Epidermal Growth Factor Induces p11 Gene and Protein Expression and Down-regulates Calcium Ionophore-induced Arachidonic Acid Release in Human Epithelial Cells*

Xiu-li Huang‡, Rafał Pawliczak‡‡, Mark J. Cowan‡, Mark T. Gladwin‡, Patricia Madara‡, Carolea Logun‡, and James H. Shelhammer‡¶

From the ‡Critical Care Medicine Department of the Warren G. Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland 20892 and the §Department of Clinical Immunology and Allergy, Medical University of Lodz, Lodz, 92213, Poland

The S-100 protein family is a multigenic family of low molecular mass (9–11-kDa) calcium-binding proteins (1). S-100A10, known as p11 or calpactin I light chain, is a distinct member of the S-100 family, because its two EF-hands carry mutations that limit its ability to bind calcium (2, 3). p11 is a natural ligand of annexin II, forming an annexin II-p11 heterotetramer (AIIt)1 (2, 4, 5), which may have a variety of functions in different cell types. AIIt regulates exocytosis and endocytosis by reorganization of F-actin. It also serves as a receptor to interact with tissue plasminogen activator, plasminogen, and procathepsin B (6, 7). The C-terminal lysine residues of the p11 subunit of the AIIt bind plasminogen and participate in the stimulation of tissue plasminogen activator-dependent plasminogen activation. A recombinant p11 subunit of AIIt stimulates tissue plasminogen activator-dependent plasminogen activation (8, 9). These data suggest that p11, acting as a regulatory protein, modulates the activity of the annexin II subunit and stimulates protease activity by AIIt.

p11 also interacts with the C terminus of cytosolic phospholipase A2 (cPLA2) and inhibits its activity, resulting in reduced arachidonic acid (AA) release (10). Antisense inhibition of p11 mRNA results in enhanced cPLA2 activity and increased AA release, whereas p11 overexpression reduces cPLA2 activity and AA release (11). Dexamethasone is known to reduce cPLA2 activity, and recent studies suggest that this effect may be mediated by up-regulation of p11 (11). Kim et al. (12) have reported that annexin I and annexin II-p11, but not annexin II alone, inhibits cPLA2 activity. Akiba et al. (13, 14) have reported that transforming growth factor-α inhibited A23187-induced AA release through increased p11 binding to cPLA2. These data suggest that p11 may play a role in inflammation by regulation of cPLA2 activity and AA release.

cPLA2, an 85-kDa enzyme, which has high selectivity to hydrolyze phospholipids containing AA esterified in the sn-2 position, has been implicated in receptor-mediated eicosanoid production and intracellular signal transduction processes (15–18). cPLA2 knock-out mice show markedly reduced production of prostaglandins, leukotrienes, and platelet-activating factor in peritoneal macrophages and in bone marrow-derived mast cells (19–22). cPLA2 is activated by physiologically relevant concentrations of calcium and by phosphorylation via mitogen-activated protein kinase ERK and p38 pathways (23–26). Maximum activation of cPLA2 also requires increased intracellular Ca2+ concentrations, which induces translocation of cPLA2 to cellular membranes. A variety of stimuli such as thrombin, platelet-derived growth factor, and fibroblast growth factors

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† To whom all correspondence and reprint requests should be addressed: Warren G. Magnuson Clinical Center, Critical Care Medicine Dept., Bldg. 10, Rm. 7D43, 10 Center Dr., MSC1662, Bethesda, MD 20892-1662. Tel.: 301-496-9320; Fax: 301-480-3389.

1 The abbreviations used are: AIIt, annexin II-p11 heterotetramer; cPLA2, cytosolic phospholipase A2; AA, arachidonic acid; EGF, epidermal growth factor; MAFP, methyl arachidonyl fluorophosphate; MAP, mitogen-activated protein; ERK, extracellular signal-regulating kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iRNA, inhibitory RNA; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; RPA, ribonuclease protection assay.

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have been found to activate cPLA₂ phosphorylation through the MAP kinase ERK pathway (27). EGF has been reported to activate a rapid cPLA₂ phosphorylation mediated by the MAP kinase ERK. EGF is also reported to regulate cPLA₂ gene expression (28–30). In this paper, we investigated the effect of EGF on p11 production, cPLA₂ activation, and AA release. We found that EGF induces p11 expression, which might contribute to the late suppression of calcium ionophore-induced AA release by EGF through increased p11 binding to cPLA₂.

