the strips were reacted with alkaline phosphatase-conjugated secondary antibodies (1:7,500 dilution, Sigma) and bound antibody visualized using a substrate solution (3.3 mg/ml nitro blue tetrazolium and 1.65 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris base, pH 9.5). For ECL method, the strips were reacted with peroxidase-conjugated goat secondary antibody, and the reaction detected by Western Blotting Luminol Reagent (Santa Crutz) and Hyperfilm ECL (Amersham Pharmacia Biotec., Buckinghamshire, UK). The density of the reactive bands on the nitrocellulose membrane was scanned and quantified by densitometry using Gel pro 3.1 (Media Cybernetics, Silver spring, MD, USA). The density of the band in the control sample was defined as 100% and the densities of the band in the test sample expressed as a percentage of this value. All experiments were performed at least three times, and the values are expressed as mean ± SD.

Immunoprecipitation

After various treatments, the adrenal cells in a 35-mm dish were washed with PBS, harvested in 300 μl of RIPA buffer (150 mM NaCl, 0.1% Nonidet-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 0.1 mM leupeptin, 0.1 mM pepstatin, pH 8.0) and ultrasonicated for 30 s on ice. After centrifugation at 12,000 *g* for 10 min at 4 °C, the cell extracts in the supernate were preincubated for 1 h at room temperature with 50 μl of protein A/G agarose slurry (Santa Crutz), the mixture centrifuged, and the supernatants collected. The supernatants were mixed for 2 h at room temperature with 10 μl of ascites containing mAb A2, then incubated with 50 μl of protein A/G agarose for another 30 min at room temperature. Agarose-bound immune complexes were collected by centrifugation at 1,000 *g* for 1 min at 4 °C; then, after washes with RIPA buffer, the bound proteins were released using 100 μl of SDS sample buffer, electrophoresed, and transferred to nitrocellulose paper, which was then probed with rabbit antiphosphothreonine antibodies (Zymed) or mAb A2. The experiments were repeated three times.

Quantitative study and image analysis

For estimation of the percentage of antibody-stained cells, all experiments were performed in triplicate cover slips, and more than 100 cells per cover slip were examined. Over 50% of the lipid droplets in one cell immunostained for p160 or ADRP was considered positive, and the percentage of positively stained cells was expressed as the mean ± SD.

For quantitative measurement of immunostaining, ten cells per cover slip and three cover slips per experimental group were used. The immunostained cells were photographed using a digital camera mounted on a Zeiss microscope and converted to black-and-white images, then the relative optical density of immunoreactivity was

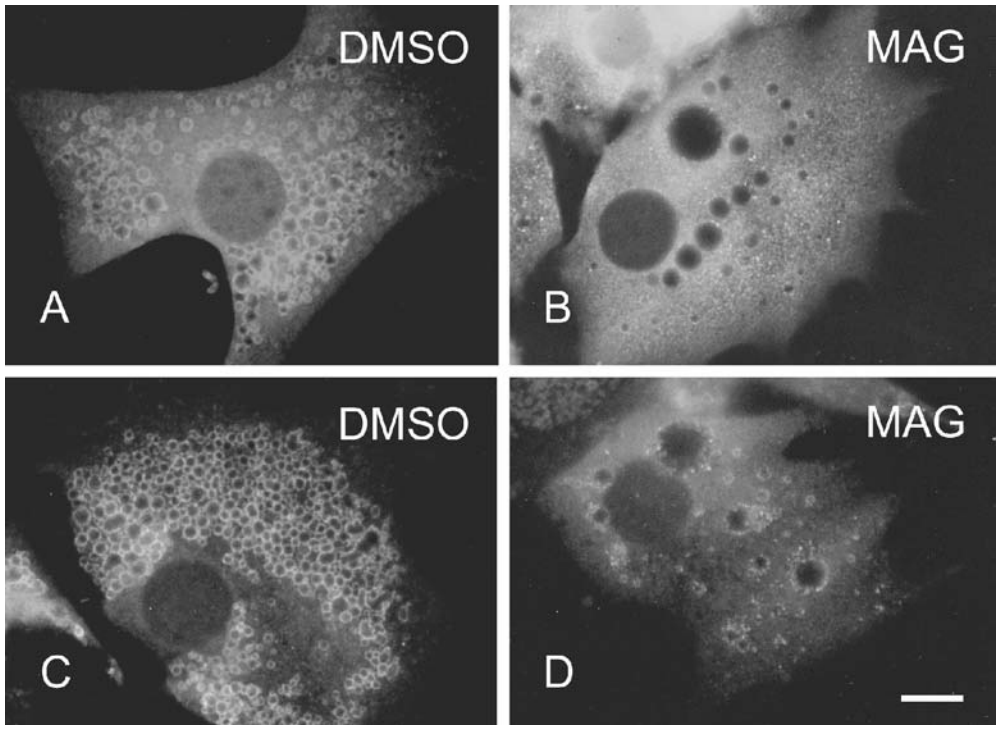


Fig. 1 Effect of magnolol on p160 and adipose differentiation-related protein (ADRP) immunostaining. Adrenal cells were incubated for 6 h with DMSO (*DMSO*; **a, c**) or 30 μM magnolol (*MAG*; **b, d**), then immunostained for p160 (**a, b**) or ADRP (**c, d**). *Scale bar*:10 μm

assessed using a PC-based image analyzer software (Image Pro 3.0 Plus, Media cybernetics, Silver Spring, MD, USA). All results were expressed as the mean ± SD. Statistical differences between means were assessed using Student's *t* test, with a *P* value less than 0.05 being considered significant.

