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Pyrrolidine dithiocarbamate differentially affects cytokine- and cAMP-induced expression of group II phospholipase A_2 in rat renal mesangial cells

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Abstract Renal mesangial cells express group II phospholipase A_2 in response to two principal classes of activating signals that may interact in a synergistic fashion. These two groups of activators comprise inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) and agents that elevate cellular levels of cAMP such as forskolin, an activator of adenylate cyclase. Using pyrrolidine dithiocarbamate (PDTC), a potent inhibitor of nuclear factor NFκB, we determined its role in cytokine – and cAMP – triggered group II PLA_2 expression. Micromolar amounts of PDTC suppress the IL-1β- and TNFα-dependent, but not the forskolin-stimulated group II PLA_2 activity in mesangial cells. Furthermore, PDTC inhibited the increase of group II PLA_2 mRNA steady state levels in response to IL-1β and TNFα, while only marginally affecting forskolin-induced PLA_2 mRNA levels. Our data suggest that NFκB activation is an essential component of the cytokine signalling pathway responsible for group II PLA_2 gene regulation and that cAMP triggers a separate signalling cascade not involving NFκB. These observations may provide a basis to study the underlying mechanisms involved in the regulation of group II PLA_2 gene expression.

Key words: Phospholipase A_2; Interleukin-1; Cyclic AMP; Nuclear factor B; Mesangial cell

1. Introduction

Phospholipase A_2 (PLA_2) comprises a group of lipolytic enzymes that specifically release fatty acids, often arachidonic acid, from the sn-2 position of membrane phospholipids for production of important lipid mediators such as eicosanoids and platelet activating factor [1,2]. It has become evident that PLA_2s are a heterogenous family of enzymes that can be classified into two classes. A class of high molecular weight PLA_2s of 60–110 kDa, also referred to as cytosolic PLA_2s or $cPLA_2$s, and a class of low molecular weight PLA_2s of 14 kDa referred to as secretory PLA_2s or $sPLA_2$s. Members of both classes from human and rat sources have recently been cloned [2]. Based on their primary structure, the $sPLA_2$s are further classified into two groups [3]. Mammalian group I PLA_2 comprises the pancreatic type of PLA_2 and mammalian group II PLA_2 is synthesized and secreted from many cell types and is believed to play a role in the initiation and propagation of inflammatory processes (for review see [4]).

Two potent proinflammatory cytokines, IL-1β and TNFα, have been shown to induce the synthesis and secretion of group II PLA_2 in rabbit and rat chondrocytes [5,6], human synovial cells [7], rat mesangial cells [8,9], MDCK cells [10] and other cell systems [2]. The cytokine effect is blocked by actinomycin D and cycloheximide, thus demonstrating a requirement for both transcription and de novo protein synthesis. This is confirmed by the observation that IL-1β and TNFα increase the level of mRNA for group II PLA_2 in rabbit articular chondrocytes [11,12], rat astrocytes [13], rat mesangial cells [14,15], rat vascular smooth muscle cells [16] and human hepatoma cells [17]. Recently, we demonstrated an increased secretion of group II PLA_2 in rat vascular smooth muscle cells [18] and rat mesangial cells [19,20] in response to cAMP-elevating agents. Furthermore, forskolin, an activator of adenylate cyclase, synergistically interacts with IL-1β to increase group II PLA_2 mRNA levels in mesangial cells [21].

At present, little is known about the mechanism(s) involved in the upregulation of group II PLA_2 mRNA levels. In addition, the nuclear factor(s) which may be important for the regulation of the group II PLA_2 gene are poorly defined. In the present study we have used pyrrolidine dithiocarbamate, an inhibitor of NFκB activation, to determine whether NFκB is required for cytokine- and cAMP- induced group II PLA_2 gene transcription.

2. Materials and methods

2.1. Cell culture

Rat mesangial cells were cultured as described previously [22]. In a second step, single cells were cloned by limited dilution using 96-microwell plates. Clones with apparent mesangial cell morphology were used for further processing. The cells exhibited the typical stellate morphology. Moreover, there was positive staining for the intermediate filaments desmin and vimentin, which are considered to be specific for myogenic cells, positive staining for Thy 1.1 antigen, negative staining for the factor VIII-related antigen and cytokeratin, excluding endothelial and epithelial contamination, respectively. The generation of inositol trisphosphate upon activation of the angiotensin II AT_1 receptor was used as a functional criterion for characterizing the cloned cell line. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μ/ml) and bovine insulin at 0.66 units/ml (Sigma). For the experiments passages 6–16 of mesangial cell were used.

