Retinoic acid reduces p11 protein levels in bronchial epithelial cells by a posttranslational mechanism


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Retinoic acid reduces p11 protein levels in bronchial epithelial cells by a posttranslational mechanism. Am J Physiol Lung Cell Mol Physiol 279: L1103–L1109, 2000.—p11 is a member of the S100 family of proteins, is the cellular ligand of annexin II, and interacts with the carboxyl region of 85-kDa cytosolic phospholipase A2 (cPLA2), inhibiting cPLA2 activity and arachidonic acid (AA) release. We studied the effect of retinoic acid (RA) on PLA2 activity in human bronchial epithelial cells and whether p11 contributes to these effects. The addition of 10−6 M RA resulted in reduced p11 protein levels at 4 days, with the greatest effect observed on days 6 and 7. This effect was dose related (10−6 to 10−9 M). RA treatment (10−6 M) had no effect on cPLA2 protein levels. p11 mRNA levels were unchanged at 6 and 8 days of treatment (correlating with maximum p11 protein reduction). Treatment with RA reduced p11 levels in control cells and in cells transfected with a p11 expression vector, suggesting a posttranslational mechanism. Lactacystin (10−6 M), an inhibitor of the human 26S proteasome, blocked the decrease in p11 observed with RA treatment. Compared with control cells (n = 3), RA-treated cells (n = 3) had significantly increased AA release after treatment with the calcium ionophore A-23187 (P = 0.006). Therefore, RA reduces p11 protein expression and increases PLA2 activity and AA release. 

P11 IS A UNIQUE MEMBER of the S100 family of calcium-binding proteins and often forms a heterotetramer with 36-kDa annexin II. p11 interacts with the carboxy terminus of cytosolic phospholipase A2 (cPLA2), inhibiting cPLA2 activity and arachidonic acid (AA) release. Antisense inhibition of p11 mRNA results in enhanced cPLA2 activity and AA release without changing cPLA2 protein levels. Dexamethasone is known to reduce cPLA2 activity, and recent studies suggest that this effect may in part be mediated by upregulation of p11. These data provide evidence that p11 may have a physiological role in the modulation of cPLA2 activity.

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All-trans and 9-cis-retinoic acid (RA) bind to RA receptors (RAR and RXR, members of the steroid hormone nuclear receptor superfamily), regulating early development, tissue differentiation, adult reproduction, vision, and maintenance of numerous tissues (10). RA administration is used in the treatment of acute promyelocytic leukemia and may result in a syndrome of lung injury referred to as the RA pulmonary syndrome. The central role of AA metabolites in lung injury and airway inflammation argues for an improved understanding of the effects of RA on AA metabolic pathways in lung tissues.

Recent evidence suggests that RA modulates arachidonate metabolic pathways. A number of studies have demonstrated that the effects on arachidonate metabolic pathways appear to be dose, time, and tissue dependent. Hamasaki and colleagues (7) treated rat basophilic leukemia-1 (RBL-1) cells with RA for 16 h. Calcium ionophore stimulation resulted in significant increases in leukotriene C4 (LTC4) and LTD4 but no change in AA, 5-hydroxyeicosatetraenoic acid, or LTB4. Other authors have described RA induction of the cyclooxygenase-2 (COX-2) gene using a luciferase reporter construct (1). Hill and colleagues (8) examined RA effects on rat tracheal epithelial cells over a 23-day course. RA treatment resulted in rapid (24-h) down-regulation of COX-1 (PGHS-1) mRNA expression and a delayed (>3 days) increase in expression of cPLA2 and COX-2 protein and mRNA. Expression of cPLA2 coincided with differentiation from a squamous to mucociliary phenotype. PGE2 was the only eicosanoid formed and began to rise after 7 days in culture. Recent work by Hill and colleagues (9) in normal human tracheal bronchial cells demonstrated that cPLA2 protein levels were unchanged by RA treatment while 15-lipoxygenase and PGH synthase isoform expression were RA (and stage of differentiation) dependent. Whereas it appears that cPLA2 protein levels are stable during RA treatment, to our knowledge the effects of RA on human bronchial epithelium p11, cPLA2 activity, and arachidonate release have not been explored.

