Pulmonary Inflammation and Edema Induced by Phospholipase A2
GLOBAL GENE ANALYSIS AND EFFECTS ON AQUAPORINS AND Na⁺/K⁺-ATPase

Received for publication, March 10, 2003, and in revised form, April 23, 2003
Published, JBC Papers in Press, May 12, 2003, DOI 10.1074/jbc.M302446200

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Victims of snakebite quickly succumb to severe respiratory failure, which can be fatal if left untreated. One of the most toxic components of snake venom is phospholipase A₂ (PLA₂; EC 3.1.1.4). PLA₂ isolated from the elapid, Naja sputatrix, induced pulmonary inflammation and edema when administered intravenously and intratracheally to rats. Analysis of pulmonary gene expression profiles using oligonucleotide microarrays revealed 60 genes whose expression was altered by at least 3-fold in response to intratracheal instillation of PLA₂ for 3 h as compared with controls. In addition to genes encoding cytokines and chemokines responsible for inflammatory processes, the Na⁺/K⁺-ATPase gene has been found to be involved in edema formation. Real-time PCR, Western blot, and immunohistochemical analyses confirmed that the expression of AQP1 and AQP5 mRNAs and proteins was decreased. Besides providing an experimental model for studies on the pathophysiology of the lung, this investigation yields a clue to the mechanisms by which endogenous PLA₂s could mediate inflammation in conditions such as allergy and rheumatoid arthritis.

Snake venoms are chemically complex mixtures of pharmacologically active proteins, many of which also have enzymatic properties (1). Because venoms serve not only as a source of digestive enzymes but also as a defense mechanism, these proteins can target multiple tissues, causing a simultaneous poisoning of multiple physiological systems. From these venoms, hundreds of toxins have been purified and characterized. One of the components that contributes significantly to the lethality of snake venoms is phospholipase A₂ (PLA₂; EC 3.1.1.4). PLA₂ found in crotalid venom and mammalian cells (3, 8). In our study, we investigated the mechanisms of nsPLA₂-induced inflammation and fluid accumulation by studying the gene expression profiles of lungs from rats treated intravenously and intratracheally with nsPLA₂ for 10 and 1 and 3 h, respectively, using oligonucleotide microarrays. This technology allows the simultaneous quantitation and comparison of the expression of ~8000 genes and expressed sequence tags found in rat genome arrays. Here, we show that the expression of inflammatory cytokines and related genes (e.g. cyclooxygenase-2; IL-1α, interleukin; TNF-α, tumor necrosis factor-α, Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

This work was supported in part by research Grant RP-183-000-067-213 from the National Medical Research Council, Singapore. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† Supported by a research scholarship from the National University of Singapore.

‡ Supported by a research scholarship from the National University of Singapore.

§ Supported by a research scholarship from the National University of Singapore.

The abbreviations used are: PLA₂, phospholipase A₂; AQP, aquaporin; nsPLA₂, Naja sputatrix phospholipase A₂; COX-2, cyclooxygenase-2; IL, interleukin; TNF-α, tumor necrosis factor-α, Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Additionally, the data may provide evidence for a physiological role played by water and ion channels in the maintenance of lung fluid balance.

**MATERIALS AND METHODS**

**Purification of nsPLA2**—N. sputatrix crude venom was obtained from Sigma. The nsPLA2 was purified using Sephadex G-100 gel filtration chromatography (Amersham Biosciences) followed by reverse phase high performance liquid chromatography and then characterized as described (16). The purified fractions were quantitated using the Bradford assay (Bio-Rad).

**Experimental Groups**—All animals were handled according to the guidelines for animal use set by the Council for International Organization of Medical Sciences (CIOMS) on animal experimentation (WHO, Geneva, Switzerland). Male Sprague-Dawley rats (200–220 g) were maintained on an ad libitum intake of standard laboratory chow and drinking water prior to and after treatments. Various doses of nsPLA2, which has an LD50 value of 125 μg/200 g body weight in Sprague-Dawley rats, were administered either intravenously or intratracheally. For the intratracheal administration, the rats were restrained and nsPLA2 was delivered via tail vein injection. For intratracheal administration, the rats were anaesthetized intraperitoneally with a mixture (100 μl/100 g body weight) consisting of an equal volume of hypnorm (0.315 mg of fentanyl and 10 mg of fluanxone; Jansen Pharmaceutica, Beerse, Belgium) and midazolam (5 mg of dormicum and 2 ml of water; Roche Diagnostics). An anterior midline incision was made and nsPLA2 was instilled into the trachea using a 27[1,2]-gauge needle and a 1-ml syringe (BD Biosciences). The tracheal site was closed using 3/0 silk sutures (Aesculap AG & Co. KG, Tuttingen) and the rats were allowed to recover. Consequently, there were a total of 3 treatment groups. (a) Intravenous administration of 240 μg/200 g body weight of nsPLA2 for 1 h (PLA2 IV; n = 6); (b) intratracheal administration of 48 μg/200 g body weight of nsPLA2 for 1 h (PLA2 IT1hr, n = 6); and (c) 3 h (PLA2 IT3hr, n = 9). Control rats (n = 15) were treated similarly but with 100 μl of vehicle (0.9% NaCl) instead. All rats were sacrificed at the various time points by cervical dislocation and whole lungs were rapidly removed, pooled, and stored in frozen in liquid nitrogen. Frozen lungs were stored at −70°C until use.

