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Signaling Pathway of Magnolol-Stimulated Lipolysis in Sterol Ester-Loaded 3T3-L1 Preadipocyes

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Abstract The aims of the present study were to examine the effect of magnolol on lipolysis in sterol ester (SE)-loaded 3T3-L1 preadipocytes and to determine the signaling mechanism involved. We demonstrate that magnolol treatment resulted in a decreased number and surface area of lipid droplets, accompanied by release of glycerol. The lipolytic effect of magnolol was not mediated by PKA based on the facts that magnolol did not induce an elevation of intracellular cAMP levels, and protein kinase A (PKA) inhibitor KT5720 did not block magnolol-induced lipolysis. Calcium/calmodulin-dependent protein kinase (CaMK) was involved in this signaling pathway, since magnolol-induced a transient rise of intracellular [Ca^{2+}] and Ca^{2+} influx across the plasma membrane, and CaMK inhibitor significantly abolished magnolol-induced lipolysis. Moreover, magnolol increased the relative levels of phosphorylated extracellular signal-related kinases (ERK1 and ERK2). In support of the involvement ERK, we demonstrated that magnolol-induced lipolysis was inhibited by PD98059, an inhibitor of mitogen-activated protein kinase kinase (MEK), and PD98059 reversed magnolol-induced ERK phosphorylation. Further, the relationship between CaMK and ERK was connected by the finding that CaMK inhibitor also blocked magnolol-induced ERK phosphorylation. Taken together, these findings suggest that magnolol-induced lipolysis is both CaMK- and ERK-dependent, and this lipolysis signaling pathway is distinct from the traditional PKA pathway. ERK phosphorylation is reported to enhance lipolysis by direct activation of hormone sensitive lipase (HSL), thus magnolol may likely activate HSL through ERK and increase lipolysis of adipocytes. J. Cell. Biochem. 91: 1021–1029, 2004.
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Key words: sterol ester-loaded preadipocyte; lipolysis; magnolol; signaling pathway

Magnolol, a compound purified from *Magnolia officinalis*, inhibits acyl-CoA: cholesterol acyltransferase, which is essential for the formation of lipid droplets [Lo et al., 1994; Kwon et al., 1997], stimulates adrenal steroidogenesis via a protein kinase A (PKA)- and protein kinase C (PKC)-independent pathway [Wang et al., 2000], and attenuates the thickness of the aortic intima and reduces monocyte chemotactic protein-1 mRNA levels in rabbits rendered atherosclerotic by high cholesterol feeding [Chen et al., 2001]. Whether magnolol exerts a universal effect on lipolysis in other cell types needs to be investigated. In this study, we tested the hypothesis that magnolol also stimulates lipolysis in sterol ester (SE)-induced adipocytes.

The traditional pathway of epinephrine-mediated lipolysis involves the cAMP-PKA cascade [Egan et al., 1992]. Activated PKA phosphorylates hormone sensitive lipase (HSL), which is subsequently translocated to the surface of lipid droplets to catalyze lipid hydrolysis [Clifford et al., 2000]. HSL can be activated by ERK activation, which results in lipolysis of adipocytes [Greenberg et al., 2001]. Moreover, a recent study has shown that growth

Abbreviations used: CaMK, calcium/calmodulin-dependent kinase; ERK, extracellular signal-related kinase; HSL, hormone sensitive lipase; MEK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate-13 acetate; SE, sterol ester.

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hormone-mediated lipolysis does not involve PKA, PKC, phospholipase C, and ERK, but is mediated by the JAK-STAT pathway [Asada et al., 2000]. One of our aims was therefore to elucidate the signaling pathway involved in the magnolol-induced reaction.

In the present study, we investigated the effects of magnolol on lipolysis and the possible pharmacological mechanism of this drug. We first induced lipid accumulation in 3T3-L1 preadipocytes by incubation with SE [Jepson et al., 1996], then treated the SE-induced adipocytes with magnolol. Lipolytic function of magnolol was evaluated by a decrease in total lipid droplet area per cell and increased glycerol release from the cells. Signaling mechanisms were investigated using specific kinase inhibitors.

MATERIALS AND METHODS

Cell Culture

3T3-L1 preadipocytes, purchased from the American Type Culture Collection (Rockville, MD), were grown in Dulbecco's modified Eagle's medium containing 10% calf serum, 4 mM glutamine, 1 mM sodium pyruvate, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a CO_2 incubator. All materials and chemicals for cell culture were obtained from Gibco BRL (Grand Island, NY).