Results

Our previous study demonstrated that magnolol stimulates corticosterone production in a time- and dose-dependent manner; the corticosterone increases significantly between 3 h and 24 h after magnolol treatment (Wang et al. 2000). Since lipid-associated proteins are proposed to act as a barrier on the lipid droplet surface, we first examined the distribution of these lipid-associated proteins by immunofluorescence staining. The results showed that after 30-μM magnolol treatment for 1 h, p160 immunostaining of the droplet surface became discontinuous (data not shown) and was almost undetectable after 6 h of treatment (Fig. 1b); meanwhile, p160 cytosolic staining was significantly increased (Fig. 1b) in contrast to the bright ring-staining pattern for p160 on the lipid droplet surface in DMSO-treated adrenal cells (Fig. 1a). In addition, magnolol treatment resulted in discontinuous dotted ADRP immunostaining around lipid droplets and very weak cytoplasmic staining (Fig. 1d) compared with the complete ring-staining pattern in DMSO-treated cells (Fig. 1c). It is noted that magnolol treatment induced phosphorylation of ERK1 and ERK2, which started at 5 min and peaked at 15 min after treatment (Fig. 2). In order to correlate the relation between ERK activation and changes in p160 staining at the lipid droplet surface, the effects of pretreatment with MAPK-ERK kinase (MEK) inhibitors on p160 distribution were examined. Treatment with PD98059 or U0126 alone had no effect on the ring staining of p160 and ADRP on the lipid droplet surface, respectively (Fig. 3a, c). Pretreatment of PD98059 or U0126 with

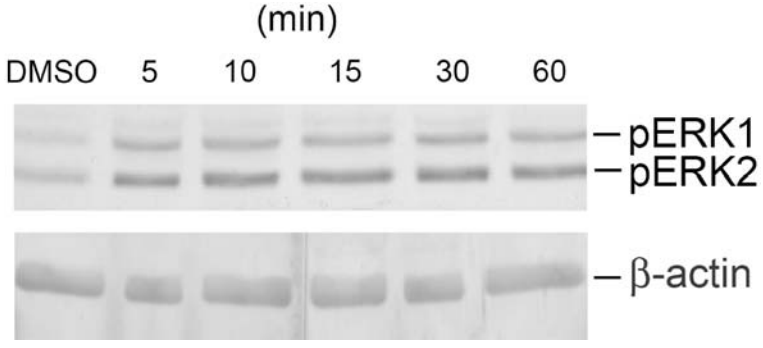


Fig. 2 Time-course study of magnolol-induced p42 (*pERK 1*)/p44 (*pERK2*) phosphorylation. Adrenal cells were incubated for 0 min (DMSO), 5, 10, 15, 30, or 60 min with 30 μM magnolol, then cell lysates were analyzed for *β*-actin (internal standard) and pERK1 and pERK2 by immunoblotting. The figure shows a typical experiment, which was repeated twice with similar results

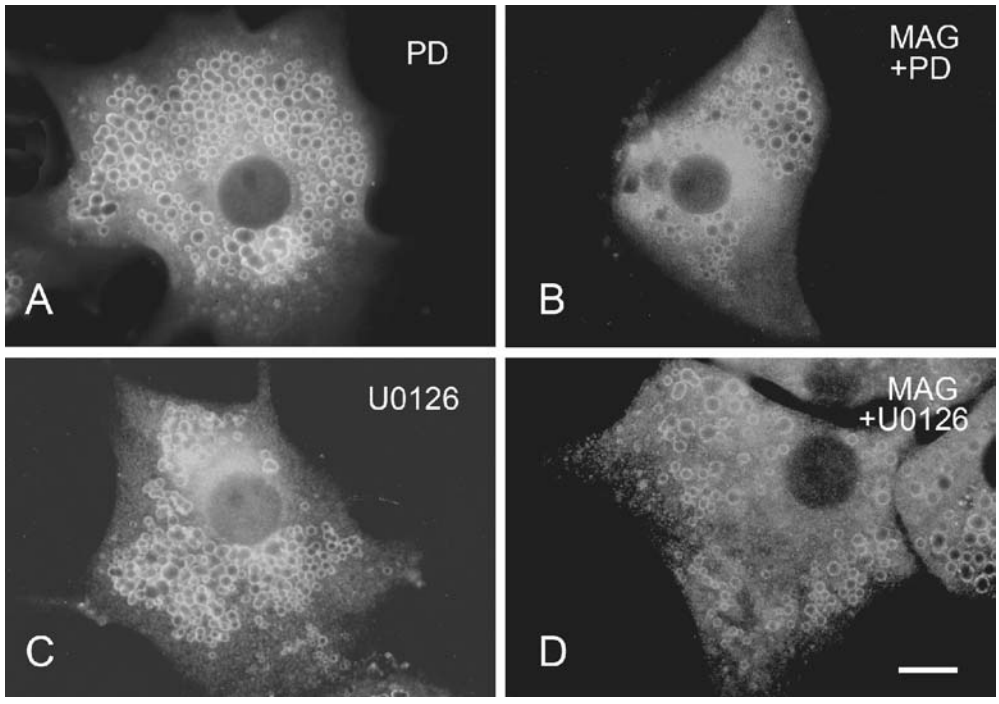


Fig. 3 Effect of MAPK-ERK kinase (MEK) inhibitor pretreatment on the magnolol-induced change in p160 distribution. Cells were incubated with 30 μM PD98059 (*PD*) for 6 h (**a**), with 30 μM PD98059 for 30 min, then with 30 μM magnolol plus PD98059 (*MAG + PD*) for 6 h (**b**), with 10 μM U0126 (*U0126*) for 6 h (**c**), or with U0126 for 30 min, then with magnolol plus U0126 (*MAG + U0126*) for 6 h (**d**), then immunostained for p160. *Scale bar*: 10 μm