2.2. Phospholipase A_2 assay

Phospholipase A_2 activity was determined with [1-^{14}C]oleate-labelled *Escherichia coli* as substrate as described previously [23]. The substrate was prepared by growing *E. coli* in the presence of [1-^{14}C]oleate, followed by autoclaving to inactivate endogenous phospholipases. Over 95% of the label incorporated by *E. coli* was in phospholipid and, as demonstrated by hydrolysis with snake venom (*Crotalus adamanteus*),

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more than 95% of the [1-^{14}C]oleate was in the sn-2 position of the phospholipids. Assay mixtures (1.0 ml) contained 100 mM Tris-HCl buffer (pH 7.0), 1 mM $CaCl_2$, 2.5×10^8 [1-^{14}C]oleate-labelled *E. coli* (5 nmol of phospholipids, 5000–8000 cpm) and the enzyme to be tested at a dilution producing 5% substrate hydrolysis. Reaction mixtures were incubated for 1 h at 37° C in a shaking water bath. The reaction was stopped by the addition of 5 ml of propan-2-ol/*n*-heptan/1 M H_2SO_4 (40:10:1 v/v) followed by 2 ml of heptane and 3 ml of water. After vigorous shaking and phase separation, a portion (2.5 ml) of the upper phase was passed over a column of silicic acid (3.5 × 0.5 cm). Free [1-^{14}C]oleic acid was quantitatively eluted with 1 ml of ethylacetate. Radioactivity was determined in a scintillation counter. Phospholipase A_2 activity is expressed as [1-^{14}C]oleate radioactivity released by 100 μl of cell culture supernatant.

2.3. Northern blot ananlysis

Confluent mesangial cells were washed twice with PBS and incubated in DMEM, supplemented with 0.1 mg/ml of fatty acid-free bovine serum albumin, with or without agents for the indicated time periods. Cells were washed twice with PBS and harvested using a rubber policeman. Total cellular RNA was extracted from the cell pellets using the guanidinium thiocyanate/cesium chloride method [24]. Samples of 20 μg RNA were separated on 1% agarose gels containing 0.66 M formaldehyde prior to the transfer to gene screen membranes (New England Nuclear). After UV-crosslinking and prehybridization for 2 h, the filters were hybridized for 16–18 h to a ^{32}P-labelled *Eco*RI/*Hin*dIII-cDNA insert from p139-1 coding for rat group II phospholipase A_2 [25]. To correct for variation in RNA amount, the PLA_2 probe was stripped with boiling 0.1 × SSC/1% SDS and the blots were rehybridized to the ^{32}P-labelled *Eco*RI genomic DNA-fragment coding for 28S ribosomal RNA or the *Bam*HI/*Sal*I cDNA insert from clone pEX 6 coding for human β-actin. DNA-probes ($0.5–1 \times 10^6$ dpm/ml) were radioactively labelled with [^{32}P]dATP by random priming (Boehringer-Mannheim). Hybridization reactions were performed in 50% (v/v) formamide, 5× SSC, 5× Denhardt's solution, 1% (w/v) SDS, 10% (w/v) dextran sulfate and 100 μg/ml salmon sperm DNA. Filters were washed 3 times in 2× SSC/0.1%SDS at room temperature for 15 min, and then 2 times in 0.2× SSC/1% SDS at 65°C for 30 min. Filters were exposed for 24–48 h to Kodak X-Omat XAR-film using intensifying screens.

