Epidermal growth factor and phorbol myristate acetate increase expression of the mRNA for cytosolic phospholipase A_2 in glomerular mesangial cells

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We have previously shown that phospholipase A_2 (PLA_2) activity is rapidly activated by epidermal growth factor (EGF) and phorbol 12-myristate 13-acetate (PMA) in renal mesangial cells and other cell systems in a manner that suggests a covalent modification of the PLA_2 enzyme(s). This PLA_2 activity is cytosolic ($cPLA_2$) and is distinct from secretory forms of PLA_2, which are also stimulated in mesangial cells in response to cytokines and other agonists. However, longer-term regulation of $cPLA_2$ in renal cells may also occur at the level of gene expression. Cultured rat mesangial cells were used as a model system to test the effects of EGF and PMA on the regulation of $cPLA_2$ gene expression. EGF and PMA both produced sustained increases in $cPLA_2$ mRNA levels, with a parallel increase in enzyme activity over time. Inhibition of protein synthesis by cycloheximide increased basal $cPLA_2$ mRNA accumulation in serum-starved mesangial cells, and the combination of EGF and cycloheximide resulted in super-induction of $cPLA_2$ gene expression compared with EGF alone. Actinomycin D treatment entirely abrogated the effect of EGF on $cPLA_2$ mRNA accumulation. These findings suggest that regulation of $cPLA_2$ is achieved by factors controlling gene transcription and possibly mRNA stability, in addition to previously characterized post-translational modifications.

INTRODUCTION

Arachidonic acid release from membrane-bound phospholipids represents an essential component of the intracellular response to ligand–receptor interactions implicated in the overall regulation of mitogenic and non-mitogenic responses in a variety of cell systems. Mesangial cells of the renal glomerulus have been intensively studied with respect to the role of arachidonic acid and its metabolites in terms of mitogenic and contractile responses to growth factors and vasoconstrictor hormones [1,2]. Activation of both secretory and cytosolic forms of phospholipase A_2 (PLA_2) in response to such agonists results in the release of arachidonic acid and the production of cyclo-oxygenase, lipoxygenase and cytochrome *P*-450 eicosanoid metabolites with diverse vasoactive and inflammatory properties, which modulate glomerular function [3–5]. We and others have previously shown that both epidermal growth factor (EGF) and phorbol 12-myristate 13-acetate (PMA) activate a cytosolic PLA_2 activity in mesangial cells by a mechanism that appears to involve covalent modification, and we characterized the structural requirements within the EGF receptor for rapid activation of PLA_2 [6–9]. More recently, Clark and colleagues have isolated a form of cytosolic PLA_2 with a molecular mass of 110 kDa which has been termed $cPLA_2$ [10]. Murine and human cDNA clones encoding this enzyme were isolated and characterized [11]. These investigators also demonstrated phosphorylation of $cPLA_2$ in response to Ca^{2+}-mobilizing agonists, PMA and certain growth factors. This phosphorylation was correlated with increased enzymic activity, which could be observed within minutes of the receptor–ligand interaction [12].

In considering the pathobiology of inflammatory and proliferative renal disease, we sought to examine the longer-term regulation of $cPLA_2$ at the level of gene expression in mesangial cells. The results of these studies indicated that both EGF and PMA increased $cPLA_2$ mRNA accumulation. We also found that increased mRNA for $cPLA_2$ correlated with increased $cPLA_2$ enzymic activity in mesangial cells. Furthermore, we found that $cPLA_2$ gene expression could be super-induced after treatment with cycloheximide.

EXPERIMENTAL

Materials

Cell culture media, antibiotics, transferrin, insulin, selenium, glutamine, trace-element mixture and Albumax were obtained from Gibco BRL, Burlington, Ontario, Canada. Fetal-bovine serum was obtained from ICN Flow, Mississauga, Ontario, Canada. PMA, cycloheximide and actinomycin D were obtained from Sigma, St. Louis, MO, U.S.A. EGF was obtained from Boehringer Mannheim, Laval, Quebec, Canada.