SE Treatment of 3T3-L1 Preadipocytes

3T3-L1 preadipocytes were incubated with SE (100 μg/ml of oleic acid, 7.5 μg/ml of 25-hydroxycholesterol, and 2 mg/ml of bovine serum albumin) for 48 h to induce lipid accumulation, as described by Jepson et al. [1996]. Phase images of at least 10 different cells obtained from each experiment group were recorded using a Nikon inverted microscope and the total area of the intracellular lipid droplets within a cell calculated using Program Gel Pro-plus, version 3.1 (Media Cybernetics, MD).

Drug Treatments

Magnolol was purified from the barks of *Magnolis officialis* by HPLC column chromatography (Cosmosil 5C 18-AR and Nucleosil 10 μm NH_2), with purity over 99% judged and was purchased from the Pharmaceutical Industry Technology and Development Center (Taiwan). 3T3-L1 preadipocytes, cultured in 35 mm dishes, were incubated with SE for 48 h. After changing to the fresh culture medium, cells were treated for 8 h with 45 μM magnolol alone or together with a PKA inhibitor (300 nM KT 5720), a PKC activator (100 nM phorbol 12-myristate-13 acetate [PMA]), a PKC inhibitor (500 nM calphostin C), CaMK inhibitors (2 μM KN-62 or 10 μM KN-93), or a MEK inhibitor (40 μM PD98059). All inhibitors were obtained from Biomol Research Laboratory (Plymouth Meeting, PA) or Calbiochem (La Jolla, CA) and added 30 min before magnolol application. The culture supernatants were then collected and the cells resuspended and sonicated, then the culture medium (extracellular concentration) were assayed for glycerol using the triacylglycerol reagent (GTO-Trinder, Sigma, St. Louis, MO) and cholesterol reagent (Sigma), respectively. The protein concentrations in the samples were determined using the Biorad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Each experimental group consists of triplicate samples, and three separate experiments were performed. The result from one typical experiment was represented. The data were expressed as micromole of glycerol or cholesterol per mg of protein.

Measurement of Cytotoxicity

SE-loaded preadipocytes grown in 24-well plates were treated with different concentrations of magnolol (15–60 μM) for 8 h or 45 μM magnolol for different time periods (0–36 h), and then subjected to MTT assay. Mitochondrial dehydrogenase activity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay described by Welder [1992]. After washes with PBS, 500 μl of MTT medium (0.5 mg/ml MTT in culture medium) was added to each well and reacted for 4 h at 37°C in a CO_2 incubator. After removal of the MTT medium, 500 μl DMSO was added to each well for 5 min to dissolve the reaction products. The absorbance at 590 nm of each well was measured and recorded.

Measurement of cAMP Levels

The cAMP assay was performed as described previously [Wang et al., 2000]. Briefly, cells in 35 mm dishes were treated for 30 min with various agents in the presence of 500 μM 3-isobutyl-1-methylxanthine (Sigma) to inhibit phosphodiesterase activity. After removal of the culture medium, the cells were washed with PBS, extracted with 0.5 ml of 0.01 N HCl to

release intracellular cAMP, and the supernatant collected by aspiration, neutralized with 5 μl of 1 N NaOH, and centrifuged at 13,000*g* for 5 min. Intracellular cAMP levels were measured using the Amersham Pharmacia [^{3}H]-cAMP assay system (Amersham, Arlington Heights, IL).

Western Blot Analysis

After various treatments, lysates of SE-loaded preadipocytes (70 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% non-fat milk in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris base, pH 8.2), then incubated overnight at 4°C with mouse monoclonal anti-β actin (Sigma) and mouse anti-phosphorylated ERK antibodies (Santa Crutz Biotechnology, Inc., Santa Crutz, CA) diluted in PBS. After washes with TBS-0.1% Tween, 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). The density of the bands on the nitrocellulose membrane was quantified by densitometric scanning using an Image Master (Pharmacia Biotech., Hong Kong). The density of the control group is defined as 100 and the densities of the other bands were expressed relatively. The densitometric results were shown as the mean ± SD from three independent experiments.