2.4. Chemicals

Recombinant human IL-1β was generously supplied by Dr. Klaus Vosbeck, Ciba-Geigy Ltd., Basel, Switzerland; recombinant human TNFα was from Boehringer-Mannheim. The genomic clone coding for 28S ribosomal RNA was kindly provided by Dr. Thomas Geiger, Ciba-Geigy Ltd., Basel, Switzerland; the cDNA-clone pEX 6, coding for human β-actin was a gift from Dr. U. Aebi, Basel, Switzerland. PDTC was from Sigma, Buchs, Switzerland; forskolin was from Calbiochem, Lucerne, Switzerland. [1-^{14}C]Oleic acid and [^{32}P]dATP (specific activity 3000 Ci/mmol) were from Amersham, Dübendorf, Switzerland. Nylon membranes (Gene Screen) were purchased from DuPont de Nemours Int., Regensdorf, Switzerland. Cell culture media and nutrients were from Gibco BRL, Basel, Switzerland and other chemicals used were either from Merck, Darmstadt, Germany or Fluka, Basel, Switzerland.

3. Results

Incubation of mesangial cells for 24 h with either IL-1β (1 nM), TNFα (1 nM) or forskolin (10 μM) increased PLA_2 activity secreted by mesangial cells as shown in Table 1. Stimulation with IL-1β increased PLA_2 activity about 13-fold, whereas TNFα was less potent, enhancing PLA_2 activity only about 5-fold. Forskolin induced PLA_2 activity by about 4-fold. The increase in PLA_2 activity is preceeded by an increase in steady state mRNA levels for $sPLA_2$ as shown in the time course experiments in Fig. 1. Mesangial cells were incubated for various time points ranging from 2 h up to 24 h with IL-1β (1 nM), TNFα (1 nM) or forskolin (10 μM), respectively. Total cellular RNA was extracted and examined by Northern blot analysis. Stimulation with IL-1β or TNFα (1 nM) resulted in a rapid accumulation of group II PLA_2 mRNA levels detectable already 2–4 h post-stimulation (Fig. 1A and B). Upon incubation of mesangial cells with forskolin (10 μM) group II PLA_2 mRNA levels could be detected already at 2 h after stimulation (Fig. 1C). Simultaneous incubation with NFκB inhibitor PDTC dose-dependently inhibited PLA_2 activity in response to IL-1β and TNFα, but not to forskolin (Table 1). Half-maximal inhibition of IL-1β- and TNFα-stimulated PLA_2-activity was observed at about 39 μM and 46 μM of PDTC, respectively. In contrast, forskolin-induced PLA_2 activity was virtually not affected by PDTC up to concentrations of 100 μM (Table 1). In order to determine whether the inhibition of the IL-1β and TNFα-dependent stimulation by PDTC was due to the down-regulation of group II PLA_2 steady-state mRNA levels, we performed Northern blot analysis. Total cellular RNA was isolated from mesangial cells after coincubation with IL-1β (1 nM), TNFα (1 nM) or forskolin (10 μM) in the presence of 100 μM PDTC. The data in Fig. 2 demonstrate down-regulation of group II PLA_2 mRNA levels upon simultaneous incubation of cells with cytokines and PDTC. Coincubation of mesangial cells with IL-1β (Fig. 2A) or TNFα (Fig. 2B) with PDTC causes an almost complete suppression of the induction of group II PLA_2 mRNA levels after 6 h (lanes 4 in Fig. 2A and B) or results in a drastically reduced induction after a 12 h coincubation period (lanes 6 in Fig. 2A and B). In contrast, PDTC did not or only sligthly attenuate group II PLA_2 mRNA increase observed after stimulation of cells with forskolin (Fig. 2C).