Cell culture

Homogeneous cultures of rat renal glomerular mesangial cells were obtained and characterized as previously described [13]. All experiments described were performed with confluent rat mesangial cells between passages 20 and 40. Cells were maintained in culture in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal-bovine serum with 50 units/ml penicillin and 50 μg/ml streptomycin in 5% CO_2 in air at 37 °C. Cells were rendered quiescent before each experiment by first rinsing with PBS and then incubating for 24 h in a serum-free medium consisting of DMEM/Ham's F12 containing 10 μg/ml transferrin, 10 nM sodium selenite, 1 × trace-element mixture, 1 mg/ml Albumax, 0.1 unit/ml insulin and 1 mM glutamine. Mesangial cells remain viable in this medium for up to 72 h.

Determination of $cPLA_2$ gene expression by Northern blotting

After incubation in the serum-free medium noted above for 24 h, mesangial cells were incubated for various times in the

Abbreviations used: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; GAPD, glyceraldehyde phosphate dehydrogenase.

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absence or presence of EGF (100 nM) or PMA (1 μg/ml). Where indicated in the Results section, in some experiments cells were also incubated in the absence or presence of cycloheximide or actinomycin D. Concentrations of EGF and PMA were chosen after initial concentration–response experiments indicated that these concentrations elicited a consistent and robust increase in $cPLA_2$ mRNA accumulation over 24 h. After incubation, mesangial cells were washed three times with ice-cold PBS before being scraped directly into 0.3 ml of guanidinium thiocyanate. RNA was isolated as previously described [14]. A 20 μg portion of total RNA was loaded in each lane of a formaldehyde denaturing agarose gel, and after electrophoresis each gel was photographed with Polaroid 665 film. RNA was transferred for 16 h on to Gene Screen Plus membranes (NEN Research Products–DuPont, Boston, MA, U.S.A.) and cross-linked with u.v. light in Stratalinker 2400 (Stratagene, La Jolla, CA, U.S.A.). A murine $cPLA_2$ cDNA probe was generated by *Nco*I/*Sal*I digest of plasmid PMT-2 containing $cPLA_2$ cDNA [11]. Human glyceraldehyde phosphate dehydrogenase (GAPD) cDNA probe was generated by *Pst*I/*Bgl*I digestion of pBR322 containing GAPD cDNA. The cDNA probes were labelled by random priming with [^{32}P]dCTP (Amersham International, Oakville, Ontario, Canada). The blots were hybridized in Quickhyb (Stratagene) as per the manufacturers' instructions. The blots were washed in 1 × SSC (0.15 M NaCl/0.015 M sodium citrate)/0.5 % SDS at 55 °C. Autoradiograms of the blots were developed after exposure at −70 °C to Kodak X-O-Mat film using one intensifying screen. Scanning laser densitometry (Ultrascan; LKB, Bromma, Sweden) was employed to quantify signals on autoradiograms and the loading of RNA gels by scanning Polaroid negatives. Levels of $cPLA_2$ mRNA are expressed as relative absorbance (A_{633}) units determined in each blot by attributing to the control serum-free $cPLA_2$ mRNA signal a value of 1 unit.

Assay of PLA_2 activity

This was done as previously described [6,7]. Mesangial cells were grown to confluency on Corning 100 mm-diameter dishes. The cells were washed with sterile PBS and then placed in serum-free media as described above for a further 24 h. EGF was added for 1 h, 3 h, 6 h, 12 h and 24 h and 37 °C. Cell-free extracts were prepared, and the protein concentration of each extract was adjusted to 1 mg/ml before being added to ^{14}C-labelled arachidonoyl phosphatidylcholine substrate. Labelled arachidonic acid released was resolved by t.l.c., scraped into Beckman Ready Protein scintillation fluid and counted in a Beckman LS701 liquid-scintillation counter. Each assay was performed in triplicate. Results are expressed as a percentage increase over baseline PLA_2 activity at each time point.

Data analysis

Results are presented as means ± S.E.M. and were compared by Student's t test using Statview II (Abacus Concepts Inc.).