Intracellular Ca^{2+} Measurement

Spectrofluometer was purchased from Cairn Research Company (Kent, England). The method was described previously by Wu et al. [1997]. Cells were incubated with 5 μM fura-2 AM (Molecular Probe, Eugene, OR) in standard Tyrode solution (118 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 11 mM glucose, 10 mM HEPES, pH 7.0) for 60 min at room temperature. Magnolol was applied initially in Ca^{2+}-free Tyrode solution and then changed to the standard Tyrode solution. For each experiment, 5–10 cells on the coverslip were selected and excited alternately with 340 and 380 nm wavelength light, then the ratio of the emission at 510 nm at the excitation wavelengths of 340 and 380 nm was recorded. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was calculated using the following equation [Grynkiewicz et al., 1985]: $[Ca^{2+}]_i = Kd\ (R - R_{min})/(R_{max} - R)\ (S_{f2}/S_{b2})$, where R is the ratio of the 510 nm fluorescence at 340 nm excitation over that at 380 nm excitation. Calibration constants were obtained by adding 5 μM ionomycin to 10 mM Ca^{2+}-containing solutions for obtaining R_{max} or to 10 mM EGTA in Ca^{2+}-free solutions for obtaining R_{min}. A Kd of 224 nm was used [Grynkiewicz et al., 1985]. S_{f2}/S_{b2} is the ratio of the 510 nm emissions at 380 nm excitation measured at R_{min} and R_{max}, respectively.

RESULTS

Evidence for a Lipolytic Function of Magnolol

Incubation of preadipocytes with SE for 24 h was enough to induce lipid droplet formation. There was a time-dependent effect of magnolol on lipolysis up to 24 h (data not shown). However, 8 h-period was enough to observe the stimulatory result. Magnolol stimulated the glycerol release from SE-loaded preadipocytes in a dose-dependent manner, with the saturation dose being 45 μM for 8 h-period (Fig. 1). Exposure of SE-loaded preadipocytes to magnolol from 15 to 60 μM for 8 h did not affect the cell viability, as judged by MTT test (data not shown). Extension of the incubation time to 24 h showed no effect on the cell viability, however, when it was extending to 36 h, about 5–6% cell

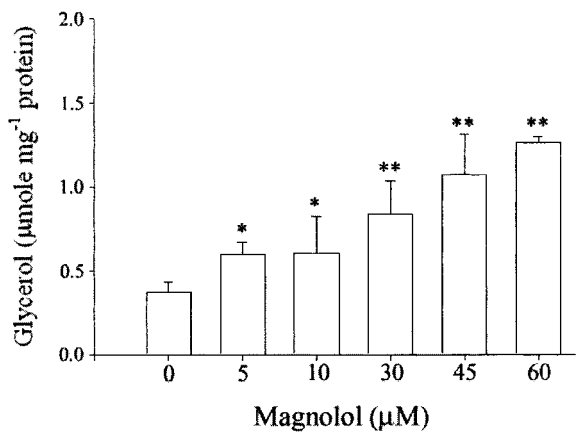

Fig. 1. Dose-response for magnolol on glycerol release in sterol ester (SE)-loaded preadipocytes. Cells were treated with DMSO (0 μM) or magnolol ranging from 5 to 60 μM for 8 h and then measured for glycerol release. Values are the mean ± SD. n = 3. *, $P < 0.05$, **, $P < 0.01$, as compared to 0 μM-group.

loss was noted. When these SE-loaded preadipocytes were incubated for 24 h with DMSO (control conditions; 1 μl/ml) (Fig. 2A), the lipid droplets increased in size and number (Fig. 2C, 0 h control 2A) and the total area of lipid droplets per cell increased to $123.9 \pm 41.5\%$ ($n = 11$) of that at 0 h. In contrast, treatment with magnolol (45 μM) for 24 h resulted in a decrease in the number of lipid droplets (Fig. 2D, 0 h control 2B) and in the total area of lipid droplets per cell ($43.9 \pm 15.1\%$).

We chose 45 μM magnolol and 8 h-incubation period in the following glycerol assay experiments. To examine the lipolytic effect of magnolol, extracellular and intracellular levels of glycerol and cholesterol were measured. After 8 h of magnolol treatment, SE-loaded preadipocytes released six times as much glycerol as DMSO-treated SE-loaded preadipocytes (Fig. 3), while the intracellular glycerol concentration of the magnolol-treated SE-loaded preadipocytes was four-fold lower than in the controls ($P < 0.01$). However, similar amounts of cholesterol were released from both groups of cells (data not shown), indicating that magnolol mobilized mainly the intracellular pool of triacylglycerol.

