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J Clin Invest. 1993;[92\(4\)](#):1974-1980. <https://doi.org/10.1172/JCI116791>.

Research Article

An important mechanism for the antiinflammatory effect of pharmacological doses of glucocorticoids is the inhibition of arachidonic acid release from phospholipids by phospholipase A2 (PLA2). As a corollary, one might predict that low endogenous concentrations of glucocorticoids favor inflammatory disease states. Indeed, clinical and experimental observations revealed an association between glucocorticoid deficiency and disease states caused by immunological and/or inflammatory mechanisms. The purpose of the present investigation was to study the regulation of PLA2 mRNA, protein, and enzyme activity in adrenalectomized (ADX) rats where glucocorticoid concentrations were below physiological levels. The mRNA of group I and II PLA2 were measured by PCR. Group II PLA2 mRNA was increased by 126 +/- 9% in lung tissue of ADX rats, whereas group I PLA2 was increased only by 27 +/- 1.5%. The increase in group II mRNA in ADX rats was reflected by a corresponding increase of group II PLA2 protein (70-100%) in lung, spleen, liver, and kidney. This increase was reversed by the administration of exogenous corticosterone. After ADX, the percentage increase in total PLA2 activity was higher than that of mRNA or PLA2 protein, suggesting that the activity of the enzyme was modulated by inhibitors or activators. The concentration of lipocortin-I, an inhibitor of PLA2 enzyme was strongly correlated with the activity of PLA2 in the tissues (lung, spleen, liver, [...])

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Glucocorticoid Deficiency Increases Phospholipase A₂ Activity in Rats

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Abstract

An important mechanism for the antiinflammatory effect of pharmacological doses of glucocorticoids is the inhibition of arachidonic acid release from phospholipids by phospholipase A₂ (PLA₂). As a corollary, one might predict that low endogenous concentrations of glucocorticoids favor inflammatory disease states. Indeed, clinical and experimental observations revealed an association between glucocorticoid deficiency and disease states caused by immunological and/or inflammatory mechanisms. The purpose of the present investigation was to study the regulation of PLA₂ mRNA, protein, and enzyme activity in adrenalectomized (ADX) rats where glucocorticoid concentrations were below physiological levels. The mRNA of group I and II PLA₂ were measured by PCR. Group II PLA₂ mRNA was increased by 126±9% in lung tissue of ADX rats, whereas group I PLA₂ was increased only by 27±1.5%. The increase in group II mRNA in ADX rats was reflected by a corresponding increase of group II PLA₂ protein (70–100%) in lung, spleen, liver, and kidney. This increase was reversed by the administration of exogenous corticosterone. After ADX, the percentage increase in total PLA₂ activity was higher than that of mRNA or PLA₂ protein, suggesting that the activity of the enzyme was modulated by inhibitors or activators. The concentration of lipocortin-I, an inhibitor of PLA₂ enzyme was strongly correlated with the activity of PLA₂ in the tissues (lung, spleen, liver, and kidney). In all these tissues, the concentrations of lipocortin-I declined after ADX. Thus upregulation of PLA₂ enzyme and downregulation of lipocortin-I might account for the enhanced inflammatory response in hypogluco-corticoid states. (*J. Clin. Invest.* 1993. 92:1974–1980.) Key words: phospholipase A₂ • glucocorticoids • adrenalectomy • inflammation • lipocortin

Introduction

The biological and clinical relevance of high endogenous or exogenous glucocorticoid concentrations in mammals has been widely discussed (1–4). During stress, for example, increased concentrations of glucocorticoid hormones were hypothesized to turn off the defensive inflammatory reactions that may overshoot after noxious stimuli (4). This effect is used in clinical practice by prescribing pharmacological doses of glucocorticoids to patients with inflammatory diseases. The

antiinflammatory effect of glucocorticoids is mediated through a number of mechanisms, among which the inhibition of eicosanoid production by phospholipase A₂ (PLA₂)¹ enzyme appears to play a key role (5). PLA₂ enzymes characterized so far are classified into group I and group II based on their primary and secondary structures (6). Group II (but not group I PLA₂) has been suggested to be involved in the pathogenesis of inflammatory reactions (7–11). Several lines of evidence reveal that high doses of glucocorticoids decrease PLA₂ activity by two different mechanisms: (a) by generating proteins such as lipocortin-I, which were shown to inhibit PLA₂ activity in vitro (for a review, see reference 5), through mechanisms that are not yet well defined (12–13), and (b) by reducing the expression of group II PLA₂ enzyme (14–15).