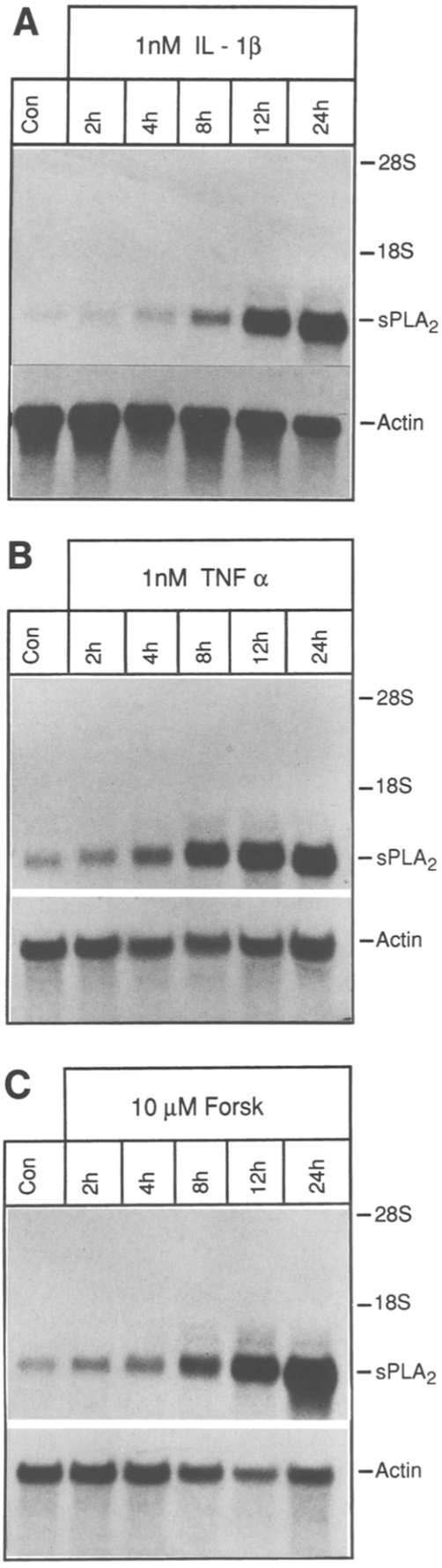
Table 1
Effects of PDTC on IL-1β, TNFα and forskolin-stimulated group II PLA_2 secretion from mesangial cells

Addition		PLA_2 secretion (cpm/100 μl)
Control		441 ± 31
PDTC (100 μM)		614 ± 38
IL-1β (1 nM)		5749 ± 262
TNFα (1 nM)		2088 ± 148
Forsk (10 μM)		1611 ± 75
IL-1β (1 nM)	+ PDTC (100 μM)	1260 ± 156***
IL-1β (1 nM)	+ PDTC (50 μM)	1751 ± 91***
IL-1β (1 nM)	+ PDTC (20 μM)	4785 ± 175*
TNFα (1 nM)	+ PDTC (100 μM)	622 ± 53***
TNFα (1 nM)	+ PDTC (50 μM)	939 ± 21***
TNFα (1 nM)	+ PDTC (20 μM)	1720 ± 150
Forsk (10 μM)	+ PDTC (100 μM)	1546 ± 140
Forsk (10 μM)	+ PDTC (50 μM)	1568 ± 282
Forsk (10 μM)	+ PDTC (20 μM)	1823 ± 83

Confluent mesangial cells were incubated with the indicated concentrations of IL-1β, TNFα, forskolin (Forsk) and PDTC for 24 h. Thereafter, the medium was withdrawn and PLA_2 activity determined as described in section 2. Results are means ± S.D. ($n = 4$). Significant differences from the corresponding control stimulation in the absence of PDTC: $^*P < 0.05$; $^{***}P < 0.001$, by Student's *t*-test.

4. Discussion

Potent proinflammatory cytokines, such as IL-1β and TNFα, have been found to increase group II PLA_2 gene expression in various cell-types [11–17]. We were the first to report that



cytokines such as IL-1β or TNFα as well as cAMP-elevating compounds are capable of inducing the synthesis and release of group II PLA_2 in cultured rat mesangial cells [8,9,19,20]. We have recently shown that IL-1β dose-dependently increases group II PLA_2 mRNA levels in mesangial cells and that forskolin synergistically interacts with IL-1β to increase group II PLA_2 mRNA steady state levels [21]. These data suggest that there exist at least two distinct activation mechanisms for the induction of group II PLA_2, one is activated by cAMP and the other is triggered by cytokines such as IL-1β.