**EXPERIMENTAL PROCEDURES**

Materials—Recombinant human EGF was purchased from R&D (Minneapolis, MN). AG 1478, A23187, PD 98059, SB 203580, and methyl arachidonyl fluorophosphate (MAFP) were obtained from Calbiochem. The mouse anti-human annexin II light chain (anti-p11) monoclonal antibody and anti-EGF receptor antibody were from Transduction Laboratories. The sheep anti-human-cPLA₂ antibody was from Binding Site (Birmingham, UK). The rabbit anti-p38, anti-p44/42, anti-phosphoserine-cPLA₂ (Ser-505), anti-pp38, and anti-pp44/42 were purchased from Cell Signaling Technology (Beverly, MA). Anti-phosphoserine antibody was from Sigma. Horseradish peroxidase-conjugated donkey anti-mouse IgG or goat anti-rabbit IgG were from Jackson ImmunoResearch Laboratory, Inc. (West Grove, PA). [3H]AA (1 mCi/ml) and the ECL Western blotting detection system were from Amersham Biosciences. BCA reagents were from Pierce.

Cell Culture and Preparation of ³H-Labeled Cells—HeLa cells (American Type Culture Collection, Manassas, VA) were grown in DMEM with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂ in 175-cm² tissue culture flasks or six-well plates. Cells were seeded in six-well plates in DMEM with 10% fetal calf serum until 60% confluence and then incubated in the presence or absence of [³H]AA (1 µCi/ml) for 16 h. The labeled cells were washed three times with DMEM without serum. The cells were incubated in 2 ml of DMEM with 10% fetal calf serum containing EGF at a final concentration for the following experiment. All experiments were performed when cells were 80–90% confluent.

Experimental Design—For the time course experiments, after replacing culture medium at the same time, the cells were treated with or without 20 ng/ml EGF at 4, 12, 24, or 48 h prior to harvest at the 48-h time point. For the dose-response experiments, cells were treated with or without 0.2, 2, or 20 ng/ml EGF and harvested at 12 h. All inhibitors were preincubated for 2 h before treatment with or without EGF. Inhibitors were maintained for the incubation period.

Cellular Arachidonic Acid Release—The [³H]AA-labeled HeLa cells were stimulated with EGF for 30 min or 24 h, washed, and placed in 2 ml of medium in the presence or absence of A23187 (10⁻⁶ M) for 30 min. The [³H]-labeled HeLa cells were preincubated with MAFP (50 µM) (cPLA₂ inhibitor), tyrphostin AG 1478 (10 µM) (EGF receptor tyrosine kinase inhibitor), PD 98059 (25 µM) (ERK inhibitor), or SB 203580 (10 µM) (p38 pathway inhibitor) for 2 h, and the medium was replaced with fresh medium containing EGF for 30 min. The supernatants were collected, centrifuged at 750 × g for 10 min, and counted in a scintillation counter (Beckman, Fullerton, CA) for quantification of AA release.

Immunoblot of p11 and cPLA₂—HeLa cells grown in six-well plates were treated with or without EGF for the indicated time and dose. Cells were washed three times with cold PBS and lysed in 100 µl of homogenization buffer: 50 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM EGTA, 100 µM leupeptin, 1 mM diithiothreitol, 10 mM phenylmethylsulfonyl fluoride, 0.5 mM soybean trypsin inhibitor, 15 mM aprotinin, and 0.25% Triton X-100. The cells were then sonicated three times for 15 s and centrifuged at 14,000 rpm for 10 min. Total protein was measured using the BCA method. Ten micrograms of crude cell lysate proteins were separated on 16% Tris-glycine SDS gels for cPLA₂ using Tris-glycine SDS running buffer. The separated proteins were electrothermally transferred onto a nitrocellulose membrane (Novex), which was then blocked with 5% nonfat dry milk with 0.1% Tween 20 for 1 h. p11 or cPLA₂ protein expression was detected by immunoblotting with a 1:2000 dilution of mouse-anti-human annexin II light chain monoclonal antibody (anti-p11 antibody) or sheep anti-human cPLA₂ and a 1:5000 dilution of horseradish peroxidase-conjugated donkey anti-mouse IgG or goat anti-sheep IgG as the second antibody. The blot was developed using the ECL Western blotting detection system and exposed to Eastman Kodak Co. MR radiographic film.

**Ribonuclease Protection Assay (RPA)—**HeLa cells grown in T-175 cm² flasks were treated with and without EGF (0.2, 2, and 20 ng/ml) for 4–48 h. The cells were washed three times with cold PBS and then

**FIG. 1.** EGF induces an increase in p11 protein levels. Cells treated with or without EGF were lysed, and 10 µg of crude cell lysates were studied by Western blot analysis using anti-annexin II light chain monoclonal antibody. A, time course (4–48 h) effect of EGF (20 ng/ml) on HeLa cells showing increased p11 protein levels, with peak levels at 12 h. EGF treatment did not effect cPLA₂ protein expression. The result shown is representative of three separate experiments. B, densitometry measurements from three time course experiments of EGF (20 ng/ml) and control cells demonstrating increased p11 protein levels (data presented as mean ± S.E., *p < 0.05 compared with control value). C, EGF (0.2, 2, and 20 ng/ml) treatment for 12 h induces a dose-dependent increase in p11 protein level. The result shown is representative of three separate experiments. D, densitometry measurements from three dose-response (0–20 ng/ml) experiments demonstrating a dose-related increase in p11 protein levels (data presented as mean ± S.E., p < 0.001 for dose-related effect by single factor analysis of variance).
EGF Induces p11 Gene and Protein Expression

protected fragments of p11 (319 bp) and GAPDH were visualized by p11-specific radiolabeled cRNA, respectively, and subjected to RPA. The micrograms of total cellular RNA were hybridized to GAPDH and time and dose, followed by extraction of total RNA. Ten and fifty cells were incubated in the presence or absence of EGF at the indicated EGF treatment increases p11 steady state mRNA levels.