In contrast to the high glucocorticoid levels, the relevance of abnormally low endogenous glucocorticoid concentrations to the inflammatory response has rarely been addressed. Previous investigations have shown an increased duration and magnitude of inflammatory reactions and higher concentrations of various prostaglandins after adrenalectomy (ADX) or treatment with a glucocorticoid receptor antagonist in animals (16–20). It was hypothesized that the increased concentrations of prostaglandins were caused by an increased activity of PLA₂ (17, 20). In the present study, we report the effect of glucocorticoid deficiency induced by ADX in rats on mRNA, protein levels of group II PLA₂, and total enzyme activity of PLA₂ in different tissues. The results revealed an upregulation of group II PLA₂ after ADX, which was reversed after the administration of corticosterone.

Methods

Preparation of ADX rats. ADX with and without replacement of corticosterone pellets and sham-operated ADX (SHAM-ADX) were performed as described previously (21). Male Sprague Dawley rats, weighing 160–180 g (Holtzman Laboratory Animals, Madison, WI), were kept in a temperature-, humidity-, and light (12 h on)-controlled room and were allowed food and water ad libitum. The rats were anesthetized with ether and treated in the following ways: Group 1: SHAM-ADX. Adrenal glands were exposed but not touched. Group 2: SHAM-ADX + V. Adrenal glands were exposed but not touched (vehicle [V] = DMSO). Group 3: ADX + corticosterone. Rats were ADX and given a subcutaneous 20% corticosterone/80% cholesterol pellet that created plasma corticosterone values of ~ 25 µg/liter. Group 4: ADX. Adrenal glands were removed completely, resulting in plasma corticosterone concentration < 1 µg/liter.

After surgery, all rats were provided with 0.5% saline for their drinking solution. Starting on the evening after the surgery, all rats, except those in group 4, were injected with DMSO three times a day to act as controls for a separate experiment. When the plasma adrenocorticotrophic hormone and corticosterone concentrations were analyzed in the SHAM-ADX rats that received either subcutaneous injections of

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Received for publication 11 March 1993 and in revised form 28 May 1993.

J. Clin. Invest.

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0021-9738/93/10/1974/07 \$2.00

Volume 92, October 1993, 1974–1980

1. Abbreviations used in this paper: ADX, adrenalectomy; CORT, corticosterone; PLA₂, phospholipase A₂; SHAM, sham-operated; TBS, Tris-buffered saline; V, vehicle.

DMSO or no injections, there were no differences, indicating that the stress involved with the injections did not affect corticosteroid concentrations. On the morning and evening of the 5th d after the surgery, the rats were decapitated, and lung, spleen, kidney, and liver tissues were collected, immediately frozen in liquid nitrogen, and stored at -80°C until further use. This protocol was approved by the University of California, San Francisco Committee on Animal Research.

RNA extraction and modification of group II PLA₂ transcript. Total RNA from the tissues of different experimental rats was extracted by the lithium chloride-urea procedure (22). The RNA concentration was determined by absorbance at 260 nm. The quality of RNA was controlled by running an aliquot on a 1% agarose formaldehyde gel. Full-length group II PLA₂ cDNA (23) (750 bp) inserted in pGEM-3Z plasmid was obtained as gift from J. Ishizaki (Shinogi and Co. Ltd., Osaka, Japan). For the quantitative measurements of group II PLA₂ mRNA, this plasmid was modified as follows to construct an internal standard. An XbaI restriction site was introduced at the 5' end of the PLA₂ cDNA by PCR to remove the BamHI site in the polylinker of pGEM-3Z plasmid. A BamHI fragment of 446 bp from positions 170–616 was removed from the PLA₂ cDNA resulting in a smaller group II PLA₂ cDNA of 254 bp. A transcript of this modified PLA₂ cDNA was prepared using SP6 RNA polymerase. This transcript was used as an internal standard for the quantitative measurement of group II PLA₂ mRNA. The transcript was constructed such that for reverse transcription and PCR the same primers could be used for the mRNA of both group II PLA₂ and the internal standard (24).

Reverse transcription of mRNA. The reverse transcription reaction mixture of 20 μl contained 50 mM Tris-HCl pH 8.2, 6 mM MgCl₂, 10 mM DTT, 100 mM NaCl, 200 μM dNTPs, 11 U RNase inhibitor, 10 pmol 25-mer primer (3' primer of the corresponding cDNA, position 695–719 of group II PLA₂ cDNA from Microsynth (Windisch, Switzerland), 100–1600 ng total RNA and 18 fg modified group II PLA₂ transcript, 1 U avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Initially, the 3' primer and total RNA, with and without modified group II PLA₂ transcript, were incubated together for 5 min at 65°C , then cooled at room temperature for 15 min. The remaining reaction components, including avian myeloblastosis virus reverse transcriptase, were then added and incubated at 42°C for 60 min.