magnolol prevented magnolol-induced decreased expression of p160 at the lipid droplet surface, as shown by intact ring staining seen in the majority of the lipid droplets in the cells (Fig. 3b, d). The observations were further confirmed by quantitative analysis. We arbitrarily defined that over 50% of the lipid droplets in one cell immunostained for p160 was considered positive. The data showed that the percentage of p160-positive cells in the groups treated with PD98059 or U0126 alone was 78.3 ± 3.1% or 73.3 ± 5.7%, respectively, comparable to the control value of 69.6 ± 0.5 % (DMSO in Fig. 4). Magnolol treatment decreased the percentage of p160-positive cells to 35.68 ± 0.96% (MAG in Fig. 4) whereas PD98059 or U0126 pretreatment restored to a percentage of 69.9 ± 4.6% or 65.6 ± 6.9%, respectively. Thus, the decreased expression of p160 at the lipid droplet surface induced by magnolol was mediated by ERK signaling.

To study the possible mechanism for decreased staining of p160 on lipid droplet surface, Western blot analyses were performed to determine whether p160 expression was down-regulated. The time-course study of p160 expression showed that magnolol treatment did not significantly change the total p160 protein levels (Fig. 5a). Based on the immunofluorescence observation, which shows the increase in the cytosolic p160

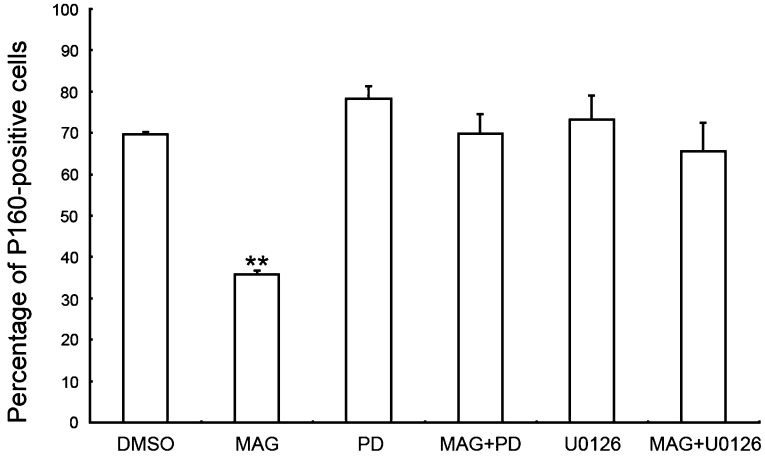


Fig. 4 Effect of MAPK-ERK kinase (MEK) inhibitor pretreatment on the magnolol-induced change in the percentage of p160-positive cells. Cells were either incubated for 6 h with DMSO (*DMSO*), 30 μM magnolol (*MAG*), 30 μM PD98059 (*PD*), or 10 μM U0126 (*U0126*) or preincubated with the inhibitor for 30 min, then incubated for 6 h with magnolol in the presence of the inhibitor (*MAG + PD*; *MAG + U0126*) and immunostained for p160. More than 100 cells per coverslip were examined, and each experiment was performed in triplicate coverslips. The percentage of stained cells is expressed as the mean ± SD. $^{**}P < 0.01$ versus the DMSO-treated group