Left panel

A, ribonuclease protection assay.

B

EGF Induces p11 Gene and Protein Expression

strates a significant effect of EGF on p11 steady state mRNA levels with control value.

D

Right panel

Lanes 3, probe without RNase; lanes 4, control cellular RNA; lanes 5, total RNA from EGF-treated cells).

E

Immunoprecipitation of p11 and cPLA2 Protein

HeLa cells cultured in six-well plates were treated with or without EGF for 5 min, 10 min, 30 min, 1 h, or 2 h. The cells were washed three times with cold PBS, and the medium was aspirated completely. 100 μl of 1× protein loading buffer was added into each well and immediately scraped and collected into microcentrifuge tubes. After sonication for 20 s, the samples were boiled for 10 min and cooled on ice. 20 μl of each sample was separated on a 4–20% Tris-glycine gel, electrophoretically transferred onto a nitrocellulose membrane, and blocked with 5% nonfat milk. The blots were then probed with a 1:2000 dilution of antisense or sense riboprobe. The mixture was hybridized at 45 °C overnight and digested by the addition of a 1:100 dilution of RNase A/T1 at 37 °C for 60 min. Digestion was terminated by the addition of the RNase inactivation and precipitation mixture. The protected fragments were separated on 6% polyacrylamide 8M urea gels (Novex) and visualized by autoradiography.

Transient Transfection Assay—HeLa cells were maintained at 37 °C under 5% CO2 in DMEM with 10% FBS. The day before transfection, 5 × 104 cells were seeded into six-well plates. 1.8 μg of p11 promoter constructs were co-transfected with 0.1 μg of the pCMV/β-galactosidase construct (CLONTECH, Palo Alto, CA) into serum-free cells using 6 μl of LipofectAMINE reagent (InverGen) in each well. After 5 h of transfection, cells were incubated in fresh DMEM medium with 10% FBS for 36 h. The transfected cells were stimulated with EGF (20 ng/ml) for the indicated times and lysed. pCAT and β-galactosidase activity was measured with pCAT and β-galactosidase enzyme-linked immunosorbent assay kits (Roche Molecular Biochemicals). The pCAT activity was normalized to β-galactosidase activity to represent relative p11 promoter activity.

Immunoblot Analysis of Phosphorylation of p44/42, p38, and cPLA2—HeLa cells cultured in six-well plates were treated with or without EGF for 0.2, 2, and 20 ng/ml treatment for 12 h shows a dose-dependent effect on p11 mRNA levels. Data are representative of three different experiments. C, densitometry measurement from three time course experiments showing a time-dependent effect of EGF (20 ng/ml) on the steady state p11 mRNA expression at 4–12 h. GAPDH mRNA levels are presented as an internal control to evaluate RNA loading. Data are representative of three different experiments. D, densitometry measurement from three dose-response experiments of EGF-treated cells demonstrates a significant effect of EGF on p11 steady state mRNA levels (data presented as mean ± S.E., *p < 0.001 for dose effect by one-factor analysis of variance).

Immunoprecipitation of p11 and cPLA2 Protein—HeLa cells grown in 175-cm2 tissue culture flasks were washed three times with cold PBS

FIG. 2. EGF treatment increases p11 steady state mRNA levels. Cells were incubated in the presence or absence of EGF at the indicated time and dose, followed by extraction of total RNA. Ten and fifty micrograms of total cellular RNA were hybridized to GAPDH and p11-specific radiolabeled cRNA, respectively, and subjected to RPA. The protected fragments of p11 (319 bp) and GAPDH were visualized by autoradiography. A, ribonuclease protection assay. Left panel, total cellular RNA was incubated with a p11 antisense or sense riboprobe. Lane 1, molecular mass markers; lanes 2–5, antisense probe (lane 2, probe plus RNase; lane 3, probe without RNase; lane 4, control from control cells; lane 5, total RNA from cells treated with EGF (20 ng/ml, 12 h)); lane 6, empty; lanes 7–10, sense probe (lane 7, probe plus RNase; lane 8, probe without RNase; lane 9, total RNA from control cells; lane 10, total RNA from EGF-treated cells).