PCR. PCR was performed in a total volume of 30 μl with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTPs, 50 pmol of the appropriate 3' and 5' cDNA primers (5' primer: position 58–82 of PLA₂ cDNA) 6.0 μg BSA, 1 μCi [α -³²P]dCTP; 4 μl reverse transcribed group II PLA₂ cDNA, 1 U *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus Corp. Norwalk, CT). Before adding the enzyme and cDNA the samples were irradiated under ultraviolet light for 20 min to eliminate any contaminating DNA (25). The mixture was overlaid with mineral oil, and cDNA was then amplified with a DNA thermal cycler (Perkin Elmer Cetus Instruments) for 35 cycles. The amplification profile involved denaturation at 94°C for 1 min and 15 s, primer annealing at 72°C for 2 min and elongation of annealed primers at 72°C for 2 min. For group I PLA₂ (26) 100–1,600 ng total RNA was reverse transcribed (3' primer: position 340–359) without internal standard. 6 μl of reverse transcribed material was used as a template for cDNA amplification by PCR, using the same conditions as for group II PLA₂ cDNA (5' primer: position 43–62). 10 μl of each PCR reaction mixture were mixed with 2 μl of sixfold concentrated loading buffer and applied on 1.5% agarose gel containing ethidium bromide. Electrophoresis was carried out in Tris-borate-EDTA buffer, pH 8.0 with a constant voltage of 50 V for 90 min. Bands were visualized under ultraviolet light and excised from the gel. The radioactivity of the bands was measured in a scintillation counter using a Cerenkov program.

Protein extraction and quantitative analysis of group II PLA₂. A small amount of tissue frozen at -80°C was powdered, and acid was extracted according to the method of Märki and Franson (27). The protein concentration was determined using the bicinchonic acid protein assay reagent (Pierce Chemical Co., Rockford, IL).

Western blotting. For Western blots, 2–10 μg of total protein (these protein samples were not boiled, and the treatment buffer did not contain any reducing agents) were loaded and resolved on a 12.5% SDS-polyacrylamide gel (28). The proteins were transferred to nitrocellulose membrane by electroblot using Tris-glycine buffer containing 20% methanol. The transfer was performed at a constant voltage of 60 V for 60 min. Protein transfer was monitored by staining the nitrocellulose membrane with Ponceau S (Sigma Chemical Co., St. Louis, MO) and the gel with Coomassie blue. The blots were agitated with Tris-buffered saline (TBS), pH 7.5, containing 5% BSA for 60 min at room temperature, washed three times with TBS and incubated with primary antibody (2E7 and 2B9 hybridoma supernatants specific for group II PLA₂ enzyme, a gift from Henk van den Bosch, State University of Utrecht, Utrecht, The Netherlands) diluted 1:5,000 in TBS containing 0.5% Tween 20 and 0.01% BSA. The incubation with monoclonal antibodies was performed for 4 hours at 37°C . Membranes were washed three times with TBS containing 0.05% Tween 20 and incubated with peroxidase conjugated rabbit anti-mouse IgG (H + L) (Sigma Chemical Co.) for 1 h at room temperature diluted 1:6,000 in TBS containing 0.05% Tween 20 and 0.01% BSA. Membranes were washed three times with TBS containing 0.05% Tween 20 and three times with TBS only to remove Tween 20. After washing, the peroxidase activity was detected using the enhanced chemiluminescence Western blotting detection system (Amersham International, Aylesbury, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. The bands on the x-ray films were scanned with a transmittance scanning densitometer (Camag TLC Scanner II; Camag, Muttentz, Switzerland) to get an absorbance profile across each band. The peak areas were weighed for measurements.

Assay of PLA₂ activity. PLA₂ activity was assayed using [³H]-oleate-labeled, autoclaved *Escherichia coli* as the substrate (29). The reaction mixture of 350 μl contained 100 mM Tris-HCl pH 8.0; 5 mM Ca²⁺, 11.4×10^9 cells of autoclaved *E. coli* cells (corresponding to 10,000 cpm and 4.6 nmol phospholipid), and 0.3–70 μg tissue acid extract. The amount of tissue protein was chosen such that 6–15% hydrolysis of substrate was obtained when incubated at 37°C for 30 min. The reaction was terminated by adding 100 μl of 2 N hydrochloric acid. 100 μl of fatty acid-free BSA (100 mg/ml) was added, and the tubes were vortexed and centrifuged at 13,000 g for 5 min. An aliquot (140 μl) of the supernatant containing released [³H]oleic acid was mixed with scintillation cocktail (Dynagel, J. T. Baker, B. V. Deventer, Holland) and counted in a liquid scintillation counter.