Possible Mechanisms of Magnolol-Induced Lipolysis

Magnolol treatment did not affect intracellular cAMP levels (data not shown). Cotreatment with magnolol and KT 5720 failed to block magnolol-induced lipolysis (Fig. 4). Treatment with PMA or Calphostin C alone increased lipolysis in different degrees (Fig. 5). Cotreatment with PMA appeared to partially block magnolol-induced lipolysis. It is possible that activation of PKC may inhibit some steps in the lipolytic cascade initiated by magnolol. Next, We measured $[Ca^{2+}]_i$ after magnolol treatment. To analyze the contribution of intracellular Ca^{2+}

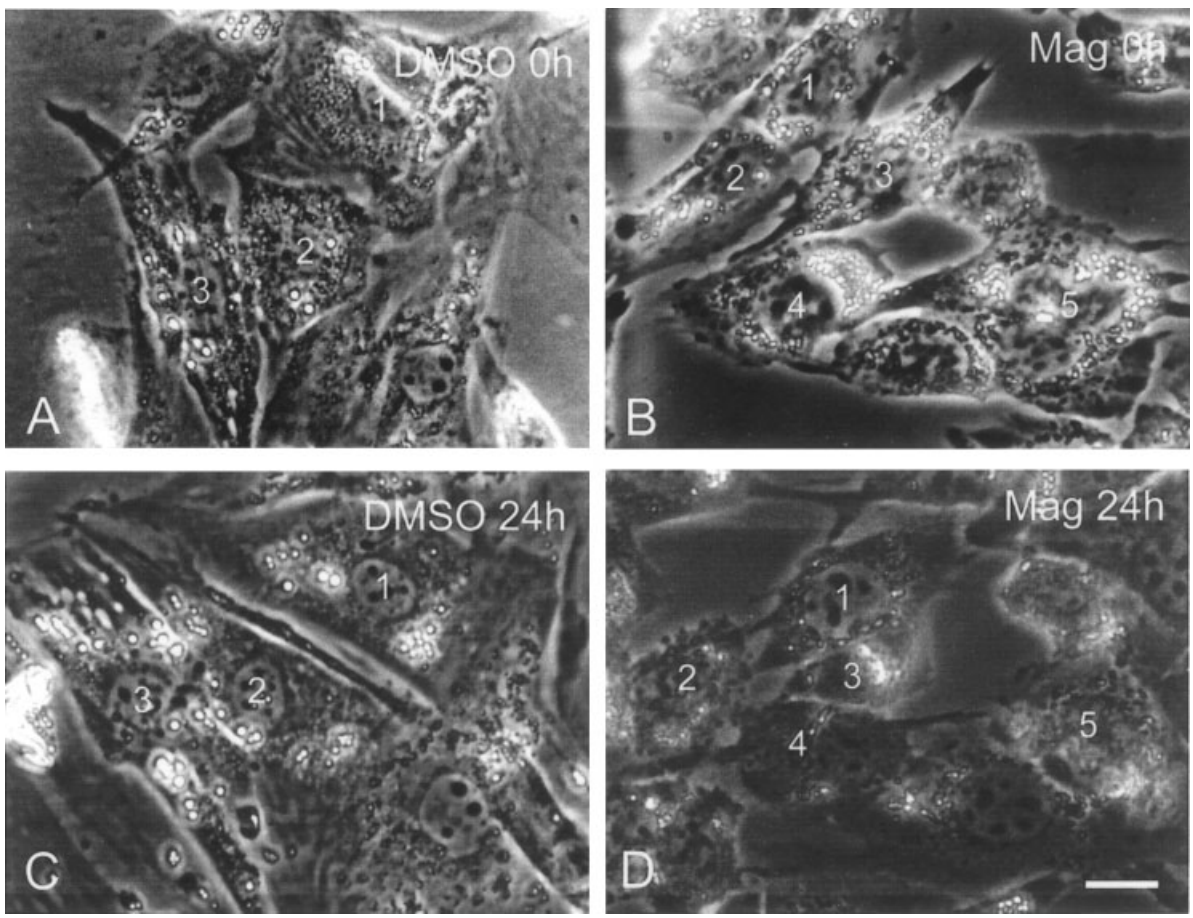

Fig. 2. Effect of magnolol on the morphology of SE-loaded 3T3-L1 preadipocytes. **A**–**D**, phase images. A, C: Cells treated with DMSO for 0 or 24 h, respectively. B, D: SE-loaded preadipocytes treated with 45 μM magnolol for 0 or 24 h, respectively. Bar = 25 μm.