Right panel, GAPDH antisense riboprobe. Lane 1, molecular mass markers; lane 2, probe plus RNase; lane 3, probe without RNase; lane 4, control cellular RNA; lane 5, EGF-treated cellular RNA. E, EGF (20 ng/ml) treatment for 4–48 h resulted in an increased p11 steady state mRNA expression at 4–12 h. GAPDH mRNA levels are presented as an internal control to evaluate RNA loading. Data are representative of three different experiments. C, densitometry measurement from three time course experiments showing a time-dependent effect of EGF (20 ng/ml) on the steady state p11 mRNA levels (data presented as mean ± S.E.; *p < 0.05 compared with control value). D, EGF (0.2, 2, and 20 ng/ml) treatment for 12 h shows a dose-dependent effect on p11 mRNA levels. Data are representative of three different experiments. E, densitometry measurement from three dose-response experiments of EGF-treated cells demonstrates a significant effect of EGF on p11 steady state mRNA levels (data presented as mean ± S.E., *p < 0.001 for dose effect by one-factor analysis of variance).
and lysed in 0.5 ml of homogenization buffer as described above. 500 μg of crude cell lysate was added to 200 μl of immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl, 0.05% Nonidet P-40) containing 10 μl of sheep anti-human cPLA₂ antibody and incubated at 4°C on a rotator at medium speed for 4 h. Thirty microliters of protein G-agarose (Pierce) beads were then added to each sample, and the mixture was incubated at 4°C for 2 h, followed by centrifugation in a microcentrifuge at 2000 rpm for 5 min at 4°C. The supernatants were aspirated, and the pellet was washed four times with immunoprecipitation buffer. The pellet was then suspended in 20 μl of protein loading buffer and boiled for 10 min before electrophoresis on 16% Tris-glycine gels for p11 and 8% Tris-glycine gels for cPLA₂. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane blocked with 5% nonfat milk and then probed with a 1:2000 dilution of anti-p11 antibody or 1:500 dilution of anti-phosphoserine antibody for 2 h. After three washings, the blots were incubated with a 1:2000 dilution of second antibody and developed by using the ECL Western blotting detection system.

Transfection of HeLa Cells with iRNAs—iRNAs were prepared by IDT (Coralville, IA) and targeted the coding region 4–24, relative to the start codon of the cPLA₂ gene. Single-stranded RNAs were annealed by incubating a 20 μM concentration of each strand in annealing buffer (100 mM potassium acetate, 30 mM HEPES buffer at pH 7.4, 2 mM magnesium acetate) for 2 min at 90°C. The iRNA sequences used in this study are 5’-GUAAGGAUCCUAUAAGATT-3’ and 5’-UCAUUU-UAUAUAUGCCUACTT-3’. HeLa cells were grown as described above and were transfected with 10 nM iRNA duplexes using LipofectAMINE reagent (Invitrogen). Four hours after transfection, media were changed, and cells were incubated with or without EGF (20 ng/ml) or with media. After 12 h of incubation with or without EGF, cPLA₂ and p11 proteins were detected using immunoblotting as described above.

Quantification of Autoradiographs—An Amersham Biosciences 301 computing densitometer was used to digitize images. The optical density of bands was analyzed with background subtraction using ImageQuant software (Amersham Biosciences).

Statistical Analysis—The dose-related effects were analyzed with one-way analysis of variance, and comparisons were performed using two-tailed paired Student’s t tests. Values of p < 0.05 were considered statistically significant.

RESULTS

EGF Increases the Level of the p11 Protein in Human Epithelial Cells—The effect of EGF on p11 expression was studied. HeLa cells were treated with or without EGF, and cellular p11 protein levels were assessed by Western blot analysis. Fig. 1, A and B, demonstrates that p11 is constitutively expressed in untreated HeLa cells. EGF (20 ng/ml) increased p11 protein expression over a 4–24-h period, with a maximum effect observed at 12 h. However, over the incubation period, EGF had no effect on cPLA₂ protein expression. Treatment of cells with 0.2, 2, or 20 ng/ml EGF for 12 h resulted in a dose-dependent increase in p11 protein levels (Fig. 1, C and D).

The Effect of EGF on p11 Steady-state mRNA Levels in HeLa Cells—To determine whether the observed increase in p11 protein levels correlates with p11 mRNA expression, HeLa cells were incubated with or without EGF (20 ng/ml) for 4–48 h or EGF (0.2, 2, or 20 ng/ml) for 12 h. Total RNA was isolated, and the steady-state p11 mRNA expression was studied by an RPA. The specificity of the p11 antisense riboprobe is presented in Fig. 2A. The antisense riboprobe produced a protected band at ~300 bases. A sense riboprobe produces no protected band. Cellular RNA incubated with a GAPDH riboprobe is presented in the right panel of Fig. 2A. As shown in Fig. 2, B and C, EGF treatment induced an increase in p11 steady-state RNA levels from 4–12 h, compared with the untreated control cells. Cells treated with 0.2, 2, or 20 ng/ml EGF for 12 h demonstrated a dose-related increase in the steady state p11 mRNA levels in HeLa cells as shown in Fig. 2, D and E. GAPDH mRNA levels are presented as an internal control for equivalent RNA loading and normalization of p11 mRNA expression.