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We therefore sought to characterize the effects of RA on p11, cPLA₂, and AA release in the human bronchial epithelial cell line BEAS-2B. The effects of RA on p11 provide novel insight into posttranslational regulation of protein expression by RA and the mechanisms of disease states such as the RA pulmonary syndrome.

MATERIALS AND METHODS

Cell culture. The human bronchial epithelial cell line (BEAS-2B) was obtained from the American Type Culture Collection (Manassas, VA) and grown in 175-cm² flasks coated with rat-tail collagen in LHC-8 medium (Biosource, Rockville, MD). LHC-8 medium does not contain RA. Immediately on passage, the cells were treated with all-trans RA in DMSO (Biosource) (10⁻⁴ to 10⁻² M) or DMSO control for 3–8 days. Cells became 80–90% confluent by 8 days, and, therefore, the experiment was not continued beyond this time point. For time-course experiments, cells were treated with 10⁻⁶ M RA or DMSO control, the medium was changed at the same time, and all cells were harvested at the same time. Time-course experiments were restricted to a single cell passage.

Effect of RA treatment on normal human bronchial epithelial cells. Normal human bronchiolar epithelial cells (Clonetech, San Diego, CA) were grown in LHC-8 medium and treated with all-trans RA in DMSO (Biosource; 10⁻⁴ to 10⁻⁹ M) or DMSO control for 4–8 days. RA (10⁻⁵ M) proved cytotoxic to these cells, and, therefore, data are presented for 10⁻⁶, 10⁻⁷, and 10⁻⁹ M.

Immunoblot of p11, cPLA₂, and actin proteins. At the indicated harvest times, cells were rinsed two times with cold PBS and detached from the flasks with trypsin (E-PET, Biosource) followed by trypsin inhibitor (SBTI, Biosource) and gentle scraping. After being washed three times, cells were transferred to 0.4 ml of homogenization buffer: 50 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM EGTA, 100 µM leupeptin, 1 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, 0.5 mM soybean trypsin inhibitor, 15 mM aproargin, and 0.5% Triton X-100. Cells were then sonicated twice for 15 s and centrifuged at 14,000 rpm for 10 min. Total protein in the supernatant was assayed using the bicinchoninic acid reagent.

To test the role of the ubiquitin-proteasome pathway in retinoate-induced p11 protein levels after RA treatment. To test the role of the ubiquitin-proteasome pathway in retinoate-induced p11 protein reduction, BEAS-2B cells were treated with all-trans RA in DMSO (Biosource; 10⁻⁶ and 10⁻⁷ M) or DMSO control for 6 days. The cells were then treated with and without lactacystin (Calbiochem; 10⁻⁶ and 10⁻⁷ M), an inhibitor of the human 26S proteasome, for 8 h.

AA release from RA-treated cells. BEAS-2B cells were grown on 150-mm rat tail collagen-coated culture flasks in the presence (n = 6) and absence (n = 6) of 10⁻⁶ M RA for 7 days. Cells were labeled on day 5 for 24 h with 1 µCi/ml [5,6,8,9,11,12,14,15-³H]AA ([³H]AA; 214 Ci/mmol; Amer sham) in LHC-8 medium with and without RA. On day 6, the medium was removed, cells were washed three times, and cellular AA was extracted from the 175-cm² culture flasks by the single-step guanidinium thiocyanate-phenol-chloroform extraction method (TRI Reagent, Molecular Research, Cincinnati, OH). The RNA pellet was precipitated with isopropanol, washed with 70% ethanol, and redissolved in diethyl pyrocarbonate water. To construct the probe for p11 mRNA, a 320-bp product of p11 cDNA was amplified by PCR using the following set of primers: 5’ primer, 5’-ACCAAC-CAAAATGGCATCTCT-3’ (bases 101–121 of Genbank accession number M38591), and 3’ primer, 5’-CTGCTCATTTCT-GCTACTT-3’ (bases 400–419; Genosys Biotechnologies, The Woodlands, TX). The product for p11 was cloned into the pGEM-T Easy Vector (Promega, Madison, WI). Proper orientation of the insert was confirmed by DNA sequencing. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA probe was prepared by in vitro transcription using T7 polymerase with [α-³²P]CTP. The p11 RNA probe was prepared by in vitro transcription using SP6 polymerase with [α-³²P]CTP. The ribonuclease protection assay (RPAl; Ambion, Austin, TX) was performed. Hybridization was performed at 45°C for 16 h, and 10 µg (for GAPDH) or 20 µg (for p11) of total RNA. For GAPDH 10⁴ dpm and for p11 2 × 10⁴ dpm of [³²P]-labeled RNA probe were used. After hybridization, the unhybridized RNA was digested by addition of 1:100 diluted RNaseA/T1 mix at 37°C for 60 min. Digestion was terminated by the addition of RNase inactivation and precipitation mixture. The protected RNA fragment was analyzed by autoradiography after separation on 6% polyacrylamide-8 M urea gels (Novex).