RESULTS

Incubation of serum-starved rat mesangial cells with either EGF or PMA over a 24 h period resulted in a time-dependent increase in $cPLA_2$ mRNA accumulation (Figure 1). Figure 1(a) is a representative Northern blot of RNA from PMA-stimulated mesangial cells showing increasing amounts of $cPLA_2$ mRNA with prolonged incubation. Figure 1(b) is the same blot re-hybridized with a human GAPD cDNA probe to demonstrate uniformity of RNA loading on the original gel. The $cPLA_2$

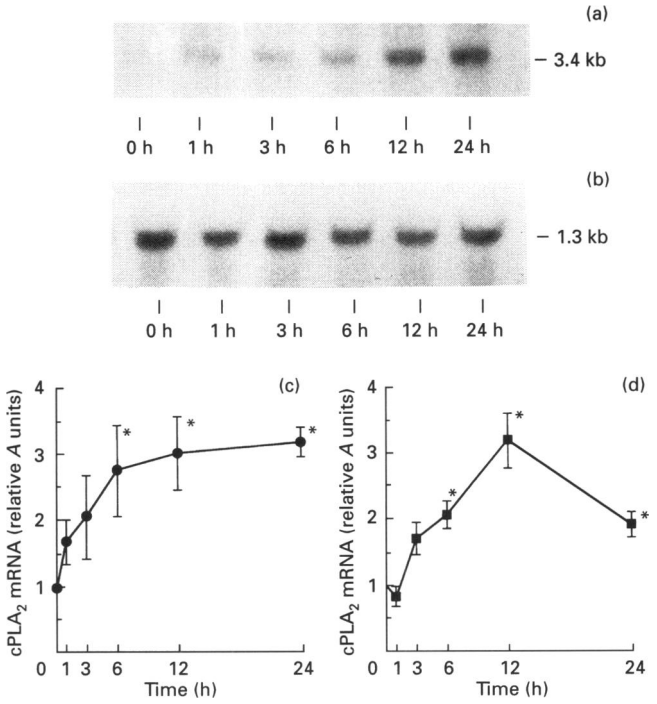

Figure 1 Time course for $cPLA_2$ mRNA response to PMA and EGF

Cells were serum starved then incubated with PMA or EGF for the time periods indicated beneath each lane. (**a**) Representative Northern-blot analysis of PMA-stimulated rat mesangial cells. Each lane was loaded with 20 μg of total RNA and hybridized as described in the Experimental section. (**b**) The same blot re-hybridized with a probe for GAPD. (**c**) and (**d**) Quantification of mesangial-cell $cPLA_2$ mRNA response to PMA (**c**) and EGF (**d**) by densitometry scanning. In each case, results are expressed as relative A units and represent the mean of 5 separate experiments normalized for RNA loading as described in the Experimental section.

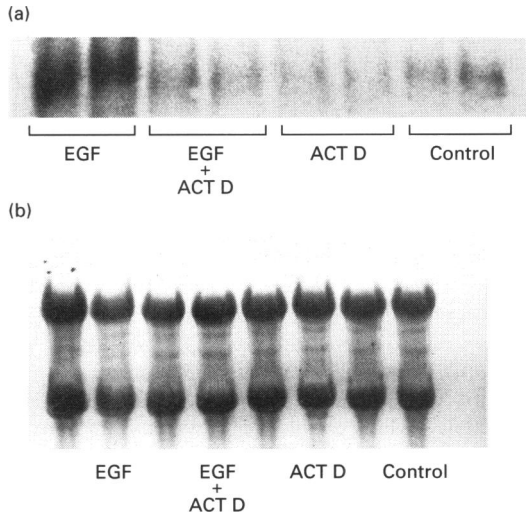

Figure 2 Effects of actinomycin D on $cPLA_2$ mRNA levels

(**a**) Incubation of serum-starved mesangial cells was carried out for 6 h with EGF alone (EGF), EGF with 6 μg/ml actinomycin D (EGF + ACT D), actinomycin D alone (ACT D) or vehicle (Control) and Northern-blot analyses were carried out as described in the Experimental section. Results of duplicate experiments for each incubation condition are shown. (**b**) Original RNA gel stained with ethidium bromide, demonstrating uniformity of RNA loading.

Table 1 Effect of EGF on PLA_2 activity in rat mesangial cells

Cytoplasmic PLA_2 activity is reported against duration of incubation with EGF measured by assay of arachidonate release in cell-free extracts from serum-starved mesangial cells. Values represent means ± S.E.M. for 15 determinations in five separate experiments: $^*P < 0.05$ versus 1 h.