Results

The mRNA of group II PLA₂ was quantified by PCR using a constant amount of a modified group II cDNA transcript as an internal standard. Both specific target mRNA and internal standard were coamplified in one reaction using the same primers (24). Fig. 1 shows the PCR products of group II PLA₂ cDNA and the internal standard of rat lung tissue separated on an agarose gel; three concentrations of total RNA from control (SHAM-ADX) rats, SHAM-ADX + V, ADX + CORT, and ADX rats were used. Visual observation of the bands revealed that group II PLA₂ mRNA was increased in ADX rat lungs compared with the values in the other three groups. Similar results were obtained in two additional experiments. The inclusion of an internal standard excluded the possibility that increased concentrations of group II PLA₂ in ADX rats were caused by an artifact during the PCR amplification procedure (24).

To assess quantitatively the effect of ADX on group II PLA₂ mRNA, [α -³²P]dCTP was included during the PCR. The incorporation of radioactivity into group II PLA₂ cDNA was determined at 20, 80, and 320 ng of RNA, with and without the internal standard (Fig. 2). Three independent experiments re-

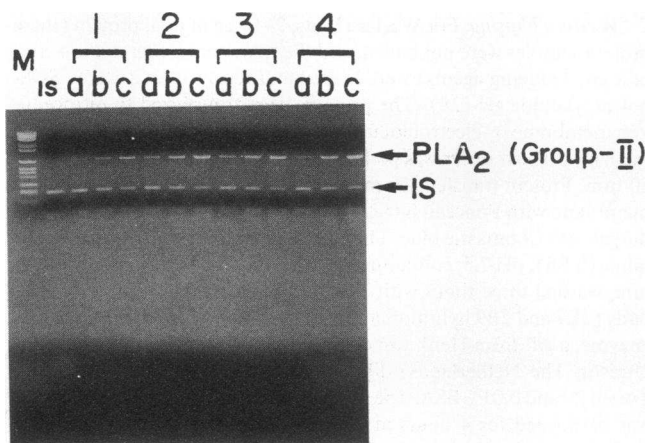


Figure 1. Agarose gel (1.5%) electrophoresis of PCR products of group II PLA₂ from rat lung tissue. Group II PLA₂ mRNA was reverse transcribed in the presence of 18 fg internal standard (IS) and 20 ng (lane a), 80 ng (lane b), and 320 ng (lane c) of reverse transcribed material was used as template for PCR reactions. M, DNA molecular weight marker VI (Boehringer Mannheim Biochemicals); 1, SHAM-ADX; 2, SHAM-ADX + V; 3, ADX + CORT; 4, ADX.

vealed that the internal standard did not affect the generation of group II PLA₂ cDNA. In all experiments, no significant difference in the group II PLA₂ mRNA was observed between SHAM-ADX and SHAM-ADX + V groups (Fig. 2, A and B). In ADX rats compared to SHAM-ADX + V rats, an increase of $126 \pm 9\%$ of group II PLA₂ mRNA was observed at 320 ng total RNA. In ADX rats substituted with glucocorticoids (ADX-CORT), group II PLA₂ mRNA was decreased by $33 \pm 15\%$ when compared with ADX rats. These results were further-

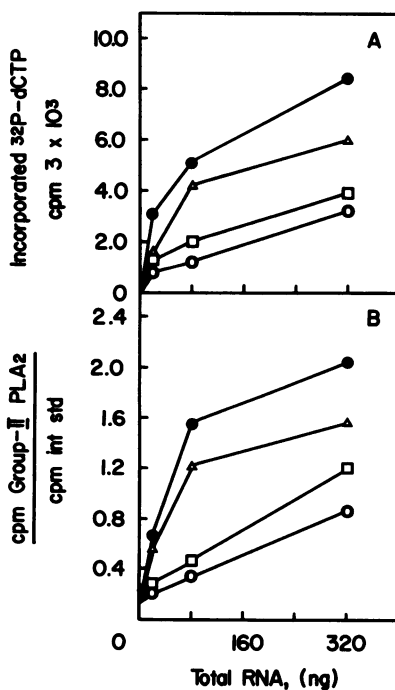


Figure 2. (A) Incorporation of [α -³²P]dCTP (cpm) into cDNA of group II PLA₂ as a function of total RNA used for reverse transcription. To exclude potential competition of primers for group II PLA₂ cDNA and internal standard during PCR, no internal standard was added to this reverse transcription. \circ , SHAM-ADX; \square , SHAM-ADX + V; \triangle , ADX + CORT; \bullet , ADX. Each symbol represents one animal. (B) Incorporation of [α -³²P]dCTP (cpm) into cDNA of group II PLA₂ in the presence of 18 fg internal standard. The ratio of group II PLA₂ cpm/internal standard

cpm is plotted against the total RNA concentration used during reverse transcription reaction. The symbols are the same as those used in A.

more supported by the analysis of the ratios of cpm of group II PLA₂/cpm of internal standard. The results of one of three identical experiments are shown in Fig. 2 B.