**RNA Isolation**—Total RNA was isolated from nsPLA2-treated and control lungs by a single-step method using Trizol reagent (Invitrogen) from lung tissues. The RNA samples were subsequently treated with RNase-free DNase I at 37°C for 20 min and stored at −70°C until use.

**Real-time Quantitative PCR**—Real-time PCR was carried out as described (17) with the following modifications. The PCR amplification for AQP5 was performed for 40 cycles with each cycle at 94°C for 20 s, 50°C for 20 s, and 60°C for 40 s. The forward and reverse primers for the AQP1 gene were 5'-GGCTTCAATATTACCATGGA-3' and 5'-CCACGGACACACCCATTT-3', respectively; for the AQP5 gene, 5'-GGTCCATGATCGGTGTCGTT-3' and 5'-GCCTCGTGTGGCTGCTGCT-3', respectively; and for Na/K-ATPase a1 gene were 5'-GGGTGGGTACTCTT-3' and 5'-CGGGTGGCAGAACA-3', respectively. The Taqman® probes for AQP1, 5, and Na/K-ATPase a1 genes were 5'-AAAGCAAGCCCTGCTGAG-3', 5'-TCAAGGGCCATATGAC-3', and 5'-ATCCTGAGCCAAATGCGGGA-3', respectively. Ribosomal RNA was used as an internal calibration. The probes for the AQP and ribosomal RNA genes had been labeled with reporter fluorescent dyes, 6-carboxyfluorescein and VIC, respectively, at their 5' and 3' ends while their 3' ends were labeled with a quencher dye, 6-carboxytetramethylrhodamine. All primers and probes were synthesized by Applied Biosystems.

**Protein Isolation**—Lung tissue from nsPLA2-treated and control rats were isolated and placed in chilled isolation buffer containing 0.9% NaCl and 50 mM EDTA. Whole tissues were homogenized in 3 ml of ice-cold homogenization buffer containing 50 mM Tris-HCl adjusted to pH 7.4, 300 mM mannitol, and 50 mM EDTA. Homogenates were centrifuged at 100,000 × g at 4°C for 20 min at 4°C. The supernatant was harvested and centrifuged at 14,000 × g for 30 min. The resulting supernatant was collected and the total protein concentration in each sample was measured using the Bradford assay (Bio-Rad).

**Western Blot Analysis**—Total membrane proteins (70 μg/sample) were separated by Tris-Tricine SDS-PAGE as described (18). Electrophoresis was carried out for 1 h on ice onto nitrocellulose membranes (Polyvinylidene difluoride; Millipore & Schleicher). Membranes were blocked for 1 h in 5% nonfat milk in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 3 mM KCl, and 0.1% Tween 20) followed by incubation overnight at 4°C with affinity purified, polyclonal anti-AQP1 (a kind gift from Dr. Mark Knepper, National Institutes of Health) at a dilution of 1 μg/ml and anti-AQP5 at a dilution of 2 μg/ml (Alpha Diagnostics International, San Antonio, TX) in 0.5% blocking solution. After three washes of 10 min each in TBST, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit (AQP1s 1 and 5; Bio-Rad) secondary antibody at a dilution of 1:50,000 in 0.5% blocking solution, washed twice for 10 min each in TBST, and visualized via enhanced chemiluminescence (SuperSignal; Pierce) with various exposures (Kodak-MF film). To confirm equivalent loading of samples, separate sets of gels were run simultaneously and stained with Coomassie Brilliant Blue. Films of Western blots were scanned (Acer SW23300U) and labeling intensities of the bands were quantitated using A1S software (Synoptics Imaging Systems).

**Histology and Immunohistochemistry**—Histopathologic changes and immune reactions for AQP1 and AQP5 were evaluated in nsPLA2-treated and control lungs fixed in 4% paraformaldehyde. Fixed lung tissues were embedded in paraffin, sectioned at 5 μm and processed as described (19) for immunohistochemical staining using the same AQP1 and AQP5 antibodies as those for Western blot analysis.

**Microarray GeneChip™ Analysis**—Total RNA isolated from rat lungs was processed and hybridized to each array of the RG_U34A GeneChip™ Array Set according to the protocols described in the GeneChip™ expression analysis technical manual (Affymetrix, Santa Clara, CA). Two chips were used for each experimental group. Data from each treatment were scaled to an average intensity of 800 and a comparison of signal intensities using Microsoft Excel showed results from duplicate chips highly correlated with R2 values = 0.98. Relative mRNA expression levels were expressed as plus or minus fold changes compared with saline-treated controls using Microarray Suite software 5.0 (Affymetrix). All genes showing a change of 3-fold or more in at least one experimental condition were included in subsequent analyses. Self-organizing map clustering was performed using GeneSpring 5.1 (Silicon Genetics, Redwood City, CA). Values below zero were set to 0 and per chip normalization was carried out by dividing each measurement by the 50th percentile of all measurements in that sample. Per gene normalization was performed by dividing each gene by the median of its normalized signal in each sample. Normalized profiles in all samples were based on the basis of similarity in their temporal expression patterns using the self-organizing map. Cluster members were classified according to their biological functions as described in the NetAffx data base (Affymetrix).