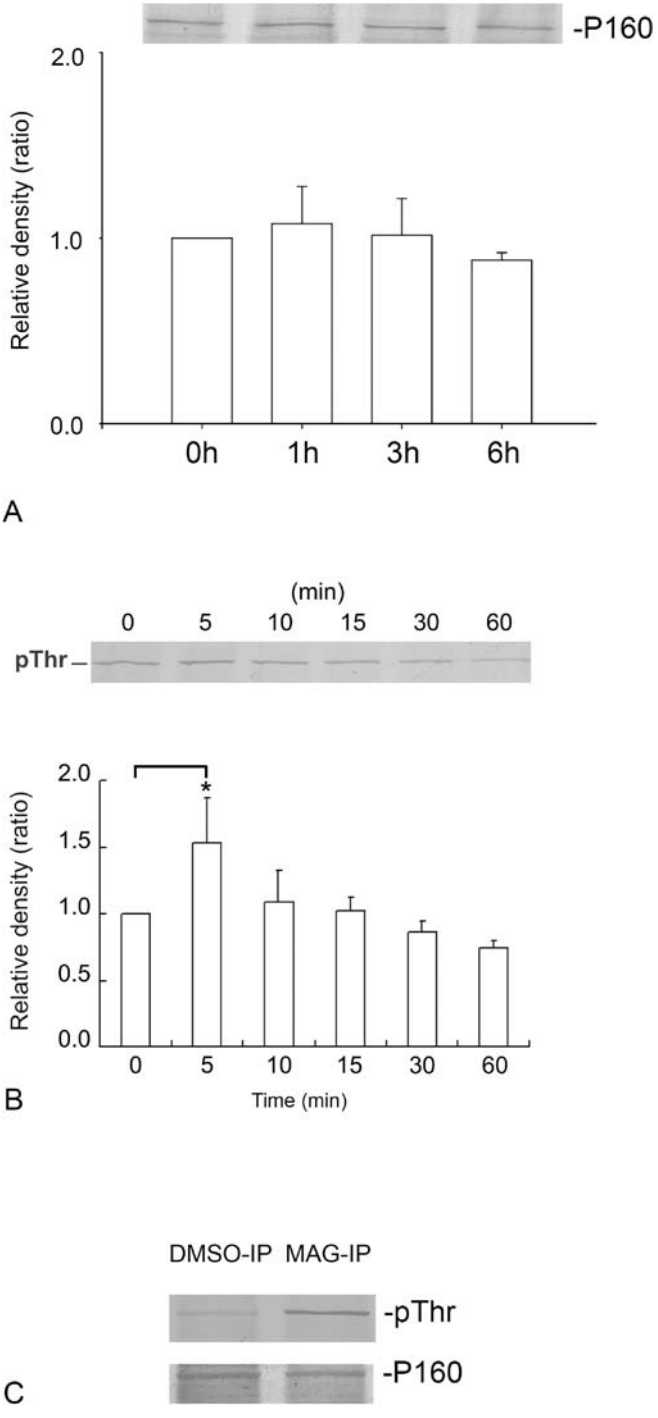


Fig. 5 Effect of magnolol treatment on p160 expression and phosphorylation. **a** Adrenal cells were treated with DMSO (0 h) or with 30 μM magnolol for 1, 3, or 6 h, then the cell lysates were analyzed for p160 by Western blotting (*upper pane*). *Lower pane*: densitometric data for the p160 bands from triplicate separate blots, presented as the ratios of the control values. **b** Cells were treated with DMSO (0) or with 30 μM magnolol for 5, 10, 30, or 60 min. Cell homogenates were then analyzed for phosphothreonine (*upper panel*). *Lower pane*: densitometric data for the stained phosphorylated p160 bands from triplicate separate blots, presented as the ratios of the control values; $n = 3$. **c** p160 proteins were immunoprecipitated from cell extracts of DMSO-treated (*DMSO-IP*) and magnolol-treated cells (*Mag-IP*; 30 μM magnolol for 5 min) with anti-p160 antibody, then immunoblotted with antiphosphothreonine antibody (*upper pane*) and anti-p160 antibody (*lower pane*); $n = 3$

staining concomitantly with the decreased staining of p160 at the lipid droplet surface after magnolol treatment, it is speculated that p160 protein is redistributed from the lipid droplets to the cytoplasm. To test whether this translocation is due to biochemical modification of p160, we examined the type of phosphorylation (serine, threonine, or tyrosine) required for p160 translocation. When adrenal cells were incubated with 1 mM sodium orthovanadate (a tyrosine phosphatase inhibitor) to increase cellular tyrosine phosphorylation, p160 staining at the lipid droplet surface was not affected (Fig. 6a) whereas application of either one of the serine/threonine phosphatase inhibitors, calyculin A (5 nM) or tautomycin (10 nM), resulted in the loss of p160 staining in the majority of lipid droplets due to hyperphosphorylation of serine/threonine (Fig. 6c, e). In order to identify serine or threonine phosphorylation on p160, specific antibodies against phosphorylated serine and threonine were used for Western blot analyses. A detailed time-course study of p160 phosphorylation upon magnolol treatment was shown in Fig. 5b. Magnolol induced threonine phosphorylation, but not serine phosphorylation (data not shown), of p160 in a time-dependent manner, reaching the peak at 5 min after treatment. This magnolol-induced threonine phosphorylation of p160 was reduced by pretreatment with the MEK inhibitor U0126 (data not shown). Furthermore, magnolol resulted in a $120 \pm 38\%$ increase ($P < 0.05$) in threonine phosphorylation of p160, which was estimated from the immunoprecipitates obtained with mAb A2 and then reprobed with antiphosphothreonine antibody (Fig. 5c, upper panel). In contrast, there was no significant difference in total protein levels of p160 in immunoprecipitates obtained from DMSO- and magnolol-treated cells (Fig. 5c, lower panel). Thus, the distributional change of p160 at the lipid droplet surface might be related to magnolol-induced p160 phosphorylation.