The impact of glucocorticoids on group I PLA₂ has not been investigated previously. Therefore, we extended our study to group I PLA₂ also. Quantitative studies with [α -³²P]dCTP-labeled PCR products revealed that in ADX compared to SHAM-ADX + V rats, there was an increase of $27 \pm 1.5\%$ of group I PLA₂ mRNA (Fig. 3 A). In all ADX rats treated with glucocorticoids (ADX + CORT) the levels of group I PLA₂ mRNA decreased by $71 \pm 4\%$ below that of control groups; no signal was detected on agarose gel after PCR (Fig. 3 A).

To establish whether the increase of group II PLA₂ mRNA is associated with increased group II PLA₂ protein, lung, spleen, liver, and kidney tissues were analyzed by Western blot using specific group II PLA₂ monoclonal antibodies (Fig. 4, A and B). In all these tissue samples ADX resulted in an increased concentration of group II PLA₂ protein ranging from 70 to 100% depending on the type of tissue analyzed (Fig. 4 B). In these tissues of ADX rats substituted with glucocorticoids, group II PLA₂ protein was decreased by 64–85%, when com-

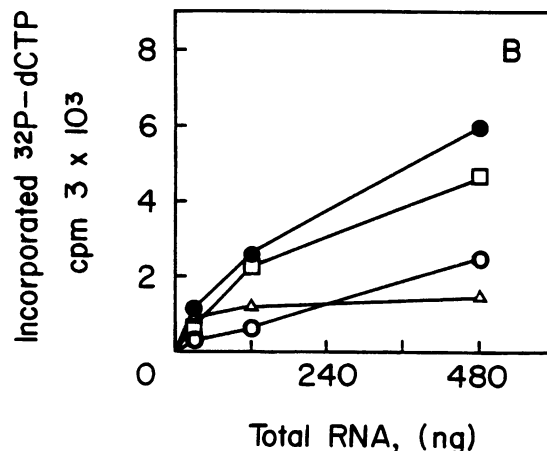
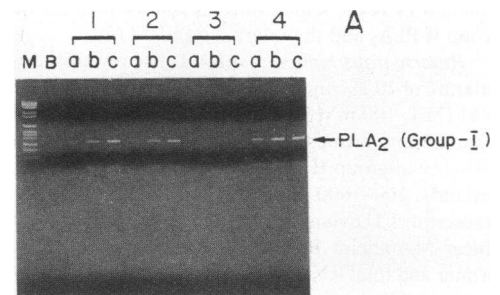


Figure 3. (A) Agarose gel (1.5%) electrophoresis of PCR products of group I PLA₂ from rat lung tissue. Group I PLA₂ mRNA was reverse transcribed and 30 ng (lane a), 120 ng (lane b), and 480 ng (lane c) of reverse transcribed material was used as template for PCR reactions. M, DNA molecular weight marker IV (Boehringer Mannheim Biochemicals); 1, SHAM-ADX; 2, SHAM-ADX + V; 3, ADX + CORT; 4, ADX. (B) Incorporation of [α -³²P]dCTP (cpm) into cDNA of group I PLA₂ cDNA as a function of total RNA used for reverse transcription. \circ , SHAM-ADX; \square , SHAM-ADX + V; \triangle , ADX + CORT; \bullet , ADX.