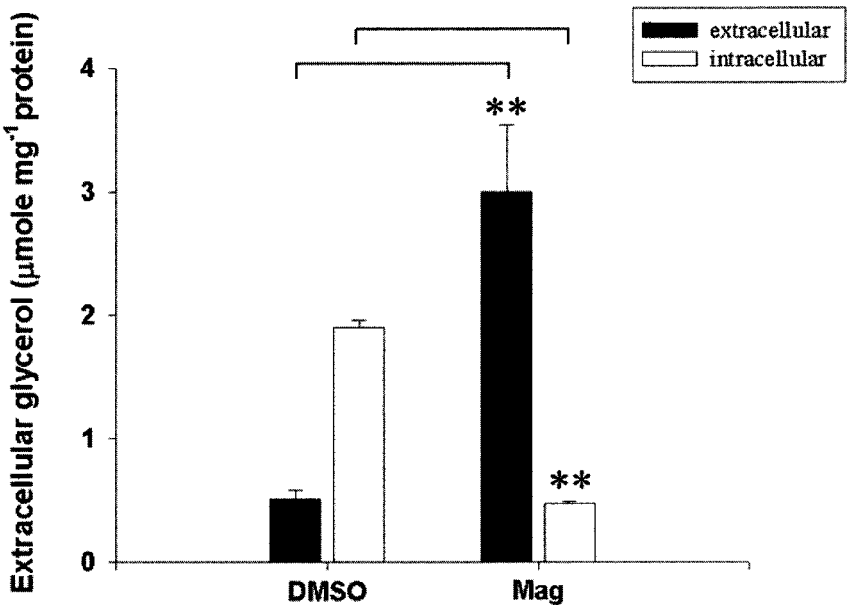

Fig. 3. Stimulation of glycerol release from SE-loaded 3T3-L1 preadipocytes by magnolol. Cells were treated with DMSO (DMSO) or 45 μM magnolol (Mag) for 8 h, then the amounts of extracellular and intracellular glycerol were measured. **, $P < 0.01$, as compared to the DMSO group. n = 3.

stores and the extracellular Ca^{2+} pool, we performed the converse experiment in which the cells were stimulated in Ca^{2+}-free medium, then switched to Ca^{2+}-containing medium. Magnolol induced a small, transient increase in the $[Ca^{2+}]_i$ in the absence of Ca^{2+}, followed by a large Ca^{2+} influx from the extracellular Ca^{2+} pool on transfer to Ca^{2+}-containing medium (Fig. 6). This indicates that magnolol initiated the release of Ca^{2+} from intracellular stores, which may subsequently induce an influx of extracellular Ca^{2+}. Since CaMK is activated by increased $[Ca^{2+}]_i$, we then examined the involvement of CaMK in magnolol function. As

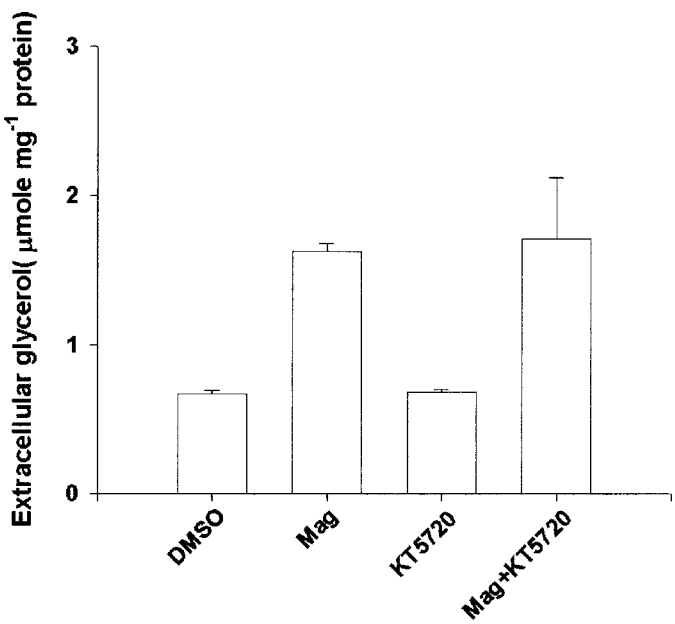

Fig. 4. Effect of protein kinase A (PKA) inhibition on magnolol-induced glycerol release. SE-loaded preadipocytes were treated for 8 h with DMSO (DMSO), 45 μM Mag or 300 nM KT5720 (KT) alone or in combination (Mag + KT), then glycerol release was assayed. n = 3.