Little is known about the underlying mechanism(s) of the up-regulation of group II PLA_2 mRNA levels and about the transcription factor(s) which may be involved in the regulation of the group II PLA_2 gene. The control of transcription in response to inflammatory cytokines has been intensively studied, and it has become clear that a number of ubiquitous transcription factors are involved in many different cellular systems. Very prominent under these factors is NFκB a multisubunit transcription factor that is activated in response to IL-1β, TNFα, bacterial endotoxin and various other stimuli and plays a pivotal role in the development of the cellular immune and inflammatory responses [26]. NFκB is constitutively present in cells as a heterodimer, consisting of a p50 DNA-binding subunit and a Rel A (p65) transactivating subunit. This NFκB complex is maintained in a latent cytoplasmic form by the physical association with an inhibitor IκB [26]. The mechanism leading to NFκB activation in not fully defined, but phosphorylation and degradation of IκB seem to be the important steps in the activation process which causes dissociation of IκB from NFκB and nuclear translocation of NFκB with subsequent specific gene transcription [26]. In order to further clarify the basic mechanism of the induction of group II PLA_2 in mesangial cells by IL-1β, TNFα and cAMP, we evaluated whether the activation of NFκB participates in this process. For our experiments we took advantage of the fact that pyrrolidine dithiocarbamate efficiently suppresses the activation of NFκB in stimulated cells, while leaving the DNA-binding capacity of other transcription factors such as Sp-1, Oct 1 and CREB unaffected [27]. Expression of the group II PLA_2 gene by IL-1β and TNFα is strongly suppressed by PDTC in a dose-dependent fashion (Fig. 2A and B), suggesting that NFκB – probably in cooperation with other transcription factors – is involved in the regulation of the group II PLA_2 gene transcription in mesangial cells. In contrast , cAMP-triggered expression of the group II PLA_2 gene is not affected by PDTC, indicating that cAMP activates other nuclear factors, most probably CREB, AP-2 or related proteins. This is particularly important because it has been shown for human myeloid cells that cAMP can activate NFκB [28]. The gene coding for rat group II PLA_2 has been cloned [29–31] and 497 bp of the putative 5′ flanking region have been sequenced [31]. Its involvement in the regulation of the group II PLA_2 gene by functional analysis remains to be demonstrated. However, screening of the proximal region of the potential promoter

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Fig. 1. Time course of induction of group II PLA_2 mRNA in mesangial cells following stimulation with IL-1β, TNFα and forskolin. Mesangial cells were incubated with IL-1β (1 nM) (A), TNFα (1 nM) (B) or forskolin (10 μM) (C) for the indicated time periods. Total cellular RNA (20 μg) was hybridized successively to ^{32}P-labelled group II PLA_2 and β-actin cDNA-probe as described in section 2.