EGF Induces p11 Expression at the Transcriptional Level in HeLa Cells—To further investigate whether the observed increase in steady-state p11 mRNA levels reflects an EGF-induced increase in p11 gene transcription, a reporter gene construct containing the 1498-bp sequence of the 5′-flanking region of the p11 promoter in the pCAT vector was transfected into HeLa cells. The results are shown in Fig. 3. EGF (20 ng/ml) treatment of HeLa cells induced increased p11 gene transcriptional expression over 2–8 h. β-Galactosidase activity was used to correct for transfection efficiency. Thus, EGF induces p11 expression, at least in part, at the transcriptional level.

EGF Induces p44/42 and p38 Phosphorylation—EGF exerts its effect by binding and activating a specific 170-kDa tyrosine kinase receptor and triggers a subsequent MAP kinase signal...
transduction cascade. As shown in Fig. 4A, EGF stimulated the phosphorylation of p44/42 in a time-dependent manner with a maximum activation at 5 and 10 min. To examine whether EGF also stimulated p38 phosphorylation, the same cell lysate was used to immunoblot with phosphospecific anti-pp38 antibody. EGF-induced p38 phosphorylation was also transient, starting at 5 min and with a maximum at 10 min (Fig. 4B).

The Effect of AG 1478, PD 98059, and SB 203580 on EGF-induced p11 Expression—To examine whether EGF-induced p11 expression is mediated via the activation of EGF receptor and mitogen-activated protein kinase pathway, the effect of tyrphostin AG 1478, an EGF receptor tyrosine kinase inhibitor, on EGF-induced p11 expression was studied. As shown in Fig. 5, A and B, AG 1478 significantly inhibited EGF-induced p11 expression, whereas AG 1478 alone had a little or no effect on p11 expression. These data demonstrated that EGF-induced p11 expression is mediated via the activation of the EGF receptor.

After pretreatment with PD 98059 or SB 203580 for 2 h, HeLa cells were incubated with EGF for 12 h. As shown in Fig. 5, C–F, PD 98059 and SB 203580 significantly inhibited EGF-induced p11 expression. Cells treated with inhibitors alone exhibited no effect. These data suggest that both the ERK pathway and the p38 pathway are involved in the induction of p11 by EGF.

The Effect of EGF on cPLA2 Phosphorylation—Phosphorylation of cPLA2 was studied by Western blot and immunoprecipitation. HeLa cells were treated with EGF (20 ng/ml) for 5 min, 10 min, 30 min, 1 h, and 2 h, and the cell lysates were immunoblotted with phospho-specific anti-cPLA2 antibody. EGF induced maximum cPLA2 phosphorylation at 10–30 min. To determine whether EGF-induced phosphorylation of cPLA2 is mediated via activation of one or both of the above mentioned MAP kinase pathways, cells were preincubated with PD 98059.
EGF Induces cPLA2 phosphorylation. A, Western blot showing that EGF induces a rapid phosphorylation of cPLA2. HeLa cells grown in six-well plate were treated with or without EGF (20 ng/ml) for the indicated time and washed three times with cold PBS. The cells were lysed in 100 μl of 1× SDS loading buffer. Equal amounts of cell lysates were separated to 8% Tris-glycine gels. The Western blot was performed, and the membrane was probed with rabbit anti-phosphoserine cPLA2 antibody. Total cPLA2 protein was unchanged.

B, immunoprecipitation experiment demonstrating that EGF induced an increased cPLA2 phosphorylation and did not affect the total amount of cPLA2. HeLa cells were grown in T-175 cm² flasks and treated with or without EGF (20 ng/ml) for the indicated time. After washing with cold PBS, the cells were lysed in the lysate buffer described under "Experimental Procedures." 200 μl (500 μg) of cell lysates were incubated with anti-cPLA2 antibody for 4 h and then with 20 μl of protein G-agarose for 2 h. After repeated washing with immunoprecipitation buffer, 20 μl of protein loading buffer was added to suspend the agarose beads, and the beads were boiled for 5 min and then subjected to electrophoretic separation on an 8% Tris-glycine gel. Precipitated cPLA2 was immunoblotted with anti-cPLA2 antibody or anti-phosphoserine antibody. C, Western blot demonstrating the effect of PD 98059 on EGF-induced phosphorylation of cPLA2. Cells were treated with or without PD 98059 (25 μM) for 2 h followed by treatment with EGF (20 ng/ml).