We next examined whether the effect of magnolol on ADRP staining was also mediated via ERK signaling. Treatment with either PD98059 or U0126 exerted no effect on the distribution of ADRP on the lipid droplet surface (Fig. 7a, c), and pretreatment with PD98059 or U0126 did not block magnolol-induced decrease in ADRP staining at the lipid droplet surface (Fig. 7 b, d). These observations were confirmed by image analysis of the optical densities and the percentage of ADRP-immunostained cells. As shown in Fig. 8a, in the DMSO group, $98.9 \pm 1.8\%$ of the cells were ADRP-positive, and treatment with magnolol (MAG) for 6 h decreased this value to $51.7 \pm 0.9\%$. The percentage of ADRP-positive cells in cells treated with PD98059 or U0126 alone was $95.1 \pm 3.7\%$ or $98.8 \pm 1.6\%$, respectively, but PD98059 or U0126 pretreatment did not prevent the decrease of ADRP-positive-cell percentages

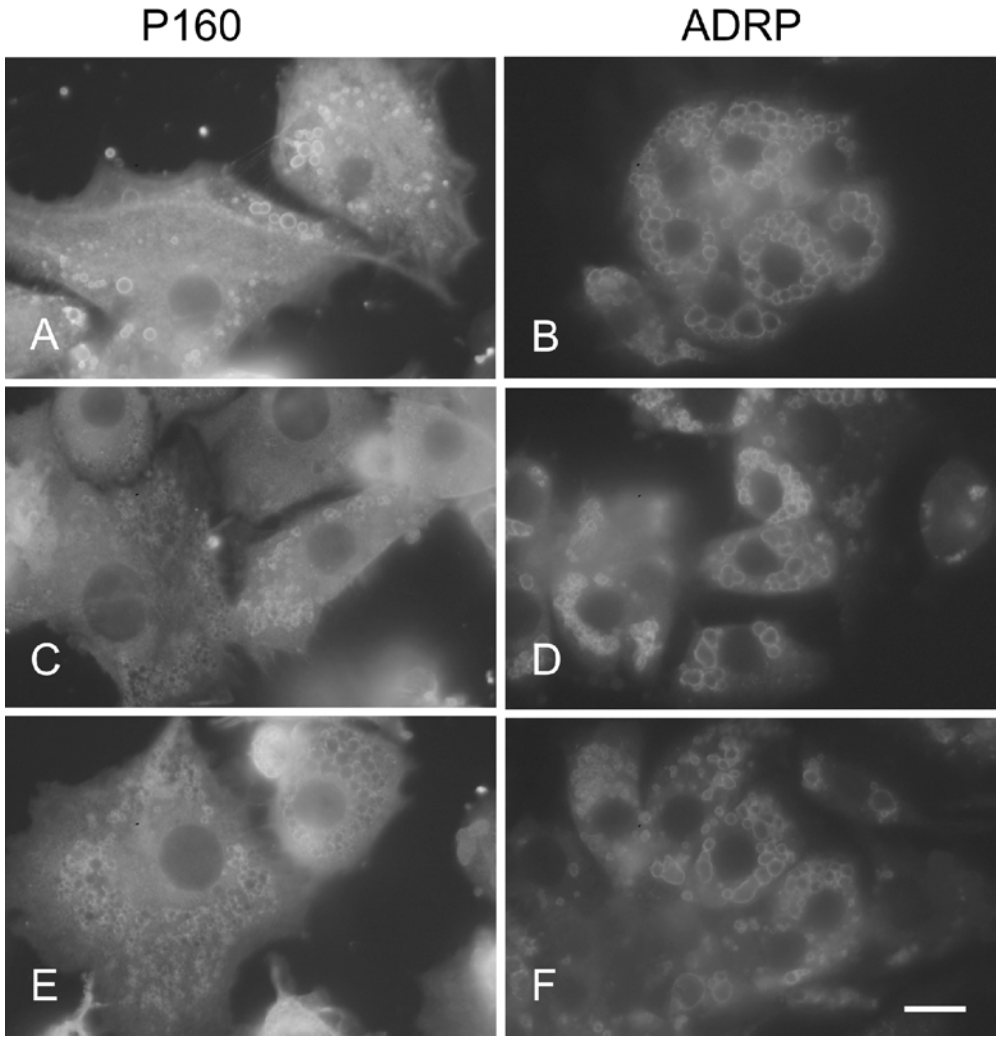


Fig. 6 Effects of different phosphatase inhibitors on p160 and adipose differentiation-related protein (ADRP) immunostaining. Adrenal cells were incubated for 1 h with 1 mM sodium orthovanadate (**a, b**), 5 nM calyculin A (**c, d**), or 10 nM tautomycin (**e, f**), then immunostained for p160 (**a, c, e**) or ADRP (**b, d, f**). *Scale bar*: 10 μm

induced by magnolol (46.7 ± 3.9% for MAG + PD group or 44.2 ± 5.9% for MAG + U0126 group, respectively). Figure 8b shows that the optical density of ADRP staining in magnolol-treated cells and in magnolol-treated cells pretreated with PD98059 or U0126 was 51 ± 8% (MAG), 51 ± 5% (MAG + PD), or 37 ± 11% (MAG + U0126) of the control values (DMSO), respectively. The optical densities of ADRP staining in cells treated with PD98059 or U0126 alone were 103% or 100% of the control values. Taken together, these data demonstrated that the magnolol-induced changes in the intensities of ADRP immunostaining were not related to ERK activity. It is unlikely that the decrease in ADRP staining is due to phosphorylation on serine, threonine, or tyrosine since ADRP is probably not phosphorylated intracellularly (Heid et al. 1996) and treatment with the phosphatase inhibitors orthovanadate, calyculin A, or tautomycin did not affect the distribution of ADRP at the lipid droplet surface (Fig. 6, b, d, f). Immunoblot analysis showed that magnolol treatment for 6 h resulted in a decrease in ADRP levels to about 49 ± 4% of the control value (Fig. 9a). To confirm the decreased protein levels of ADRP at the lipid droplet surface, we isolated lipid droplets from DMSO- and magnolol-treated cells by subcellular fractionation and then analyzed the protein contents of ADRP. The ADRP levels in lipid droplet fractions are decreased by 50% after magnolol treatment (Fig. 9b). This result agrees with the decreased immunostaining intensities of ADRP at the lipid droplet surface (Fig. 1d).