Stable transfection of a p11 expression plasmid in BEAS-2B cells. cDNA containing the coding region of the p11 gene was cloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen, Carlsbad, CA) to create p11 pcDNA3.1. The identity and orientation of the construct were confirmed by DNA sequencing. The pcDNA3.1 (+) vector carries the human cytomegalovirus (CMV) immediate-early enhancer-promoter sequences to promote constitutive expression of the cloned p11 insert in mammalian cells. The BEAS-2B cells grown in 175-cm² flasks were exposed to 120 µl of LipofectAMINE reagent (Life Technologies, Gaithersburg, MD) with 20 µg of p11-pcDNA3.1 (+) plasmid. Control cells were transfected with pcDNA3.1 (+) expression plasmid alone. Cells were exposed to the mixture of LipofectAMINE and plasmid for 4 h. After removal of the transfection reagent, fresh LHC-8 medium and 1,000 µg/ml genetin (G418 sulfate; Calbiochem, La Jolla, CA) were added to the cells. Subsequent cultures of selected BEAS-2B cells were routinely grown in the presence of selective pressure. Transfected BEAS-2B cells were cloned by limiting dilution, and clones were used for Western blot and RA treatment after four passages.

Effect of 26S proteasome inhibition with lactacystin on p11 protein levels after RA treatment. To test the role of the ubiquitin-proteasome pathway in retinoate-induced p11 protein reduction, BEAS-2B cells were treated with all-trans RA in DMSO (Biosource; 10⁻⁶ and 10⁻⁷ M) or DMSO control for 6 days. The cells were then treated with and without lactacystin (Calbiochem; 10⁻⁶ and 10⁻⁷ M), an inhibitor of the human 26S proteasome, for 8 h.
fresh medium with and without RA was added. On day 7, the medium was collected for analysis of basal AA release. Cells were then washed three times and studied for AA release after calcium ionophore stimulation. LHC-8 medium (12 ml) with the calcium ionophore A-23187 (10^{-6} M; Calbiochem) was added to each flask, and the cells were incubated at 37°C for 30 min. The supernatant was harvested for HPLC analysis. The samples for HPLC analysis were extracted by octadecylsilane C_{18} cartridges (Sep-Pak C_{18}; Waters Associates, Milford, MA) and analyzed by reverse-phase HPLC. Individual Sep-Pak C_{18} cartridges were prepared with 15 ml of methanol followed by 5 ml of 5 mM EDTA and 10 ml of water. Samples were loaded onto the cartridges, washed with 10 ml of water, and eluted with 4 ml of methanol. The methanol fraction was collected and evaporated to dryness under steady-flow nitrogen gas and resuspended in 200 μl of methanol for analysis by HPLC. An ultrasphere C_{18} column (4.7 × 250 mm; Beckman Instruments, Fullerton, CA) with 5-μm particle size was used. A gradient program was used with mobile phase A, water-acetonitrile-phosphoric acid (75:25:0.025), and mobile phase B, methanol-acetonitrile-trifluoroacetic acid (60:40:0.0016), at a flow rate of 1.5 ml/min. The AA fraction of HPLC elution was collected and measured for radioactivity. Basal and stimulated AA release into supernatants was also quantified by scintillation counting (Beckman LS 6500).