**Wet-Dry Lung Weight Ratios**—Whole lungs were excised from nsPLA2-treated and control rats and weighed to obtain lung wet weights (n = 3 per group). Lungs were dried in a 62°C oven with desiccant and weighed again after 7 days to obtain dry weights.

**Statistical Analysis**—Statistical analyses were performed using unpaired Student’s t test and results were expressed as mean ± S.E. A p value of <0.05 was considered statistically significant.

**RESULTS**

Intravenous injection of nsPLA2 (240 μg/200 g body weight; 2 × LD50) into rats caused death characterized by paralysis and severe respiratory distress after ~10 h. Morphologic observation of the lungs indicated gross swelling and inflammation. To quantitatively assess pulmonary edema in animals injected with nsPLA2 intravenously, whole lung wet-to-dry weight ratios were determined. The wet-to-dry weight ratio of whole lungs from rats 10 h after introduction of nsPLA2 intravenously proved to be significantly increased (170 ± 1% of saline-treated controls, p < 0.05; Fig. 1B). To study the effects of nsPLA2 on the lungs alone, the toxin was administered locally via intratracheal instillation (48 μg/200 g body weight, 0.4 × LD50) for 1 and 3 h. Because nsPLA2 was applied directly to the lungs by intratracheal administration, a much lower dose of nsPLA2 (0.4× LD50) was required to induce inflammation and edema as compared with that for intravenous injection (2× LD50), which would have caused nsPLA2 to enter the systemic circulation. Lung histology was used to assess inflammation in rats after intratracheal instillation of nsPLA2 for 3 h (Fig. 1A). Pulmonary infiltrate and edema fluid were observed in the alveolar airspaces 3 h after nsPLA2 administration (Fig. 1A, center and right panels) as compared with the saline-treated control (Fig. 1A, left panel), which showed clear airspaces. The infiltrate was not detected in bronchial or pulmo-
Pulmonary histology and lung wet-to-dry weight analysis after administration of nsPLA₂. Lung sections (7 μm) from rats (n = 3 per group) administered 100 μl of saline (A, left panel) and 48 μg/200 g body weight nsPLA₂ intratracheally for 3 h (A, center and right panels) were stained with hematoxylin and eosin. Control lung sections showed clear alveolar airspaces (×310) while cellular infiltrate and fluid (arrows) were observed in the alveoli in lungs sections from nsPLA₂-treated rats (center panel, ×310; right panel, ×490). Determination of wet-to-dry weight ratios from whole lungs of rats (n = 3 per group) treated intratracheally for 1 (PLA₂ IT 1hr) and 3 h (PLA₂ IT 3hr) and intravenously for 10 h (PLA₂ IT 10hr) with nsPLA₂ revealed an increase in lung fluid after nsPLA₂ administration as compared with the saline-treated controls (B). Values obtained as grams of wet weight/grams of dry weight are expressed as percentage of control ± S.E. *p < 0.05.
In recent years, the discovery of specific water channels known as aquaporins has deepened our understanding of water transport and homeostasis in cells. There are now 11 known AQPs of which AQPs 1 and 5 are found in the distal lung in rats (24). Although noticeably absent from Fig. 2 (expression of AQPs 1 and 5 was altered less than 3-fold by nsPLA2; data not shown), we decided to investigate for any significant changes in the expression of AQPs 1 and 5 in nsPLA2-treated rat lungs. Quantitative real-time PCR analysis was carried out for the same pooled RNA samples using a trio of specific primers and probe for each gene (Fig. 4). The relative expression of AQPs 1 and 5 in PLA2 IV10 hr, PLA2 IT1 hr, and PLA2 IT3 hr lungs was obtained after normalizing against an internal control (18S ribosomal RNA) and a calibrator, in this case, their saline-treated controls (relative gene expression/H110051). Both AQPs 1 and 5 were significantly down-regulated after intratracheal instillation of nsPLA2 for 3 h (AQP1, 20-fold and AQP5, 10-fold, respectively, p < 0.01) while only AQP1 expression was significantly decreased after 1 h (~1.7-fold, p < 0.05). Intravenous injection of nsPLA2 also dramatically reduced the expression of AQPs 1 and 5 after 10 h (~100 fold, p < 0.01).