Discussion

Magnolol has several known pharmacological functions, including antioxidative activity, vascular muscle relaxation, inhibition of neutrophil adherence, and antithrombotic, antiatherosclerosis, and antitumor effects (Chen et al. 2002; Lin et al. 2002; Tseng et al. 1990; Zhong et al. 2003). In the present study, magnolol at the concentration of 30 μM had no antiproliferative or apoptosis-inducing effects on adrenal cells but stimulated adrenal steroidogenesis. This result is consistent with our previous finding that magnolol stimulates lipolysis in sterol ester-loaded 3T3-L1 preadipocytes (Huang et al.

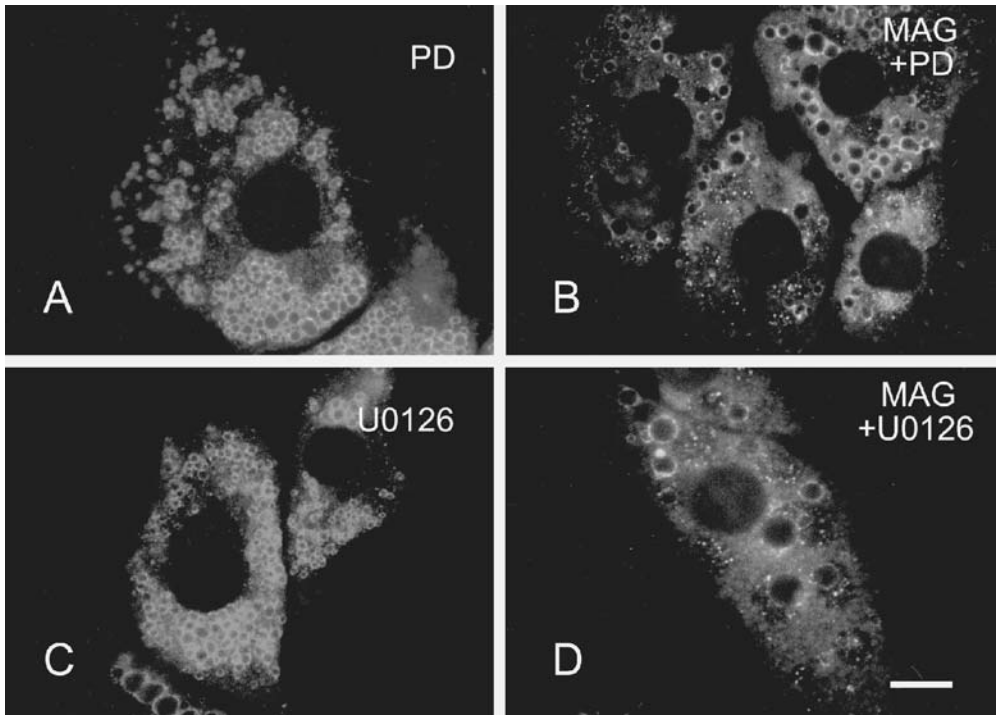
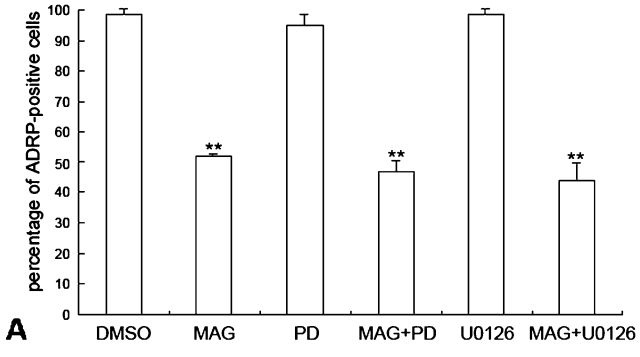
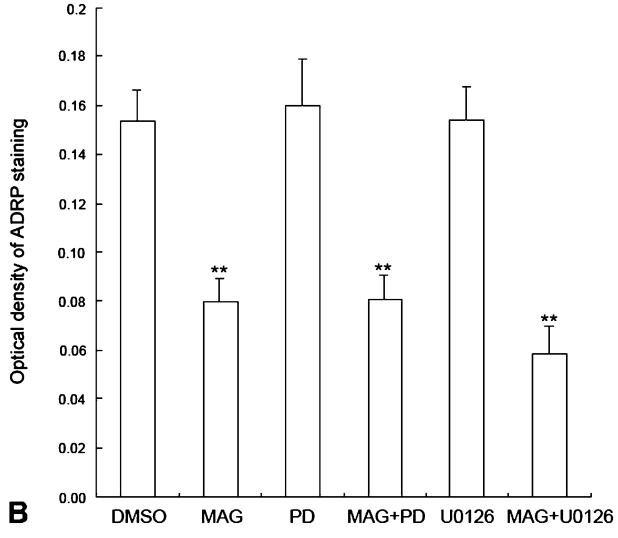