Duration of incubation with EGF	PLA_2 activity (% of control)
1 h	145 ± 13
3 h	170 ± 11
6 h	182 ± 22
12 h	168 ± 22
24 h	329 ± 67*

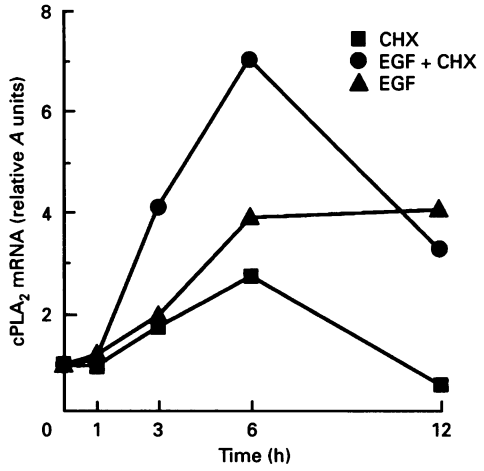

Figure 3 Effect of cycloheximide

After serum starvation, mesangial cells were incubated for the times indicated in the presence of 10 μg/ml cycloheximide alone (CHX; ■), EGF alone (EGF; ▲), or EGF plus cycloheximide (EGF + CHX; ●), and then Northern-blot analyses were performed as described in the Experimental section. Northern blots were quantified by densitometry (corrected for RNA loading), and each data point represents the mean of three results for each incubation condition and time point.

mRNA levels in response to PMA and EGF were maximal at 6 h and 12 h respectively, and increases were maintained to 24 h of incubation (Figures 1c and 1d). Actinomycin D treatment entirely abrogated the increase in $cPLA_2$ mRNA in response to EGF (Figure 2).

Measurements of PLA_2 activity were made over a 24 h period to establish the temporal relationship between agonist-induced increases in $cPLA_2$ mRNA and alterations in enzyme activity. In addition, experiments using cycloheximide were undertaken to determine if inhibition of protein translation altered the increase in $cPLA_2$ mRNA levels in response to EGF.

Table 1 demonstrates the increase in PLA_2 enzyme activity after prolonged incubation with EGF. PLA_2 enzyme activity increased to over 300% of control values after 24 h of incubation with EGF. Control values for PLA_2 enzyme activity were determined in mesangial cells which were incubated under identical serum-free conditions in the absence of EGF for the time period indicated.

Figure 3 illustrates the temporal effects of cycloheximide, EGF and the combination of EGF plus cycloheximide on $cPLA_2$ mRNA accumulation after incubation for up to 12 h with these agents. Cycloheximide alone resulted in an increase in $cPLA_2$ mRNA accumulation over baseline unstimulated serum-free conditions. Cycloheximide also significantly augmented the effect of EGF, with the maximum effect observed at the 6 h time point. At this time point, the mean absolute $cPLA_2$ mRNA levels (relative *A* units) were 2.7 for cycloheximide alone, 3.9 for EGF alone and 7.0 for the combination of EGF plus cycloheximide.

DISCUSSION

Signalling pathways for the release of arachidonic acid and its subsequent metabolism to a variety of eicosanoid products represents a subject of intensive investigation in a number of cell systems [4]. More recently, Asaoka et al. [15] have shown that lysophospholipids also serve as important factors for protein kinase C activation as well as in other transmembrane signalling pathways. We and others have shown in mesangial cells and other cell systems that PLA_2 activation represents the predominant pathway for the release of arachidonic acid and the formation of *sn*-2-position-deficient lysophospholipids [16,17]. Activation of both secretory and cytosolic PLA_2 activities in response to a variety of agonists has been described in mesangial cells [18,19]. We have previously described the rapid activation within minutes of a cytosolic phospholipase A_2 activity in response to EGF and PMA in mesangial cells [16]. More recently, Clark and colleagues and others have described a 110 kDa dithiothreitol-insensitive cytosolic PLA_2 that is activated by phosphorylation in response to a variety of agonists, and the enzyme responsible for this activity has been termed $cPLA_2$ [11,20,21]. Accordingly, we sought to determine whether long-term regulation of the expression of $cPLA_2$ in mesangial cells might also occur at the level of gene expression. Indeed, stimulation of mesangial cells with PMA or EGF resulted in a sustained increase in $cPLA_2$ mRNA over time. There was also a corresponding increase in cytosolic PLA_2 enzyme activity, indicating that agonist-induced increases in $cPLA_2$ mRNA was indeed reflected in functionally active enzyme. The delay between the maximal increase in $cPLA_2$ mRNA levels and subsequent increase in cytosolic PLA_2 activity likely reflects the requirement for both translation of the protein and subsequent post-translational modification of the enzyme. These results are consistent with recent reports which indicate that growth factors and mitogens can have long-lasting effects on cellular arachidonate release [22] and suggest that this effect may be mediated in part at the level of expression of the gene encoding $cPLA_2$. Further evidence for this mechanism is provided by the report that interleukin-1α induces the accumulation of cytosolic PLA_2 in human fibroblasts; however, in those latter studies mRNA levels were not determined [23].