To determine whether the decrease in mRNA levels of AQPs 1 and 5 resulted in a corresponding decrease in their protein levels, Western blot analysis was carried out on PLA2 IV10 hr, PLA2 IT1 hr, and PLA2 IT3 hr lungs. Using an affinity purified polyclonal antibody directed against the AQP1 carboxyl-terminal synthetic peptide, Western blot analysis revealed bands at 28 and 35 kDa, representing the native and glycosylated forms, respectively (Fig. 4). Densitometry of the 28-kDa AQP1 band showed a significant decrease in protein levels 1 and 3 h after intratracheal instillation of nsPLA2 (90.5 and 30.2% of the saline-treated controls, respectively, p < 0.01) and 10 h after intravenous injection of nsPLA2 (13 ± 1% of the saline-treated control, p < 0.01; Fig. 4). AQP5 protein expression was analyzed by Western blot using an affinity purified polyclonal antibody directed against the AQP5 carboxyl-terminal synthetic peptide and a band at 29 kDa was revealed (Fig. 4). Protein levels were significantly decreased 3 h after intratracheal instillation of nsPLA2 (29 ± 3% of the saline-treated control, p < 0.01) and 10 h after intravenous injection of nsPLA2 (4 ± 0.5% of the saline-treated control, p < 0.01; Fig. 4). AQP5 protein levels of PLA2 IT1 hr lungs were not statistically distinct from that of the control (98 ± 2%, p < 0.01; Fig. 4). Clearly, there is considerable overlap in the results obtained for both mRNA and protein determinations of changes in AQPs 1 and 5 expression in rats administered nsPLA2.

Immunohistochemistry was carried out to determine the localization and expression of AQPs 1 and 5 in the lungs of rats. Fig. 2. Self-organizing map and functional clustering of genes that exhibited a 3-fold or greater change in expression after intratracheal instillation of nsPLA2 for 1 and 3 h. Two selected self-organizing map clusters are shown and they contain genes whose expression profiles were altered in a similar pattern after nsPLA2 administration. The normalized average signal intensity for each gene was plotted against time.
treated intratracheally with nsPLA₂ for 1 and 3 h. AQP1 had previously been shown to be present in bronchial and alveolar vascular endothelium and fibroblasts in the distal rat lung whereas AQP5 was localized in the apical epithelial of Type I pneumocytes (23). The localization of AQP1 remained unchanged in nsPLA₂-treated and saline-treated control lungs. By light microscopy, staining for AQP1 was decreased in PLA₂ IT1hr and PLA₂ IT3hr lungs (Figs. 5, B and C, respectively) as compared with that for the control (Fig. 5A). AQP1 staining was decreased more prominently in PLA₂ IT3hr than in PLA₂ IT1hr lungs. Similarly for AQP5, staining was reduced in ns-PLA₂-treated lungs in comparison to the saline-treated control, with PLA₂ IT3hr lungs showing a more marked decrease in AQP5 staining (Fig. 5, D–F). Although staining for both AQP1s 1 and 5 was clearly observed in the alveolar regions, the exact cell types that were stained could not be differentiated.

DISCUSSION

The biochemical, immunological, and pharmacological properties of snake venoms have been subjects of keen research for a long time. These venoms contain many proteins and polypeptides that possess enzymatic activity as well as exhibit patho-

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**Table I**

| Category of genes | Description | Treatments |
|------------------|-------------|------------|
| **Inflammatory** | Nerve growth factor-induced gene | PLA₂ IT 1h: +52.0 ± 0.12, PLA₂ IT 3h: +55.7 ± 0.10 |
|                  | Macrophage inflammatory protein-1 | PLA₂ IT 1h: +42.2 ± 0.05, PLA₂ IT 3h: +104.0 ± 0.48 |
|                  | Adrenomedullin precursor | PLA₂ IT 1h: +17.1 ± 0.21, PLA₂ IT 3h: +27.9 ± 0.08 |
|                  | Nerve growth factor-induced factor A | PLA₂ IT 1h: +18.4 ± 0.05, PLA₂ IT 3h: +26.1 ± 0.12 |
|                  | Cyclooxygenase-2 | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +27.9 ± 0.03 |
|                  | Lipocalin | PLA₂ IT 1h: -5.1 ± 0.06, PLA₂ IT 3h: -7.33 ± 0.29 |

| **Cytokines, chemokines receptors** | | |
| Interleukin-1 α | PLA₂ IT 1h: +21.9 ± 0.16, PLA₂ IT 3h: +23.2 ± 0.19 |
| Interleukin-1 β | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +4.3 ± 0.08 |
| Interleukin-6 | PLA₂ IT 1h: +7.0 ± 0.29, PLA₂ IT 3h: +362.0 ± 0.02 |
| Interleukin-18 precursor | PLA₂ IT 1h: -3.2 ± 0.11, PLA₂ IT 3h: -4.2 ± 0.02 |
| Gro | PLA₂ IT 1h: +18.4 ± 0.11, PLA₂ IT 3h: +42.2 ± 0.07 |
| ST38 precursor | PLA₂ IT 1h: +3.2 ± 0.05, PLA₂ IT 3h: +3.7 ± 0.48 |
| Suppressors of cytokine signalling-3 | PLA₂ IT 1h: +3.5 ± 0.06, PLA₂ IT 3h: +4.6 ± 0.08 |
| Interleukin-1β receptor type II | PLA₂ IT 1h: +6.8 ± 0.01, PLA₂ IT 3h: +5.4 ± 0.06 |
| Tumor necrosis factor α | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +7.0 ± 0.07 |

| **Oxidative stress-related and oxidants** | | |
| Inducible nitric-oxide synthase | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +4.0 ± 0.17 |
| DNA-damage inducible transcript, Gadd45 | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +3.4 ± 0.03 |
| Oxidative stress-inducible protein-tyrosine phosphatase | PLA₂ IT 1h: +3.5 ± 0.03, PLA₂ IT 3h: +5.3 ± 0.07 |
| Metallothionein | PLA₂ IT 1h: +4.3 ± 0.10, PLA₂ IT 3h: +7.5 ± 0.15 |
| Glutathione S-transferase | PLA₂ IT 1h: NSC, PLA₂ IT 3h: -3.2 ± 0.08 |