Fig. 7 Effect of MEK-inhibitor pretreatment on ADRP immunostaining. Adrenal cells were incubated for 6 h with 30 μM PD98059 (**a**, *PD*), for 30 min with 30 μM PD98059, then for 6 h with 30 μM magnolol + 30 μM PD98059 (**b**, MAG + PD), for 6 h with 10 μM U0126 (**c**, *U0126*), or for 30 min with 10 μM U0126, then for 6 h with 30 μM magnolol + 10 μM U0126 (**d**, MAG + U0126), then immunostained for ADRP. *Scale bar* 10 μm

2004). Magnolol-initiated apoptosis apparently requires a high concentration of magnolol (up to 80 μM), and the signaling pathways involve a transient increase in Ca^{2+} levels as a result of PKC activation, cytosolic accumulation of cytochrome *c*, and activation of caspases 9 and 3 (Lin et al. 2002; Yang et al. 2003; Zhong et al. 2003). In addition, ERK activation may be involved in the initiation of magnolol-induced apoptosis of human lung squamous carcinoma CH27 cells (Yang et al. 2003). In adipocytes, the magnolol-induced lipolysis-signaling cascade is initiated by calcium mobilization and influx, followed by activation of calcium/calmodulin-dependent kinase and downstream ERK (Huang et al. 2004). Consistent with these results, we found that in adrenal cells, magnolol induced ERK activation, which resulted in p160 translocation from the lipid droplet surface. It seems that magnolol performs various functions, depending on the concentration used, the cell type studied, and the signaling pathways triggered.

Fig. 8 Effect of MAPK-ERK kinase (MEK)-inhibitor pretreatment on the percentage of adipose differentiation-related protein (ADRP)-stained cells and the mean optical density of ADRP immunostaining. Adrenal cells were either incubated for 6 h with DMSO (*DMSO*), 30 μM magnolol (*MAG*), 30 μM PD98059 (*PD*), or 10 μM U0126 (*U0126*) or pretreated with inhibitor for 30 min, then incubated with inhibitor and 30 μM magnolol for 6 h (*MAG + PD*; *MAG + U0126*), then immunostained for ADRP, and the percentage of stained cells (**a**) and mean optical densities (**b**) were estimated as described in the Materials and methods section. $^{**}P < 0.01$ versus the DMSO-treated group





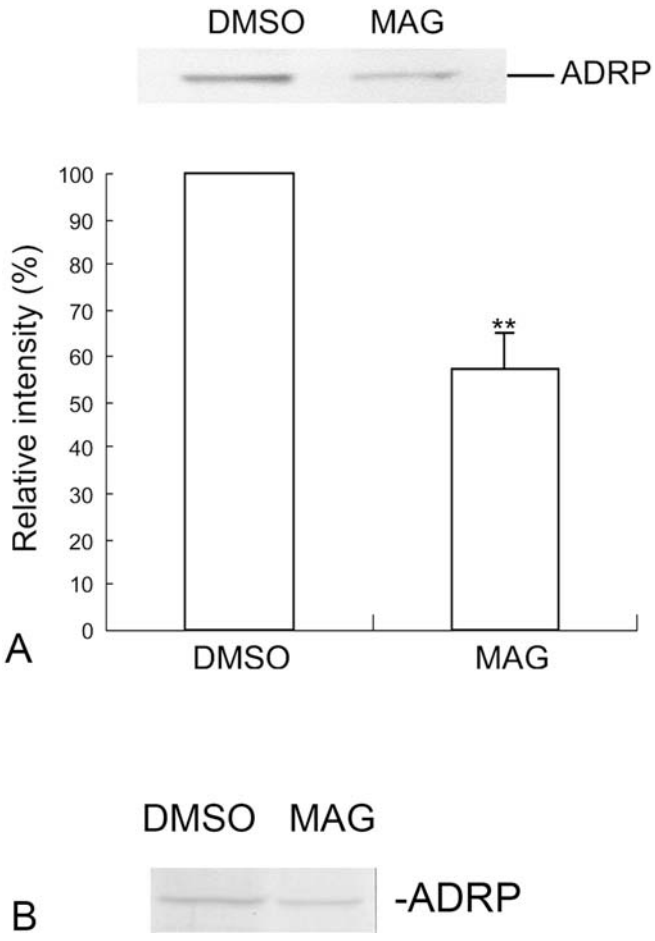


Fig. 9 Effects of magnolol on protein level of adipose differentiation-related protein (ADRP). **a** *Upper pane*: Adrenal cells were incubated for 6 h with DMSO or 30 μM magnolol, then total cell homogenates were analyzed by immunoblotting using anti-ADRP antibody. *Lower pane*: Densitometric scans of triplicate blots. Values are the mean ± SD. $^{**}P < 0.01$ versus the DMSO-treated group. **b** Lipid droplets are purified from DMSO and magnolol-treated cells by subcellular fractionation and analyzed for ADRP by Western blotting; $n = 2$