The effect of EGF on $cPLA_2$ mRNA levels in mesangial cells is particularly intriguing, since we have previously shown no coupling of the activated EGF receptor to phospholipase C-γ in mesangial cells and hence no EGF-mediated increase in diacylglycerol or intracellular Ca^{2+} [8]. Accordingly, it will be of particular interest to further investigate the signalling mechanism involved in coupling of the receptor to $cPLA_2$ gene expression, independent of PKC and Ca^{2+} signalling.

It is notable that no effect of EGF on the expression of a gene encoding a secretory form of PLA_2 ($sPLA_2$) was noted in mesangial cells by other investigators [24]. Moreover, the present observations are also consistent with the finding in other cell systems that overexpression of $cPLA_2$, but not $sPLA_2$, results in an increase in growth-factor-stimulated arachidonate release

[12], and suggests that it is $cPLA_2$ which makes the major contribution to agonist-stimulated PLA_2 activity.

Changes in steady-state levels of $cPLA_2$ mRNA could potentially be mediated at a number of levels [25]. Transcriptional control elements for the promoter may have positive or negative effects on $cPLA_2$ gene expression, and changes in transcription-factor concentrations may regulate gene transcription. In addition, mRNA-processing events, including the rate of degradation of mRNA transcripts, may influence steady-state levels of $cPLA_2$ mRNA. In the present study, actinomycin D completely inhibited $cPLA_2$ mRNA accumulation in EGF-stimulated mesangial cells, consistent with an effect mediated at a transcriptional level. Incubation with cycloheximide caused a superinduction of steady-state $cPLA_2$ mRNA levels. Cycloheximide, a protein-synthesis inhibitor, has previously been shown to superinduce immediate early-response genes, including *c-fos*, *c-jun* and *c-myc* [26]. This superinduction is achieved by alteration in mRNA stability in transcripts with AUUUA repeats in the 3′-untranslated regions of the mRNA [26]. Indeed, in the 3′-untranslated region of human and murine $cPLA_2$ multiple conserved AUUUA sequences have been identified [11], and we have confirmed this for the rat $cPLA_2$ sequence as well (results not shown). Cycloheximide could potentially alter mRNA stability by a number of mechanisms, including inhibition of the translation of a labile RNAase or the coupling of degradation of mRNAs with AUUUA-rich sequences in their 3′-untranslated regions with active translation of the mRNA. Alternatively, the effect of cycloheximide may be mediated by inhibition of the translation of transcription factors which normally provide basal inhibition of $cPLA_2$ gene expression. Taken together, these results suggest that expression of the gene encoding $cPLA_2$ is tightly regulated to avoid over-production of eicosanoids, and this is consistent with the well-recognized regulation of the multiple enzymic steps involved in eicosanoid biosynthesis [27,28].

EGF has been implicated in the regulation of glomerular filtration *in vivo*, and the effects were entirely dependent on arachidonic acid metabolites [29,30]. EGF and EGF receptors have been localized to vascular elements of the human glomerulus [31], and urinary levels of other ligands for the EGF receptor have been found to rise in some forms of human glomerular injury [32]. Sellmayer et al. [33] have demonstrated that non-cyclo-oxygenase metabolites of arachidonate modulate the mesangial cell growth response to a number of vasoconstrictor hormones and growth factors. Taken together with recent reports implicating a role for growth factors in glomerular injury [34] and increases in PLA_2 activity in glomerular dysfunction in diabetes mellitus [35], the results of the present study suggest that up-regulation of $cPLA_2$ gene expression may participate in the glomerular response to injury.

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