| **Signal transduction** | | |
| Cyclase-associated protein homolog | PLA₂ IT 1h: -3.0 ± 0.18, PLA₂ IT 3h: -6.2 ± 0.19 |
| Phospholipase C | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +3.2 ± 0.02 |
| Protein kinase, MUK | PLA₂ IT 1h: -3.0 ± 0.24, PLA₂ IT 3h: -7.2 ± 0.07 |
| SSectKs | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +3.5 ± 0.06 |
| Schwannoma-derived growth factor | PLA₂ IT 1h: +3.2 ± 0.01, PLA₂ IT 3h: +4.3 ± 0.21 |
| RAC protein kinase | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +3.5 ± 0.47 |
| cAMP phosphodiesterase | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +4.0 ± 0.08 |
| Serine/threonine kinase, Tpl-2 | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +6.5 ± 0.14 |
| Small inducible gene, JE | PLA₂ IT 1h: +3.7 ± 0.15, PLA₂ IT 3h: +6.0 ± 0.18 |

| **Transcriptional regulation** | | |
| c-jun | PLA₂ IT 1h: +4.0 ± 0.05, PLA₂ IT 3h: +3.2 ± 0.02 |
| c-fos | PLA₂ IT 1h: +4.2 ± 0.05, PLA₂ IT 3h: +5.7 ± 0.17 |
| JunB | PLA₂ IT 1h: +8.6 ± 0.03, PLA₂ IT 3h: +10.6 ± 0.01 |
| rhoB | PLA₂ IT 1h: +4.0 ± 0.08, PLA₂ IT 3h: +4.9 ± 0.02 |
| Fos-related antigen-2 | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +6.1 ± 0.01 |
| CREM | PLA₂ IT 1h: +4.0 ± 0.08, PLA₂ IT 3h: +4.9 ± 0.01 |
| Immediate-early serum responsive gene | PLA₂ IT 1h: +3.2 ± 0.15, PLA₂ IT 3h: +11.3 ± 0.16 |
| CCAAT enhancer-binding protein | PLA₂ IT 1h: -4.9 ± 0.04, PLA₂ IT 3h: -5.8 ± 0.01 |
| Nuclear receptor (steroid hormone receptor) | PLA₂ IT 1h: +5.3 ± 0.15, PLA₂ IT 3h: +9.2 ± 0.01 |
| Nuclear factor IA (steroid hormone receptor) | PLA₂ IT 1h: +3.3 ± 0.02, PLA₂ IT 3h: -4.1 ± 0.05 |
| Krox24/krox-20 | PLA₂ IT 1h: +21.1 ± 0.02, PLA₂ IT 3h: +34.3 ± 0.03 |

| **Ion channels** | | |
| Sodium/potassium-ATPase α1 | PLA₂ IT 1h: NSC, PLA₂ IT 3h: -3.7 ± 0.02 |

| **Lipid metabolism** | | |
| Pancreatic phospholipase A₂ | PLA₂ IT 1h: NSC, PLA₂ IT 3h: -4.0 ± 0.12 |
| Lysosomal acid lipase | PLA₂ IT 1h: -3.2 ± 0.01, PLA₂ IT 3h: -6.4 ± 0.14 |
| Fatty acid synthase | PLA₂ IT 1h: NSC, PLA₂ IT 3h: -5.5 ± 0.03 |

| **Coagulation system** | | |
| Plasminogen activator inhibitor-1 | PLA₂ IT 1h: +4.3 ± 0.02, PLA₂ IT 3h: +14.0 ± 0.03 |
| Tissue plasminogen activator | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +4.8 ± 0.01 |
| Coagulation factor III | PLA₂ IT 1h: +3.5 ± 0.06, PLA₂ IT 3h: +6.2 ± 0.08 |

| **Others** | | |
| Atrial natriuretic peptide clearance receptor-3 | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +3.4 ± 0.12 |
| Angiotensin receptor | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +3.2 ± 0.03 |
| Hypertension-regulated vascular factor-1C-4 | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +3.6 ± 0.2 |
| c-myc | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +4.6 ± 0.05 |
| Tumor-associated glycoprotein E4 | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +4.6 ± 0.03 |
| Tissue inhibitor of metalloproteinase I | PLA₂ IT 1h: NSC, PLA₂ IT 3h: -3.5 ± 0.01 |
| Calgranulin A | PLA₂ IT 1h: NSC, PLA₂ IT 3h: +5.3 ± 0.08 |
| Cathepsin D | PLA₂ IT 1h: NSC, PLA₂ IT 3h: -3.5 ± 0.01 |
| Eukaryotic initiation factor-5 | PLA₂ IT 1h: -5.3 ± 0.08, PLA₂ IT 3h: -7.5 ± 0.03 |
| Guanine deaminase | PLA₂ IT 1h: +3.5 ± 0.07, PLA₂ IT 3h: +6.6 ± 0.04 |
| Heat shock protein-90 | PLA₂ IT 1h: -3.2 ± 0.04, PLA₂ IT 3h: -5.0 ± 0.04 |