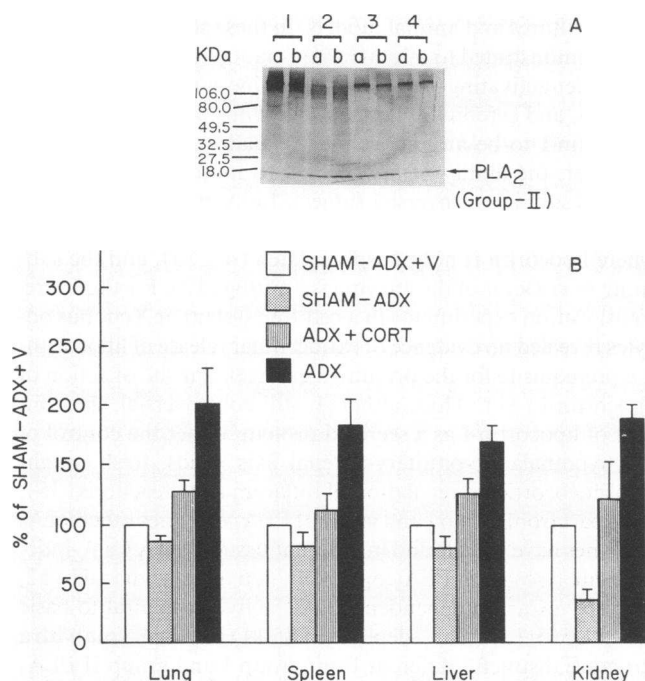


Figure 4. PLA₂ protein measurements in rat tissues. (A) Western blot analysis of group II PLA₂ in acid extracts of lung (1), spleen (2), liver (3), and kidney (4) tissues. A representative control group SHAM-ADX + V (lane a) and experimental group ADX (b) for each tissue is presented. To get a good signal different amounts of protein were used: 2 μ g for lung, 5 μ g for spleen and liver, and 10 μ g for kidney. (B) Western blot analysis of group II PLA₂ protein of different tissues. SHAM-ADX + V-treated rats were used as a reference (100%). The values are expressed as the mean \pm SD ($n = 3$).

pared with ADX rats. In lung tissue, the percentage decline in protein was more pronounced (73%) than the corresponding decline of mRNA (33%). The reason for the very low PLA₂ protein content in kidneys of SHAM-ADX is not clear.

Activity of PLA₂ was assessed in tissues of lung, liver, spleen and kidney. For that purpose, the PLA₂ enzyme was solubilized after acid extraction. The PLA₂ enzyme extracted showed a pH optimum of 7.5–9.0 and required 5 mM Ca²⁺ for optimal activity. The results from PLA₂ activity measurements are shown in Fig. 5. Interestingly different tissues showed different amounts of PLA₂ specific activity; lung tissue had the highest and kidney tissue the lowest PLA₂ activity. In correlation with increased mRNA and PLA₂ protein, the total PLA₂ activity was also increased in the above studied tissues of ADX rats (Fig. 5). In all the tissues analyzed, there was a threefold increase of PLA₂ activity in ADX rats when compared with SHAM-ADX + V rats. The increased PLA₂ activity was restored to normal level when ADX rats were substituted with glucocorticoids.

The relative increase in total PLA₂ activity in ADX rats was twofold higher compared to the relative increase of mRNA and protein suggesting that PLA₂ activity is possibly modulated by changes in activators or inhibitors of PLA₂. Lipocortin-I has shown to inhibit PLA₂ activity (12, 13, 29). Previously, we have reported decreased levels of lipocortin-I mRNA and protein in ADX rats and restoration of lipocortin-I to control levels in ADX rats treated with glucocorticoids (21). When the lipocortin-I concentrations were plotted next to the corre-

sponding PLA₂ activities (Fig. 5), a correlation was observed: at higher concentrations of lipocortin-I, a decreased PLA₂ activity was observed, and at lower concentrations of lipocortin-I an increased PLA₂ was observed when the same organ was analyzed in different groups of rats.

Discussion

The quantitative RNA-PCR study revealed that both group I and group II PLA₂ mRNA levels were elevated after ADX in lung tissue. The upregulation of PLA₂ in ADX rats was not a nonspecific phenomenon, since the mRNA levels of other proteins (metallothionein-II and *c-fos*, *c-myc*, *c-erbA β* oncogenes) have been shown to remain unchanged (21). After ADX, the increase of mRNA of group II PLA₂ was more pronounced than that of group I PLA₂, and when the ADX rats were substituted with glucocorticoids, the mRNA of group II PLA₂ was decreased less than that of group I PLA₂, suggesting that group I PLA₂ and group II PLA₂ are regulated differently by glucocorticoids. Previous studies with rat vascular smooth vessel cells indicated that group II PLA₂ mRNA was regulated by two different mechanisms: First, by cAMP as a second messenger, and second, by a cAMP-independent pathway (11). It has been shown that glucocorticoids inhibited cAMP-dependent, but not cAMP-independent expression of group II PLA₂ mRNA (15). Provided these in vitro studies reflect the in vivo