D, densitometry measurements from four experiments demonstrating that PD 98059 inhibited EGF-induced cPLA2 phosphorylation in HeLa cells (data presented as mean ± S.E.; *, p < 0.05 for EGF-treated cells versus EGF plus PD 98059-treated cells). E, Western blot showing that SB 203580 inhibited EGF-induced cPLA2 phosphorylation. Cells were treated with or without SB 203580 (10 μM) for 2 h followed by treatment with or without EGF (20 ng/ml). F, densitometry measurement from five different experiments demonstrating that SB 203580 inhibited EGF-induced cPLA2 phosphorylation (data presented as mean ± S.E.; *, p < 0.05 for EGF-treated cells versus EGF plus SB 203580-treated cells).
Effect of cPLA2-inhibitory RNAs on Arachidonic Acid Release and p11 Production in Response to EGF—Anti-cPLA2 iRNAs were utilized in order to further assess the role of cPLA2 in EGF-induced arachidonic acid release and in p11 production. Cells treated with iRNAs exhibited reduced EGF-induced arachidonate release compared with control cells treated with LipofectAMINE alone (Fig. 10A). Western blot of cell lysates from cells treated with iRNAs also demonstrated a reduction in cellular cPLA2 protein (Fig. 10B). Finally, treatment of cells with iRNAs resulted in a diminished p11 production in response to EGF compared with cells treated with the transfection reagent alone (Fig. 10C).

EGF Increases Native p11 Bound to cPLA2—To study the effect of EGF-induced p11 expression on cPLA2, immunoprecipitation complex from HeLa cells was studied. As shown in Fig. 11, A and B, treatment with 20 ng/ml EGF for 4–24 h induced an increase in p11 co-immunoprecipitated with cPLA2 in a time-dependent manner with a maximum effect at 12–24 h. There also was a dose-dependent effect of EGF treatment on co-immunoprecipitated p11-cPLA2 (Fig. 11, C and D). These results suggest that EGF-induced p11 expression is important for the activation of cPLA2.

EGF Induces p11 Gene and Protein Expression

Fig. 7. AG 1478, PD 98059, and SB 203580 inhibit EGF induced AA release. A, the expression of EGF receptor in HeLa cells. Cells treated with or without EGF (20 ng/ml) for 12 h were lysed, and 10 μg of crude cell lysates were studied by Western blot analysis using anti-EGFR antibody. B, AG 1478 abolished EGF-induced AA release. [3H]AA-labeled HeLa cells were preincubated with tyrphostin AG 1478, an EGF receptor tyrosine kinase inhibitor, for 2 h. The cells were washed and stimulated with or without EGF (20 ng/ml) for 30 min. An aliquot of medium was used to measure AA release by scintillation counter. Data are presented as mean ± S.E., n = 6, [3H]AA release from one of three experiments each with a similar result. *, p < 0.001 for EGF-treated compared with EGF plus AG 1478-treated cells.

Fig. 8. MAFP inhibits EGF-induced AA release in HeLa cells. HeLa cells grown in six-well plates were labeled with [3H]AA (1 μCi/ml) in DMEM with 10% FBS for 16 h. After washing with medium without FBS, the cells were preincubated with MAFP (50 μM) for 2 h and then with EGF (20 ng/ml) for 30 min, and the media were collected and centrifuged. An aliquot of medium was used to measure AA release by a scintillation counter. Data are presented as mean ± S.E. [3H]AA release from one of four experiments, each with a similar result. *, p < 0.001 for EGF-treated compared with cells treated with EGF plus MAFP.
results demonstrate that EGF treatment results not only in an increase of cellular p11 expression but also in an increase of p11 bound to cPLA2.

The Effect of EGF on the Cellular Arachidonic Acid Release—To study whether increased binding between p11 and cPLA2 could affect AA release, [3H]AA-labeled HeLa cells were treated with or without EGF (20 ng/ml) for 30 min or 24 h. After EGF treatment, medium was changed, cells were treated with or without A23187 for 30 min, and medium was harvested for scintillation counting. The results shown in Fig. 12. A and B, indicate that EGF treatment for 30 min increased both basal and A23187-induced AA release. Interestingly, EGF treatment for 24 h diminished A23187-induced AA release. These data suggest that the inhibitory effect of EGF on AA release at 24 h may be due to EGF-induced p11 expression, which may inhibit cPLA2 activity and reduce AA release.

DISCUSSION

p11, or calpain light chain, is a unique member of the S-100 family of calcium-binding proteins. Although it shares sequence homology with the S100 family, it does not have the ability to bind Ca2+ due to amino acid deletions and substitutions in the two EF-hand motifs. p11 is present in a variety of cells separately or as a heterotetramer with annexin II (AIIt). The expression of p11 and annexin II is not always coordinated, and the ratio of p11 to annexin II varies with different cell types. Munz et al. (31) reported that wound-derived growth factors (transforming growth factor-β1, EGF, and keratinocyte growth factor) differentially regulate p11 and annexin II expression in cultured keratinocytes during skin injury and modify the ratio between p11 and annexin II. Annexin II tetramer also can act as a surface protein receptor for plasminogen and t-PA.