* NSC, no significant change.
physiological properties. Not only have the studies of these toxins aided in the treatment and management of snake envenomation, they have also been a source of research tools crucial for the elucidation of molecular mechanisms underlying physiological processes. A component of all snake venoms, phospholipase A2, is of particular interest. PLA2 enzymes are ubiquitous in nature and can be classified into two groups: intracellular or extracellular/secretory. Intracellular PLA2 is cytosolic and can be Ca2+-dependent or independent. Extracellular PLA2 is divided into four groups and all require millimolar concentrations of Ca2+ for enzymatic activity. Venom PLA2 belongs to Group I of the secretory PLA2 family, as do pancreatic and mammalian PLA2, and is characterized by its low molecular mass and high disulfide bond content (26, 27). To date, venom PLA2 has been used to evaluate the structural function of phospholipids and their roles as mediators of biological processes like the phospholipids methylation system (28, 29), to determine asymmetric organization of phospholipids in biomembranes (30–32), as models of mammalian intracellular phospholipases that are difficult to obtain in sufficient quantities for meaningful studies and to aid in the understanding of presynaptic control mechanisms of neurotransmitter release (33–35).

The main cause of death upon cobra envenomation is peripheral respiratory paralysis because of both presynaptic and postsynaptic neurotoxins present in the venom (36). However, intravenous injection of nsPLA2, a presynaptically acting neurotoxin, into rats induced marked inflammation and edema in the lungs. Given the prolonged period of envenomation prior to death (>10 h) and the massive accumulation of pulmonary fluid during this time, inflammation must play an important role in venom-induced respiratory failure, an aspect often overlooked. This observation led us to investigate the proinflammatory and edema-inducing properties of nsPLA2. Whereas these effects are known (14, 37, 38), little had been done to decipher the molecular determinants involved in venom PLA2-induced inflammation and edema, especially in the lungs.

The results of this study had identified 60 pulmonary genes whose expression was changed by nsPLA2 (Fig. 6). A majority of the genes induced by nsPLA2 are mediators of inflammation (Fig. 2). The expression of cytokines, interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α; Fig. 2A, Cluster 1), and small inducible cytokine subfamily A20, was all increased. IL-6, in particular, was induced 362-fold above the control. IL-6 is secreted by macrophages, T cells, and mast cells and serves to enhance the maturation of activated T and B cells. IL-1β and TNF-α are known to stimulate the release of chemokines, specifically monocyte chemotactic protein-1 and IL-8 (39), which recruit neutrophils to sites of inflammation. IL-1β is also able to stimulate the secretion of macrophage inflammatory protein-1 by monocytes (40). Macrophage inflammatory protein-1 is a chemokine whose expression was increased 104-fold 3 h after intratracheal instillation of nsPLA2.

![Figure 3](http://www.jbc.org/figure3.png) **Fig. 3.** Real-time PCR analysis of Na+/K+-ATPase expression after intratracheal instillation of nsPLA2. Real-time PCR analysis of total RNA isolated from lungs of rats (n = 3 per group with each measurement performed in triplicate) treated intratracheally for 1 and 3 h showed that the expression of Na+/K+-ATPase was significantly decreased 3 h after administration of nsPLA2, as compared with the saline-treated control. No significant changes in gene expression were seen after 1 h. Values are expressed as ±S.E. *p < 0.05.

![Figure 4](http://www.jbc.org/figure4.png) **Fig. 4.** Real-time PCR and Western blot analyses of AQPs 1 and 5 expression after nsPLA2 administration. A, real-time PCR analysis of total RNA isolated from lungs of rats (n = 3 per group with each measurement performed in triplicate) treated intratracheally for 1 and 3 h and intravenously for 10 h with 48 and 240 μg/200 g body weight nsPLA2, respectively, showed that AQPs 1 and 5 mRNA expression was significantly decreased relative to the saline-treated controls. B, Western blot analysis was performed to determine the changes in AQPs 1 and 5 protein levels in lung homogenates from PLA2 IT1hr, PLA2 IT3hr, and PLA2 IV10hr rats (n = 3). Equal amounts of protein were loaded as determined by the Bradford assay. Probing with anti-AQP1 revealed 28- and ~35-kDa bands, corresponding to the native and glycosylated forms of the protein, respectively. Anti-AQP5 detected a 29-kDa band, corresponding to the AQP5 protein. C, changes in protein expression were quantitated by densitometry which showed that nsPLA2 administration reduced AQPs 1 and 5 protein levels significantly except in PLA2 IT1hr lungs. Values are expressed as ±S.E. **p < 0.01.
Recent studies (44, 45) have implicated IL-1β in yet another role during the inflammatory response. Freund et al. (43) observed that IL-1β induced an increase in both the mRNA and protein levels of nerve growth factor in human airway smooth muscle cells. It was also shown that nerve growth factor produced by macrophages and lung fibroblasts and epithelial cells augmented allergic early phase reaction (45). Analysis of gene expression in lungs of rats treated with nsPLA2 revealed that nerve growth factor-induced gene and -factor A transcripts were both substantially up-regulated (Table I). Nerve growth factor is likely to mediate interactions between immune and nerve cells during inflammation.