RESULTS

RA reduces p11 protein levels in BEAS-2B cells and normal human bronchiolar epithelial cells. p11, cPLA2, p36 (annexin II heavy chain), and actin were constitutively expressed in untreated BEAS-2B cells and normal human bronchiolar epithelial cells. The addition of 10^{-6} M RA to BEAS-2B cells (n = 5) resulted in significantly reduced p11 protein levels at 5 days, with a consistent effect observed on days 5–8 (Fig. 1, A and B; n = 5). RA treatment (10^{-6} to 10^{-9} M) demonstrated a dose-related reduction in p11 on days 5–8 (Fig. 1, C and D, shows results for day 6; n = 5, P < 0.001 for a dose-effect by one-way ANOVA). RA proved cytotoxic to normal human bronchiolar epithelial cells at a dose of 10^{-6} M but reduced p11 protein levels without cytotoxicity at a dose of 10^{-7} M (Fig. 2; n = 4). Actin levels were stable during RA treatment in both BEAS-2B and normal human bronchiolar epithelial cells (Fig. 2 shows data for normal human bronchiolar epithelial cells; n = 4).

RA did not change cPLA2 or p36 protein levels in BEAS-2B cells. The addition of 10^{-6} to 10^{-9} M RA had no consistent effect on cPLA2 or p36 protein levels from 3 to 8 days. Figure 3 shows an immunoblot of cPLA2 protein levels on days 6–8 using an anti-cPLA2 mouse monoclonal antibody (n = 5). Similar results were obtained with a rabbit polyclonal antibody (Genetics Institute; n = 5). p36 protein levels demonstrated no consistent changes with RA treatment (n = 5; data not shown).

RA does not affect p11 mRNA levels. Because p11 protein levels were reduced by treatment with RA, steady-state levels of p11 mRNA were studied. p11 mRNA levels were determined by RPA after 6–8 days of treatment and compared with GAPDH mRNA controls (n = 7). There was no change in p11 or GAPDH mRNA levels at any of these time points, correlating with maximum p11 protein reduction; (Fig. 4 shows representative data from days 6 and 8).
p11 levels are reduced with RA treatment in cells stably transfected with a p11 expression vector. Because there was no apparent change in p11 mRNA levels, posttranslational control of p11 protein was studied in two ways. First, BEAS-2B cells were transfected with a p11 expression vector under the control of a CMV promoter. These cells demonstrated increased p11 protein levels by Western blotting. Six days of RA treatment (10^{-6} M) reduced p11 protein levels in both the p11 expression vector-containing cells and in vector control cells (Fig. 5; n = 4).

Pharmacological inhibition of the ubiquitin-proteasome pathway increases p11 protein levels in RA-treated cells. To further study the posttranslational control of p11 by RA, the ubiquitin-proteasome pathway of protein degradation was inhibited. An 8-h exposure with 10^{-7} M lactacystin, an inhibitor of the human 26S proteasome, increased basal and RA-treated p11 protein levels (6 days RA treatment at 10^{-6} and 10^{-7} M; Fig. 6; n = 5).

RA increased basal and calcium ionophore-stimulated AA release from BEAS-2B cells. To determine whether the observed changes in p11 protein levels had physiological consequences, PLA2 activity during RA treatment of BEAS-2B cells was investigated. Cells were labeled on day 5 for 24 h with [3H]AA in LHC-8
medium with and without $10^{-6}$ M RA. On day 6, the medium was removed, cells were washed three times, and fresh medium with and without RA was added. On day 7, medium was collected for analysis of basal AA release, demonstrating a 29% increase ($P < 0.001$) in AA release from RA-treated cultures. The mean count in controls was 40,882 ± 1,548 dpm ($n = 6$). The mean count in RA-treated cells ($n = 6$) was 52,578 ± 1,579 dpm (Fig. 7A). Cells were then washed three times and studied for AA release (30-min collection) before and after calcium ionophore stimulation. Compared with control cells ($n = 3$), RA-treated BEAS-2B cells ($n = 3$) had significantly increased AA release over 30 min as measured by HPLC separation and scintillation counting ($P = 0.03$). This effect was magnified in cells exposed to the calcium ionophore A-23187 ($P = 0.006$; Fig. 7B).

**DISCUSSION**

All-trans and 9-cis-RA regulate early development, tissue differentiation, and maintenance of numerous tissues, including the bronchial epithelium. RA appears to modulate arachidonate metabolic pathways in the pulmonary epithelium in a tissue-, dose-, and time-dependent manner. The central role of AA metabolites in lung injury and airway inflammation, coupled with the role of retinoates in the maintenance of bronchial epithelial cell growth and differentiation, argues for an improved understanding of the effects of retinoate on AA metabolic pathways in lung tissues. We therefore sought to characterize the effects of RA on p11, cPLA2, and AA release in the human bronchial epithelial cell line BEAS-2B.