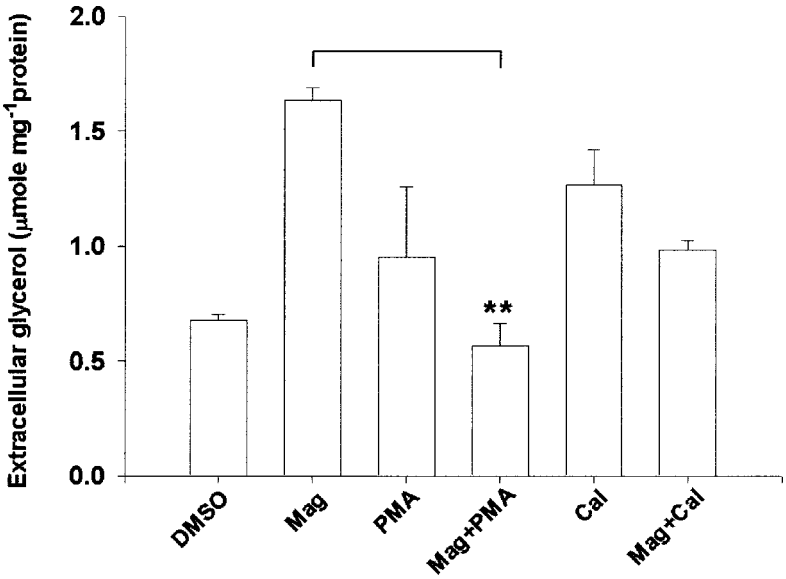

Fig. 5. Effect of protein kinase C (PKC) activation and inhibition on magnolol-induced glycerol release. SE-loaded preadipocytes were treated for 8 h with DMSO (DMSO), 45 μM Mag, 100 nM phorbol 12-myristate-13 acetate (PMA), 500 nM calphostin C (Cal) or with Mag plus either PMA or calphostin C before assaying glycerol release. Only PMA caused a significant decrease in magnolol-induced glycerol release (**, $P < 0.01$, as compared to the Mag group). n = 3.

expected, cotreatment with KN-62, a CaMK inhibitor, completely blocked magnolol-induced lipolysis (Fig. 7). ERK activation was apparently involved, since cotreatment of magnolol with MEK inhibitor PD98059 also blocked magnolol-induced glycerol release (Fig. 7). To provide further evidence on ERK activation, immunoblotting with anti-phosphorylated ERK antibody showed that increased phosphorylation of ERK1 and ERK2 was noted 5 min after magnolol application and remained at the same level up 30 min (Fig. 8A). Furthermore, magnolol-induced phosphorylation of both ERK1 and ERK2 is greatly abolished by combined treatment with PD98059 (Fig. 8B). A duplicate membrane was immunoblotted with monoclonal anti-β actin antibody in order to confirm the same loading amount in each lane (Fig. 8B). Densitometric quantitation confirmed that magnolol increased the amounts of

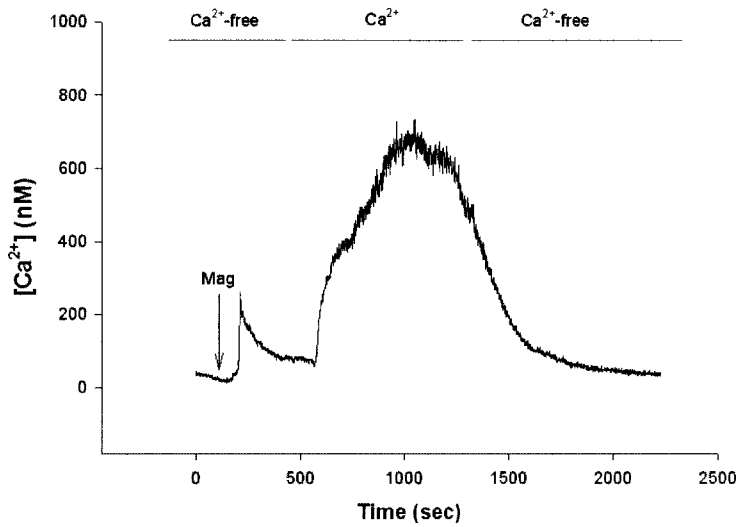

Fig. 6. Magnolol-induced increase on intracellular Ca^{2+}. The final concentration of Mag was 45 μM. Measurement of $[Ca^{2+}]$ was performed first in the absence of Ca^{2+} and then switched to Ca^{2+}-containing solution. n = 4.