Studies in cPLA2-deficient mice demonstrate that cPLA2 plays an essential role in eicosanoid production and allergic response (19). It was important to investigate the negative regulation of cPLA2 in physiological conditions. Annexin I, or lipocortin I, has been reported to suppress cPLA2 activity not only in vitro but also in cultured cells. Thus, annexin I may function as an endogenous negative regulator of cPLA2. p11 interacts with and inhibits cPLA2 activity and subsequent AA release in vitro and in vivo (10). Therefore, p11 may also function as a negative regulator of cPLA2.

p11 has been reported to be regulated in different cell types. Nerve growth factor increases p11 mRNA expression in rat pheochromocytoma (PC12) cells (32). Retinoic acid reduces p11 protein levels by a post-translational mechanism in BEAS-2B cells (33). Transforming growth factor-α or the combination of transforming growth factor-α and interleukin-1β induces an increase in p11 protein expression in rat gastric epithelial cells (14). Nitric oxide induces p11 release through a cGMP-dependent pathway in epithelial cells (34). In this study, we show that EGF induces p11 production in a time- and dose-dependent manner in human epithelial cells (HeLa cells). The effect was correlated with increased steady-state levels of p11 mRNA in response to EGF in a time- and dose-related manner. This effect of EGF on p11 expression was regulated at least in part at the transcriptional level. EGF has been reported to induce cPLA2 expression at the transcriptional level (28, 29). However, in our experiments, over an incubation up to 48 h, EGF did not appear to affect cPLA2 expression, indicating that EGF induced cPLA2 expression may be cell type-dependent.

The binding of EGF to the EGF receptor on the cell surface...
triggers receptor trans-autophosphorylation and subsequent activation of the Ras/Raf/mitogen-activated protein kinase cascade. Several experiments were done to explore the signal pathway involved in the induction of EGF-induced p11 production. First, we observed that EGF induces rapid p44/42 and p38 phosphorylation starting at 5 min and with a maximum activation at 10 min. Second, the EGF receptor tyrosine kinase inhibitor, AG 1478, significantly inhibited EGF-induced p11 expression, indicating the involvement of the activation of EGF receptor. Third, PD 98059 and SB 203580 inhibited EGF-induced p11 production, demonstrating that both MAP kinase ERK and p38 participated in this event. Thus, the addition of EGF activates the EGF receptor tyrosine kinase and subsequent MAP kinase ERK and p38 pathway, resulting in the induction of p11.

Previous work has shown that EGF stimulates AA release through the phosphorylation of cPLA2. In our studies, we observed similar results with two sets of experiments. First both Western blot and immunoprecipitation experiments showed that EGF treatment resulted in phosphorylation of cPLA2, confirming its role in AA release. The time-dependent and dose-dependent increase of p11 bound to cPLA2 is depicted in Figure 11, showing a significant rise in binding starting as early as 4 hours after EGF treatment.

EGF Induces p11 Gene and Protein Expression

Fig. 12. Effect of EGF on A23187-induced arachidonic acid release in HeLa cells. A, EGF increases A23187-induced AA release at 30 min. [3H]AA (1 μCi/ml)-labeled HeLa cells were incubated with or without EGF (20 ng/ml) for 30 min, the medium was changed, and the cells were then treated with or without EGF for 30 min. The supernatants were collected and centrifuged. AA release was measured by a scintillation counter. Data are presented as AA release from one (n = 6) of three experiments with similar results. *, p < 0.001 for A23187-induced AA release compared with non-A23187-stimulated cells treated with or without EGF. B, EGF inhibits A23187-induced AA release at 24 h. [3H]AA (1 μCi/ml)-labeled HeLa cells were incubated with or without EGF (20 ng/ml) for 24 h. The medium was changed, cells were treated with or without 10 μM A23187 for 30 min, and the supernatants were collected and centrifuged. AA release into the medium was measured by a scintillation counter. Data are presented as mean ± S.E. (n = 6). [3H]AA release from one of three separate experiments each with similar results. *, p < 0.001 for A23187-induced AA compared with control value.
starting at 5 min and with a maximum activation at 10–30 min. Second, cells pretreated with MAPF significantly inhibited EGF-induced AA release, suggesting that the activation of cPLA₂ is responsible for EGF-induced AA release. In addition, AG 1478, PD 98059, and SB 203580 significantly inhibited EGF-induced AA release, indicating that the activation of cPLA₂ by EGF is through the activation of EGF receptor and subsequent MAP kinase pathways. To further study whether these events of the activation of cPLA₂ and AA release could influence p11 production, we showed that MAPF pretreatment significantly suppressed EGF-induced p11 expression. These data suggest that the activation of cPLA₂ may play a role in the induction of p11 by EGF. We postulate that EGF induced p11 expression through the activation of EGF receptor, MAP kinase, and cPLA₂. Whether EGF-induced AA or AA metabolites contribute to the p11 expression by EGF requires further study.

In conclusion, we have demonstrated that EGF induces p11 production through EGF activation of the EGF receptor tyrosine kinase and activation of p44/42, p38, and cPLA₂.