ADRP is an early marker of adipocyte differentiation and is involved in the deposition of triacylglycerols in nascent lipid droplets in several types of cells, including macrophages, adipocytes, and hepatocytes (Gao and Serrero 2000; Heid et al. 1996; Imamura et al. 2002; Jiang and Serrero 1992; Steiner et al. 1996). It is found together with perilipin and p160 at the lipid droplet surface in adrenal cells. While PKA stimulation induces redistribution of perilipin and p160 at the lipid droplet surface, ADRP is remarkably resistant to the same stimulus (Fong et al. 2002) and was not expected to play any role in adrenal steroidogenesis. In the present study, surprisingly, we found that total ADRP protein levels and optical density of ADRP staining at the lipid droplet surface were decreased by magnolol treatment. This is the first description of distributional change for ADRP at the lipid droplet surface in adrenal cells during active steroidogenesis. Milk lipid globule kinase can phosphorylate ADRP in vitro, but ADRP does not seem to be phosphorylated intracellularly (Heid et al. 1996). Consistent with the above study, we concluded that ADRP translocation was not due to the phosphorylation of ADRP based on the fact that several phosphatase inhibitors, tautomycin, calyculin A, and orthovanadate, exerted no effect on magnolol-induced ADRP translocation. Thus, decreased ADRP expression induced by magnolol may be regulated at the translational level or may be caused by an increased degradation rate of ADRP, leading to the reduced amount of ADRP on the lipid droplet surface. The only post-translational modification of ADRP so far identified is acylation (Heid et al. 1996). Upregulation of ADRP mRNA levels can be easily manipulated using dexamethasone, isobutylmethylxanthine, or long-chain fatty acids (Gao and Serrero 2000; Jiang and Serrero 1992). Fatty acids are ligands for peroxisome proliferator-activated receptors (PPAR), and PPAR alpha and gamma may mediate ADRP expression in adipocytes, human monocytes, RAW 284.3 macrophages, and human kidney cells (Buechler et al. 2001; Hodgkison and Ye 2003; Liu et al. 2003). In contrast, studies on the mechanism of downregulation of ADRP are very limited. Jiang and Serrero (1992) reported that ADRP mRNA expression is inhibited by transforming the growth factor β, the tumor necrosis factor, and the epidermal growth factor, but no precise mechanism has been proposed. Whether magnolol-induced ADRP downregulation is mediated by PPAR expression or other mechanisms remains to be determined.

In adrenal cells, p160 is located at the lipid droplet surface and following PKA stimulation is detached from the droplet surface and redistributed to the cytosol (Fong et al. 1997; Wang and Fong 1995). By contrast, we have previously shown that magnolol treatment causes p160 translocation from the lipid droplet surface to the cytosol in a PKA-independent manner (Wang et al. 2000). The present study further identified the possible mechanism involving increased threonine phosphorylation of p160, which triggered p160 redistribution. Moreover, both p160 redistribution and p160 phosphorylation were ERK dependent. Whether ERK activation directly or indirectly increases threonine phosphorylation of p160 requires further study. The possibility that magnolol or ERK decreases serine/threonine phosphatase activity should be considered. Since perilipin A is phosphorylated by PKA (Greenberg et al. 1991) and dephosphorylated by phosphatase PP1 (Clifford et al. 1998), whether p160 is dephosphorylated by PP1 is an interesting issue. Thus, at least two different signaling pathways, one PKA dependent and the other ERK dependent, are involved in the regulation of p160 translocation in adrenal cells.

ERK1 and ERK2, members of the MAPK family, are involved in proliferation and differentiation (Chang and Karin, 2001; Seger and Krebs 1995). In addition, they are activated in TNFγ- and catecholamine-mediated adipocyte lipolysis (Greenberg et al. 2001) and steroidogenesis in adrenal glomerulosa cells

(Cherradi et al. 2003). ERK can be activated by PKA, PKC, PLC, Src-tyrosine kinase, and receptor tyrosine kinase in several cell types (Cox et al. 1996; Tian et al. 1998; Van Biesen et al. 1996). CaMK can phosphorylate ERK in aortic vascular smooth muscle (Abraham et al. 1997) and in magnolol-stimulated 3T3-L1 sterol ester-loaded preadipocytes (Huang et al. 2004), and the possibility that magnolol-induced ERK activation in adrenal cells is mediated by CaMK requires further investigation. Activation of the ERK pathway increases hormone-sensitive lipase (HSL) phosphorylation and HSL activity in adipocytes (Greenberg et al. 2001). In the adrenal gland, HSL is responsible for the major cholesterol ester hydrolase (CEH) activity (Cook et al. 1982; Kraemer et al. 2002). Several lines of evidence support the regulatory role of ERK in steroidogenesis. In adrenal glomerulosa cells, angiotensin II increases CEH activity by phosphorylation of ERK (Cherradi et al. 2003). Activation of ERK is also involved in forskolin-induced steroid production in Y-1 mouse adrenocortical cells (Gyles et al. 2000). In the adrenal gland, the phosphorylatable substrate of ERK may be CEH, as demonstrated in adrenal glomerulosa cells (Cherradi et al. 2003). ERK activation triggered by magnolol may therefore stimulate steroidogenesis both by increasing CEH activity and by inducing p160 redistribution. The simultaneous decreased expression of ADRP and p160 at the lipid droplet surface induced by magnolol may not favor lipid droplet stability, and this may increase the access of activated CEH to the cholesterol esters stored in the lipid droplets thereby accelerating lipolysis. Our results show that ERK activation is involved in magnolol-induced phosphorylation of p160 and decrease in p160 staining at the lipid droplet surface. This signaling mechanism is not responsible for the decreased ADRP levels at the lipid droplet surface, the mechanism of which is apparently mediated by downregulation of ADRP.

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