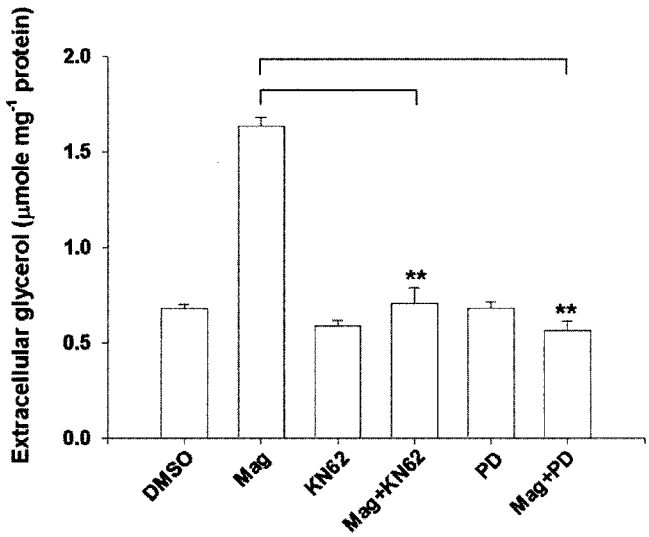

Fig. 7. Effect of KN-62 and PD98059 on magnolol-induced glycerol release. SE-loaded preadipocytes were treated for 8 h with DMSO (DMSO), 45 μM Mag, 2 μM KN62 (KN62), or 40 μM PD98059 (PD), alone or in combination, before assaying glycerol release. KN62 and PD98059 completely blocked magnolol-induced glycerol release (**, $P < 0.01$ compared to the magnolol group). n = 3.

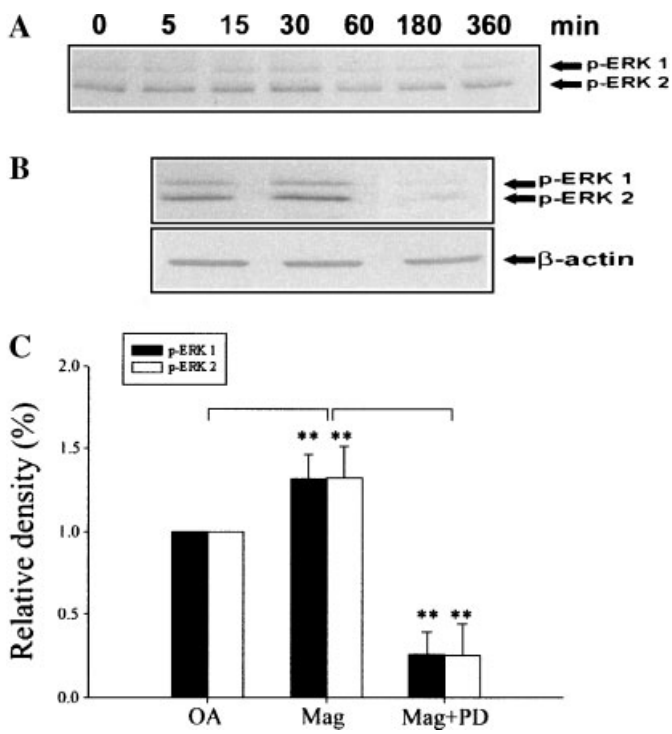

Fig. 8. Induction of ERK phosphorylation by magnolol. **A**: Time-course study of ERK phosphorylation by magnolol in SE-loaded preadipocytes. SE-loaded preadipocytes treated with 45 μM magnolol for different periods (0, 5, 15, 30, 60, 180, 360 min) and cell homogenates were subjected to electrophoresis and immunoblotting with mouse anti-phosphorylated ERK antibody. The data represent a typical example from three independent experiments (n = 3). **B**: Effect of PD98059 on magnolol-induced ERK phosphorylation in SE-loaded preadipocytes. SE-loaded preadipocytes were treated with DMSO, 45 μM Mag or 45 μM magnolol plus 40 μM PD98059 (Mag + PD) for 30 min. The cell lysates were electrophoresed and immunoblotted with mouse anti-phosphorylated ERK antibody or mouse monoclonal anti-β actin antibody (β actin, an internal standard). **C**: Densitometric scans of triplicate blots with anti-phosphorylated ERK antibody from three independent experiments (n = 3).

phosphorylated ERK1 and ERK2 by additional 30%, and cotreatment with PD98059 decreased the phosphorylation levels of ERK1 and ERK1 by 75%, as compared to the control group (Fig. 8C). In order to study the relationship between CaMK and ERK, we examined the effect of KN-93, another selective CaMK inhibitor, on magnolol-induced ERK phosphorylation. Figure 9 demonstrated that KN-93 prevented ERK phosphorylation induced by magnolol.

DISCUSSION

We have previously reported that, in adrenal cells, magnolol induces steroidogenesis, and that PKA is not involved in this response [Wang et al., 2000]. The present study on SE-loaded preadipocytes confirmed that magnolol function is not mediated via PKA, but via a distinct mechanism involving ERK and CaMK.