A gene of particular interest whose expression had been induced by nsPLA2 is cyclooxygenase-2. COX-2 and lipoygenase-5 metabolize arachidonic acid, a product of phospholipid hydrolysis by PLA2, via distinct pathways to produce eicosanoids, namely prostaglandins and leukotrienes, respectively. Both these pathways appear to operate cooperatively as inhibition of any one pathway will simply divert the arachidonic acid pool to the other. In addition, prostaglandins and leukotrienes induce the same processes and are both agonists and antagonists of the same pathological processes (27). Hence, it is intriguing to note that nsPLA2 only induced the COX-2 pathway, implying that the inflammation is mediated primarily through prostaglandins. A possible explanation is that COX-2 synthesis can be up-regulated by TNF-α and IL-1β as a means of regulating the release of several inflammatory mediators (46). Prostaglandins produce vasodilatation and increase vascular permeability. They also contribute to hyperalgesia, fever, platelet aggregation, and bronchoconstriction. In addition, prostaglandin E2 has been shown to function as a secondary messenger in the pathways regulating the expression of cytokine receptors (47). Notably, pancreatic PLA2 expression was reduced in response to nsPLA2. Pancreatic PLA2, although a mainly digestive function, is known to stimulate the expression of cytosolic PLA2 and COX-2 (48). The down-regulation of its expression during nsPLA2-induced inflammation is possibly another example of how finely tuned and closely regulated the inflammatory response is.

Other genes that were induced by nsPLA2 included inducible nitric-oxide synthase, DNA damage-inducible transcript (Gadd45), and oxidative stress-inducible protein-tyrosine phosphatase. Arachidonic acid plays an essential role in the activation of NADPH oxidase, which generates the superoxide anion by neutrophils (49). The production of reactive oxygen species is a natural consequence of inflammation and is responsible for tissue damage that occurs in the later stages of the inflammatory process. In addition, nitric oxide is a vasodilator that increases endothelium permeability during inflammation. The expression of methallothionein, an antioxidant that has the ability to scavenge superoxide anion, was reassuringly increased al-

![Fig. 5](image_url) Immunohistochemical analysis of AQP1 and 5 localization and expression in lung sections from nsPLA2-treated rats. Paraffin sections (5 µm) were stained with anti-AQP1 and -AQP5 and then counterstained with hematoxylin. AQP1 and 5 staining was decreased in lungs treated with 48 µg/200 g body weight nsPLA2 for 1 (B and E, respectively) and 3 h (C and F, respectively) as compared with the saline-treated controls (A and D, respectively). The arrows depict AQP1 (A–C) and AQP5 (D–F) labeling in the alveolar region. Data are representative of three independent experiments.

![Fig. 6](image_url) nsPLA2 alters the expression of genes during pulmonary inflammation and edema. A schematic diagram summarizing the pathways involved in nsPLA2-induced inflammation and edema in rat lungs as revealed by microarray analysis, real-time PCR, Western blotting, and immunohistochemistry. ?, possible pathway; MIP-1, macrophage inflammatory protein-1; MCP-1, monocyte chemotactic protein-1; iNOS, inducible nitric-oxide synthase.

It was one of the most highly up-regulated genes, revealing the critical role that macrophage inflammatory protein-1 plays in chemotaxis and the release of inflammatory mediators. The increase in TNF-α gene expression correlated with the decreased expression of the tissue inhibitor of metalloproteinase-1. Metalloproteinases cause TNF-α release by facilitating the processing of TNF-α by TNF-α-converting enzyme, TACE (39). Besides being an inflammatory cytokine, TNF-α has been hypothesized to be involved in the systemic manifestations of snake envenomations, especially hypotension. How TNF-α mediates the lowering blood pressure is not clear. It was also suggested that TNF-α might be responsible for the development of local edema (41), a point that is discussed further in the text. Interestingly, IL-18 precursor transcript was down-regulated while there was an increase in IL-1 receptor II transcript. During airway inflammation underlying asthma, immunoregulatory cytokines like IL-12 and IL-18 are down-regulated, presumably to strengthen the type-2 helper cell-driven inflammatory process (42). This may explain the reduced expression of IL-18 precursor in nsPLA2-induced inflammation here. IL-1 receptor II serves as a decoy receptor for IL-1β (43). Concomitant up-regulation of IL-1 β and its decoy receptor likely represents a physiological mechanism to tightly regulate the inflammatory process.
though it is unclear as to why glutathione S-transferase transcript was reduced. Various transcription factors (e.g. junB and krox24/krox20), signaling molecules (e.g. cyclase-associated protein homolog, SSEeKs, phospholipase-Cδ, kinases, and receptors), and inflammation-related genes (e.g. adenomedullin precursor, gro, plasmaminogen activator inhibitor-1, and atrial natriuretic peptide clearance receptor-3) were also differentially expressed in response to nsPLA2 and are important regulators of the inflammatory response.