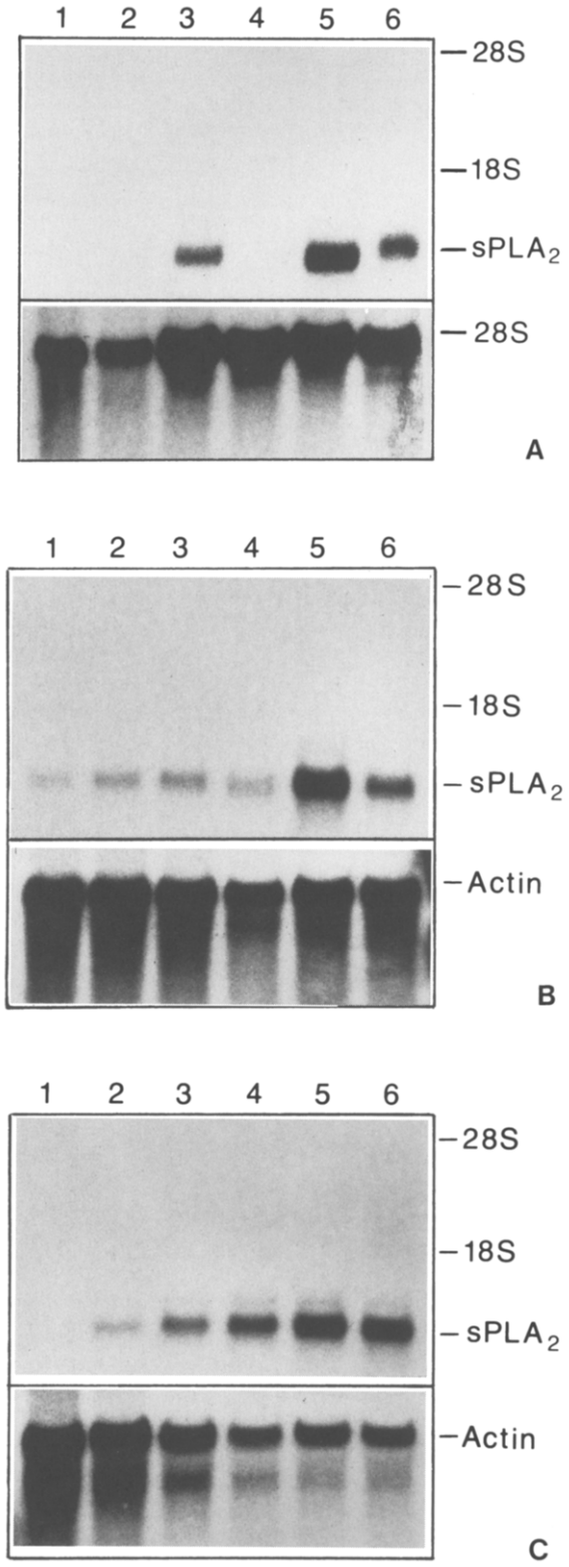

Fig. 2. (A) Effects of PDTC on IL-1β-stimulated group II PLA_2 mRNA accumulation. Mesangial cells were incubated for 6 h with vehicle (control) (1), PDTC (100 μM) (2), IL-1β (1 nM)(3), IL-1β (1 nM) plus PDTC (100 μM) (4), or for 12 h with IL-1β (1 nM) (5) or IL-1β (1 nM) plus PDTC (100 μM) (6). (B) Effects of PDTC on TNFα-stimulated group II PLA_2 mRNA accumulation. Mesangial cells were incubated for 6 h with vehicle (control) (1), PDTC (100 μM) (2), TNFα (1 nM) (3), TNFα (1 nM) plus PDTC (100 μM) (4), or for 12 h with TNFα (1 nM) (5) or TNFα (1 nM) plus PDTC (100 μM) (6). (C) Effects of PDTC on forskolin-stimulated group II PLA_2 mRNA accumulation. Mesangial cells were incubated for 6 h with vehicle (control) (1), PDTC (100 μM) (2), forskolin (10 μM) (3), forskolin (10 μM) plus PDTC (100 μM) (4), or for 12 h with forskolin (10 μM) (5) or forskolin (10 μM) plus PDTC (100 μM) (6). Total cellular RNA (20 μg) was successively hybridized to ^{32}P-labelled group II PLA_2 and β-actin cDNA probes as described in section 2.
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fragment for consensus sequences of possible *cis*-acting elements reveals the presence of the sequence-motif 5′-GGGCTTTTCC-3′ which is highly homologeous (in 8 out of 9 bases) to the consensus sequence 5′-GGGPuNNPyPyCC-3′ known to bind with high affinity the heterodimer of NFκB composed of subunits p50 and Rel A (p65) [26]. Preliminary data in our laboratory, obtained with electrophoretic mobility-shift assays using a radioactively labelled oligonucleotide containing the sequence derived from the group II PLA_2 gene, give strong evidence that this potential *cis*-acting element is in fact involved in the binding of NFκB (G. Walker, D. Kunz and J. Pfeilschifter, unpublished results).

From data present in this study it is becoming obvious that the induction of PLA_2 activity in mesangial cells is regulated by multiple signalling cascades. These may act alone or synergize with each other when stimulated in combination. It is clear from our results that PDTC can inhibit IL-1β and TNFα-stimulated PLA_2 expression in mesangial cells. Obviously IL-1β and TNFα use NFκB to trigger regulation of the group II PLA_2 gene whereas cAMP activates the gene by transcription factors different from NFκB. Glomerular mesangial cells are a specialized type of vascular smooth muscle cells participating in the regulation of the glomerular filtration rate. These cells are being increasingly recognized as important target and effector cells in the pathogenesis of acute and chronic forms of renal diseases. In addition, there is substantial evidence that extracellular group II PLA_2 plays an important role in the pathogenesis of diverse inflammatory processes [4]. Therefore, to understand the basic mechanism of PLA_2 gene regulation is an essential prerequisite for the development of new pharmacological approaches for effective treatment of patients with chronic renal diseases.

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