The effects of magnolol on $[Ca^{2+}]_i$ were different depending on the cell types. Magnolol relaxed rat thoracic smooth muscle cells by inhibiting calcium influx through voltage-gated calcium channels [Teng et al., 1990], whereas it stimulated BK (Ca) channel activity in human tracheal smooth muscle cells [Wu et al., 2002]. Induction of $[Ca^{2+}]_i$ elevation by magnolol was found to be phospholipase C-dependent manner in human hepatoma and colon cancer cell lines [Lin et al., 2001] or inositol triphosphate signaling pathway in rat neutrophils [Wang and Chen, 1998]. Our finding that magnolol induced Ca^{2+} released from intracellular stores and later the Ca^{2+} influx in SE-loaded preadipocytes agrees with previous studies in other cell types [Wang and Chen, 1998; Lin et al., 2001].

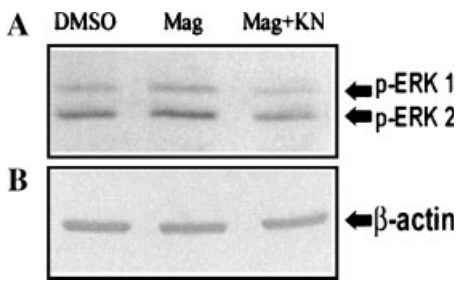

Fig. 9. Inhibition of magnolol-induced ERK phosphorylation by CaMK inihibitor KN-93. SE-loaded preadipocytes were treated with DMSO, 45 μM Mag or 45 μM magnolol plus 10 μM KN-93 (Mag + KN) for 30 min. Cell lysates were blotted with anti-phosphorylated ERK antibody (**A**) or mouse anti-β actin antibody (**B**). The data represent a typical example from three independent experiments (n = 3).

The function of CaMK in steroidogenic cells is intimately associated with the expression of steroidogenic acute regulatory protein, a key protein in the transport of free cholesterol across the mitochondrial membrane [Nishikawa et al., 1997; Kallen et al., 1998]. The present study identified a novel signalling pathway involving Ca^{2+}-CaMK-ERK in magnolol-induced lipolysis in SE-loaded preadipocytes. The present finding that either PD98059 or KN-62 could completely block magnolol-induced lipolysis and magnolol-induced ERK phosphorylation raises the possibility of the presence of a link between CaMK and ERK. Although this is the first time that this particular signaling pathway is demonstrated to be involved in the effects of magnolol on SE-loaded preadipocytes, the crosstalk between CaMK and ERK has been reported in several cell types. Soderling [1999] showed that CaMKIV can activate ERK through B-Raf activation, while Abraham et al. [1997] showed that ionomycin can induce ERK activation in rat aortic smooth muscle and that this Ca^{2+}-dependent ERK activation is mediated by CaMKII, since KN-93 abolishes ERK activation by ionomycin. Similarly, in human T lymphocytes, ionomycin-induced ERK activation is also blocked by KN-93, calmidazolium, and PP2, an inhibitor of p56 (Lck), a Src-family kinase, suggesting that increased $[Ca^{2+}]_i$ causes ERK activation mediated via sequential activation of CaMKII and phosphorylation of p56 (Lck) [Franklin et al., 2000]. As regards how p56 (Lck) phosphorylates ERK, p56 (Lck) can either phosphorylate MEK, which then phosphorylates ERK [Ettehadieh et al., 1992], or induce phosphorylation and activation of Raf-1, an upstream modulator of ERK [Hafner et al., 1994]. In fibroblasts, CaMK can also activate ERK by inhibiting the interaction between calmodulin and K-Ras [Villalonga et al., 2001]. HSL has been shown to be a substrate of phosphorylated ERK [Greenberg et al., 2001], therefore phosphorylation of ERK may increase HSL activity and promote lipolysis of SE-loaded preadipocytes.

Taken together, the stimulation of lipolysis by magnolol seen in SE-loaded preadipocytes may be mediated, in part, by activation of CaMK and ERK. Magnolol has been shown to inhibit the activity of acyl-CoA: cholesterol acyltransfer-

ase, which catalyzes the esterification of cholesterol and favors the deposition of intracellular lipid droplets [Kwon et al., 1997]. Therefore, it is possible that part of the lipolytic effect of magnolol may be due to the inhibitory action of this drug on acyl-CoA: cholesterol acyltransferase. We have previously reported that the mechanism of magnolol-induced adrenal steroidogenesis does not involve PKA or PKC [Wang et al., 2000] and have now demonstrated that the Ca^{2+}-CaMK-ERK system plays a role in magnolol-induced lipolysis of SE-loaded preadipocytes.

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