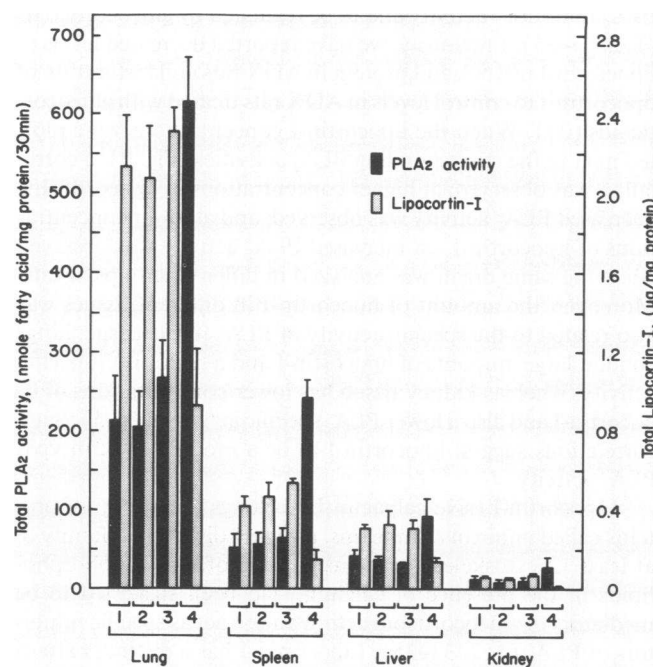


Figure 5. Total PLA₂ activity (nmol fatty acid/mg protein per 30 min) and lipocortin concentrations (μ g/mg protein) in rat tissues. Lipocortin-I data previously published were used to correlate PLA₂ activity measurements with lipocortin-I protein content (21). In this approach total lipocortin-I concentration of monomer in lung, monomer and dimer in spleen, and dimer in liver and kidney were estimated using the standard curve of recombinant lipocortin-I. Each value represents the mean (\pm SD) of three different experiments. Lane 1, SHAM-ADX; lane 2, SHAM-ADX + V; lane 3, ADX + CORT; lane 4, ADX.

situation, then one might assume that the inhibition of mRNA of group II PLA₂ by glucocorticoids in ADX rats was mediated by the cAMP pathway. Since the cAMP-independent pathway seems to be regulated by factors other than glucocorticoids (15), the absence of a complete normalization of group II PLA₂ mRNA in ADX rats with glucocorticoids is not surprising.

In lung tissue of ADX rats substituted with glucocorticoids, group II PLA₂ protein was decreased by 73% when compared with ADX rats; this percentage decline in protein was more pronounced than the corresponding decline of 33% in mRNA, suggesting that glucocorticoids decrease group II PLA₂ protein also by modulating posttranscriptional mechanisms. Separate transcriptional and posttranscriptional effects of pharmacological doses of glucocorticoids have been reported in inflammatory conditions on the synthesis of group II PLA₂, tumor necrosis factor, and interleukin-1 (14, 30, 31).

The increases of group II PLA₂ protein and mRNA in ADX rats were also reflected by increases of PLA₂ enzyme activity. The PLA₂ enzyme activity measured in these tissues exhibited biochemical properties similar to group II PLA₂ enzyme activity characterized from synovial fluid from rheumatoid patients and human platelets with regard to its optimum pH, Ca²⁺ dependence, substrate preference, and also stability towards acidic pH (32). The relative increase in total PLA₂ activity was two-fold higher compared with the relative increase of mRNA and protein following ADX, suggesting that PLA₂ activity was possibly modulated by an increase in activators or by removal of inhibitors. Lipocortins have been demonstrated to exhibit PLA₂ inhibitory activity and to be regulated by glucocorticoids (1, 2, 33–35). Previously, we have reported decreased levels of lipocortin-I mRNA and protein in ADX rats and restoration of lipocortin-I to control levels in ADX rats treated with glucocorticoids (21). When the lipocortin-I concentrations were plotted next to the corresponding PLA₂ activities (Fig. 5), a correlation was observed: at higher concentrations of lipocortin-I, a decreased PLA₂ activity was observed, and at lower concentrations of lipocortin-I, an increased PLA₂ activity was observed when the same organ was analyzed in different groups of rats. Moreover, the amount of lipocortin-I in different tissues was also related to the specific activity of PLA₂ in the tissue; lungs contain large amounts of lipocortin-I and a high PLA₂-specific activity, whereas kidney tissue has lower concentrations of lipocortin-I and also a lower PLA₂ specific activity (Fig. 5). Such correlations suggest lipocortin-I to be a modulator of *in vivo* PLA₂ activity.

Lipocortin-I (sive calpactin-II) belongs to a group of proteins called annexins. Annexins, a widely distributed family of at least 12 cytoskeletal proteins capable of binding phospholipids in the presence of calcium, had been suggested to be mediators of glucocorticoid action and to act as specific inhibitors of PLA₂ (1, 2, 33–35). Lipocortin-I has a distinct pattern of organ distribution (Fig. 5) and a discrete pattern of cellular distribution (36). For example, lipocortin-I in skin is extremely abundant in the epidermis, especially in the basal keratinocytes, but sparse in the dermis (36). There exists evidence that the synthesis of lipocortin-I can be induced in monocytes and differentiated macrophage-like cell lines by glucocorticoids (37–41); however, in certain other cell lines the synthesis of lipocortin-I does not appear to be regulated by glucocorticoids (42, 43). Several pharmacological studies have used recombinant human lipocortin-I and derived peptide fragments