REFERENCES

1. Moore, B. W. (1965) Biochem. Biophys. Res. Commun. 19, 739–744
2. Gerke, V., and Weber, K. (1984) EMBO J. 3, 227–233
3. Gerke, V., and Weber, K. (1985) EMBO J. 4, 2917–2920
4. Waisman, D. M. (1995) Mol. Cell Biochem. 148, 301–322
5. Gerke, V., and Moss, S. E. (1997) Biochim. Biophys. Acta 1357, 129–154
6. Kang, H. M., Choi, K. S., Kassam, G., Fitzpatrick, S. L., Kwon, M., and Waisman, D. M. (1999) Trends Cardiovasc. Med. 9, 92–102
7. Fitzpatrick, S. L., Kassam, G., Choi, K. S., Kang, H. M., Fogg, D. K., and Waisman, D. M. (2000) Biochemistry 39, 1021–1028
8. Kassam, G., Le, B. H., Choi, K. S., Kang, H. M., Fitzpatrick, S. L., Louie, P., and Waisman, D. M. (1998) Biochemistry 37, 16958–16966
9. Kassam, G., Choi, K. S., Ghuman, J., Kang, H. M., Fitzpatrick, S. L., Jackson, T., Zackson, S., Tobe, M., Shimosiya, A., and Waisman, D. M. (1998) J. Biol. Chem. 273, 4790–4799
10. Wu, T., Angus, C. W., Yao, X. L., Logun, C., and Shelhamer, J. H. (1997) J. Biol. Chem. 272, 17145–17153
11. Yao, X. L., Cowan, M. J., Gladwin, M. T., Lawrence, M. M., Angus, C. W., and Shelhamer, J. H. (1999) J. Biol. Chem. 274, 17202–17208
12. Kim, S., Ko, J., Kim, J. H., Choi, E. C., and Na, D. S. (2001) FEBS Lett. 489, 243–248
13. Akiba, S., Hatazawa, R., Ono, K., Hayama, M., Matsui, H., and Sato, T. (2000) Br. J. Pharmacol. 131, 1004–1010
14. Akiba, S., Hatazawa, R., Ono, K., Kitatani, K., Hayama, M., and Sato, T. (2001) J. Biol. Chem. 276, 21854–21862
15. Clark, J. D., Schiefvella, A. R., Nalefski, E. A., and Lin, L. L. (1995) J. Lipid Mediat. Cell Signal. 12, 83–117
16. Ackermann, E. J., and Dennis, E. A. (1995) Biochim. Biophys. Acta 1259, 125–136
17. Balsinde, J., Bianco, I. D., Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 92, 8527–8531
18. Leslie, C. C. (1997) J. Biol. Chem. 272, 16709–16712
19. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (1997) Nature 390, 618–622
20. Bonventre, J. V., Huang, Z., Taheri, M. R., O’Leary, E., Li, E., Moskowitz, M. A., and Sapirstein, A. (1996) Nature 390, 622–625
21. Gijon, M. A., Sanchez Mejia, R. O., Bingham, C. O., Ills, B. M., Sapirstein, A., Bonventre, J. V., Austen, K. F., and Arm, J. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4803–4807
22. Clark, J. D., Lin, L. L., Krix, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) Cell 65, 1043–1051
23. Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J. (1993) Cell 72, 269–278
24. Kramer, R. M., Roberts, E. F., Um, S. L., Borsch-Haubold, A. G., Watson, S. P., Fisher, M. J., and Jakubowski, J. A. (1996) J. Biol. Chem. 271, 27723–27729
25. Borsch-Haubold, A. G., Kramer, R. M., and Watson, S. P. (1997) Eur. J. Biochem. 245, 751–759
26. Sa, G., Murugesan, G., Jaye, M., Ivaschenko, Y., and Fox, P. L. (1995) J. Biol. Chem. 271, 2360–2366
27. Maxwell, A. P., Goldberg, H. J., Tay, A. H., Li, Z. G., Arbus, G. S., and Skorecki, K. L. (1990) Biochem. J. 265, 763–766
28. Chepenik, K. P., Diaz, A., and Jimenez, S. A. (1994) J. Biol. Chem. 269, 21786–21792
29. Marpulis, B. L., Bonventre, J. V., Kremer, S. G., Kudlow, J. E., and Skorecki, K. L. (1988) Biochem. J. 249, 587–592
30. Munz, B., Gerke, V., Gilitzter, R., and Werner, S. (1997) J. Invest. Dermatol. 108, 307–312
31. Mastakowski, P., and Shroot, E. M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1277–1281
32. Gladwin, M. T., Yao, X. L., Cowan, M., Huang, X. L., Schneider, R., Grant, L. R., Logun, C., and Shelhamer, J. H. (2000) Am. J. Physiol. 278, L1103–L1109
33. Pawlczak, R., Cowan, M. J., Huang, X., Nanavaty, U. B., Alsata, S., Logun, C., and Shelhamer, J. H. (2001) J. Biol. Chem. 276, 44613–44621
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Xiu-li Huang, Rafal Pawliczak, Mark J. Cowan, Mark T. Gladwin, Patricia Madara, Carolea Logun and James H. Shelhamer

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