The development of edema after intratracheal instillation of nsPLA2 may partly be attributed to the decreased mRNA transcripts encoding the Na+/K+-ATPase. There is clear evidence for the role of Na+/K+-ATPase in edema fluid clearance (50–52). It is now known that active Na+ transport rather than hydrostatic or protein osmotic gradients regulates in vivo alveolar fluid clearance (51). Activation of the channel by β-agonists or increasing channel expression by adenoviral transfer of either subunit (α1 or β1) enhanced edema clearance significantly (51). Consequently, a reduced expression would result in pulmonary fluid accumulation. The recent discovery of Na+/K+-ATPase in alveolar epithelial type I cells (52) may implicate aquaporins in edema formation. The physiological significance of AQPs 1 and 5 has been vigorously debated. Studies using knockout mice had concluded that aquaporins played only minor roles in airway humidification, airway surface liquid hydration, and osmotic fluid reabsorption (53, 54). However, the high water permeability of alveolar epithelia (AQP5) and vascular endothelia (AQP1) and the presence of both Na+/K+-ATPase and AQP5 in the type I cells may suggest a cooperative role in maintaining lung fluid balance. Studies demonstrating decreased pulmonary vascular permeability in AQP1-null humans (55) and reduced expression of AQPs 1 and 5 in inflamed lung after acute viral infection (56) also strengthen this view. Indeed, results from our study have shown that AQPs 1 and 5 mRNA and protein levels were significantly decreased in the edematous lungs of rats treated with nsPLA2. Active Na+ transport across the alveolar epithelium via apical sodium channels and basolateral Na+/K+-ATPase generates a vectorial Na+ flux that produces a transepithelial osmotic gradient that causes water to move passively from airspaces to the alveolar interstitium (51) via AQP5. A decrease in Na+/K+-ATPase expression reduces this Na+ flux and clearance is impaired. It is unclear if reduced expression of the sodium pump is directly regulated by nsPLA2 or by an inflammatory mediator. The corresponding decrease in AQPs 1 and 5 expression may be an adaptive response to the change in osmotic pressure or as a consequence of active signal transduction pathways. TNF-α had been shown to inhibit AQP5 expression via a molecular pathway that involves binding to the p55 TNF-α receptor and activating NF-κB transcription factor (57). Hence, the induction of TNF-α expression by nsPLA2 led to the downregulation of AQP5 expression. The discrepancy between the differential gene expression for AQPs 1 and 5 as determined by microarray analysis and real-time PCR indicates that conditions and detection sensitivities vary between techniques, despite the apparent similarity in the principles of hybridization chemistry underlying both methods. This highlights the necessity to employ additional techniques to support significant data.

The results of our study initially provide evidence that pulmonary inflammation and edema are likely causes of respiratory failure and death in victims of snake envenomation, in addition to possible airway paralysis. Subsequent analysis of pulmonary gene expression profiles in the lungs of rats intratracheally administered nsPLA2 revealed an intricate web of cross-talk among signaling pathways that mediated pulmonary inflammation and provided evidence for the physiological significance of water and ion channels in lung fluid homeostasis. Additionally, venom PLÅ2-induced inflammation may serve as an experimental model for studying the mechanisms of action of endogenous secretory PLA2s, which are implicated in conditions such as allergic airway inflammation, sepsis, acute pancreatitis, asthma, rheumatoid arthritis and eventually, adult respiratory distress syndrome.

Acknowledgment—We thank Professor Mark Knepper for the generous gift of AQP1 antibody.
PLA₂-induced Pulmonary Inflammation and Edema

15, 1834–1836
47. Tithof, P. K., Peters-Golden, M. & Ganey, P. E. (1998) *J. Immunol.* 160, 953–960
48. Factor, P. (2001) *Cell. Mol. Biol.* 47, 347–361
49. Matthay, M. A., Fukuda, N., Frank, J., Kallet, R., Daniel, B. & Sakuma, T. (2000) *Clin. Chest Med.* 21, 477–488
50. Sznajder, J. I., Factor, P. & Ingbar, D. H. (2002) *J. Appl. Physiol.* 93, 1869–1886
51. Dematte, J. E. & Sznajder, J. I. (2000) *Intensive Care Med.* 26, 477–480
52. Johnson, M. D., Widdicombe, J. H., Allan, L., Barby, P. & Dobbs, L. G. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 1961–1971
53. Song, Y., Jayaraman, S., Yang, B., Matthay, M. A. & Verkman, A. S. (2001) *J. Gen. Physiol.* 117, 573–582
54. Ma, T., Fukuda, N., Song, Y., Matthay, M. A. & Verkman, A. S. (2000) *J. Clin. Invest.* 105, 1860–1866
55. Dematte, J. E. & Sznajder, J. I. (2000) *Intensive Care Med.* 26, 477–480
56. Towne, J. E., Harrod, K. S., Krane, C. M. & Menon, A. G. (2000) *Am. J. Respir. Cell Mol. Biol.* 22, 34–44
57. Towne, J. E., Krane, C. M., Bachurski, C. J. & Menon, A. G. (2001) *J. Biol. Chem.* 276, 18657–18664
58. Howard-Jones, N. A. (1995) *WHO Chronicle* 39, 51–56
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J. Biol. Chem. 2003, 278:31352-31360.
doi: 10.1074/jbc.M302446200 originally published online May 12, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302446200

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