We report here that p11 protein expression is reduced with RA treatment, whereas cPLA2, p36, and
actin protein levels appear unchanged. The change in p11 protein levels is accompanied by an increase in AA release. The stability of cPLA<sub>2</sub> protein levels in the setting of significantly augmented AA release suggests that p11 depletion may reduce p11 protein-cPLA<sub>2</sub> interactions and upregulate cPLA<sub>2</sub> activity. These results are consistent with two previous observations about the effect of p11 protein on cPLA<sub>2</sub> activity. First, it has been shown that antisense inhibition of p11 increases AA release with no change in cPLA<sub>2</sub> protein levels (16) and that dexamethasone-driven reductions in cPLA<sub>2</sub> activity are associated with increases in p11 levels, with stable cPLA<sub>2</sub> protein levels (17). Second, although it is known that p11 forms a heterotetramer with p36, p11 alone can downregulate cPLA<sub>2</sub> activity (15). These data argue that p11 has a role in the protein-protein interactions that reduce PLA<sub>2</sub> activity, consistent with the observed isolated p11 reduction with RA treatment and accompanying increase in AA release.

Retinoids typically exert their biological effect at the transcriptional level by binding to the nuclear receptors of the superfamily of steroid-thyroid hormone nuclear receptors (RA receptor and retinoid X receptor) or by antagonizing the activator protein-1 transcription factor (2, 12). Therefore, we first measured p11 mRNA levels to determine whether these were reduced with RA treatment. However, RPAs demonstrated that mRNA levels were stable during RA treatment, suggesting that the mechanism of reduction of p11 protein appears to be posttranslational. This is further supported by p11 expression vector experiments. A cDNA containing the coding region of the p11 gene was cloned into a mammalian expression vector carrying the human CMV immediate-early enhancer-promoter sequences to promote constitutive expression of the cloned p11 insert. BEAS-2B cells transfected with this construct have elevated p11 protein levels, and these levels are reduced with RA treatment. Because the p11 expression vector used is controlled by a CMV promoter, we believe that it would be expressed at a consistently high level and not be transcriptionally regulated in BEAS-2B cells. Thus the observed change in p11 protein levels in these transfected cells during RA treatment likely represents a posttranscriptional or posttranslational effect.

Posttranslational regulation of most proteins in eukaryotic cells occurs via the ATP-dependent ubiquitin-proteasome pathway. Target proteins are conjugated to multiple ubiquitin polypeptides, marking them for hydrolysis by the 26S proteasome complex (19S particle and 20S proteasome) (6). Lactones such as the antibiotic lactacystin bind to the terminal threonine on the β-subunit of mammalian proteasomes, specifically and irreversibly inhibiting this complex (3). The observation presented here, that the reduction in p11 protein levels observed with RA treatment can be reversed with proteasome inhibition, suggests that RA treatment increases proteasome pathway protein degradation of p11. Furthermore, basal p11 levels increase with proteasome inhibition, suggesting that p11 is under steady-state posttranslational control by the ubiquitin-proteasome pathway. Similar effects of RA on this pathway were recently reported by Langenfeld and colleagues (11), who described that cyclin D1, a cyclin-dependent kinase, is degraded by the ubiquitin-proteasome pathway during RA treatment. The RA effects on p11 provide further insight into posttranslational regulation of protein expression by RA.

The critical role of cPLA<sub>2</sub> and p11 in the regulation of AA release has implications for a number of human diseases. AA metabolites have a central role in asthma, lung inflammation and injury, and cardiovascular disease. Supraphysiological levels of RA are used for the treatment of acute promyelocytic leukemia and may result in a syndrome of lung injury referred to as the RA pulmonary syndrome (4, 5, 13). Treatment with prednisolone, a corticosteroid, has been shown to prevent this syndrome (14). The data presented here that p11 modulates AA release during RA exposure and previous data that dexamethasone increases p11 protein levels provide a possible mechanism for this disease and the beneficial effect of dexamethasone prophylaxis. Further research targeting the mechanism of RA effects on ubiquitin-proteasome pathway-mediated p11 degradation may lead to novel anti-inflammatory therapies.

REFERENCES