in cell cultures and animal models. In these studies, lipocortin-I was demonstrated to inhibit inflammation (44–45), synthesis of platelet-activating factor (46), cytokine-mediated pyrogenesis (47), and cerebral ischemia (48). Thus potential exists for lipocortin-I to be an endogenous regulator of inflammation. However, the ability of lipocortin-I to inhibit the activity of PLA₂ is still a controversial subject. Lipocortin-I is indeed an inhibitor of PLA₂ *in vitro* (29), but only under conditions where lipocortin is not phosphorylated (49, 50), and the substrate or cofactor of the enzyme is limiting (12). Furthermore, translocation experiments in a cell-free system or *Xenopus* oocytes revealed no evidence of extracellular release of lipocortin-I, a prerequisite for the presumed extracellular site of action of lipocortin-I (51). Thus, although still controversial, the concept of lipocortin-I as a secreted protein, under the control of the hypothalamic-pituitary-adrenal axis yields, fresh insight into glucocorticoid regulation of inflammatory reactions (35).

Apart from group I and group II PLA₂ enzymes other PLA₂ isozymes have been found in different tissues: (a) A Ca²⁺-independent lysosomal PLA₂ optimally active at acidic pH (52, 53); (b) a Ca²⁺-independent PLA₂, active at neutral to basic pH (54, 55); (c) a Ca²⁺-dependent 85-kD PLA₂ enzyme with a structure distinctly different from group I and group II PLA₂ enzymes (56, 57). The structure of both Ca²⁺-independent PLA₂ enzymes is not known. Whether the 85-kD PLA₂ enzyme is involved in inflammatory reactions has not been determined. Most likely, different isoforms of PLA₂ enzymes may coexist in a single system, and might exert separately or cumulatively their effect in generating arachidonic acid. The other enzymes that generate arachidonic acid, depending on cell types are (a) sequential action of phospholipase C followed by diacylglycerol lipase or diacylglycerol kinase followed by phosphatidic acid specific PLA₂; and (b) phospholipase D followed by phosphatidic acid specific PLA₂ (58). It is evident that glucocorticoids inhibit the release of arachidonic acid and subsequent formation of eicosanoids, no matter which class or type of phospholipase enzyme releases the arachidonic acid. The present investigation demonstrates that for restoration of PLA₂ activity by glucocorticoids in ADX rats, apart from regulating PLA₂ enzyme at transcriptional and posttranscriptional level, an increase of an endogenous inhibitor is necessary. Lipocortin-I is a good candidate for such an inhibitory function, since lipocortin has been shown to inhibit Ca²⁺-independent PLA₂ (52), phospholipase C (59), and phospholipase D (60).

The majority of chronic inflammatory diseases are of unknown etiology, and the factors modulating their severity are largely unknown. Physiological endogenous glucocorticoid levels may protect from these disease states and low levels promote these disease states. The latter hypothesis was proposed in 1922 by Képinov, who reported an increased sensitivity to bronchial anaphylactic reactions in ADX guinea pigs (16). That observation was in line with the later findings of an association between glucocorticoid deficiency and disease states explained by allergic and/or immunological mechanisms in experimental animals and humans (17, 61–64). The upregulation of group II PLA₂ enzyme and the downregulation of lipocortin-I by low levels of glucocorticoids indicate that clinically unrecognized partial glucocorticoid deficiency, either caused by suppression of endogenous glucocorticoid production after withdrawal of exogenous glucocorticoids or caused by low cortisol synthesis for other reasons, may be relevant for the genesis of inflammatory diseases.

Acknowledgments

We thank H. van den Bosch for providing 2E7 and 2B9 hybridoma supernatants and for discussion and J. Ishizaki (Shionogi Research Laboratories, Osaka, Japan) for providing the plasmid encoding group II PLA₂ cDNA.

This project was supported by grants from Swiss National Foundation for Scientific Research (no. 32-31048-91), Wissenschaftlicher Fonds für die Forschung, an der Universität Bern and Sandoz Stiftung.

References

1. Flower, R. J., and G. J. Blackwell. 1979. Anti-inflammatory steroids induce biosynthesis of a phospholipase A₂ inhibitor which prevents prostaglandin generation. *Nature (Lond.)*. 278:456-459.
2. Blackwell, G. J., R. Carnuccio, M. Di Rosa, R. J. Flower, L. Parente, and P. Persico. 1980. Macroscortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids. *Nature (Lond.)*. 287:147-149.
3. Frey, F. J. 1987. Kinetics and dynamics of prednisolone. *Endocr. Rev.* 8:453-473.
4. Munck, A., P. M. Guyre, and N. J. Holbrook. 1984. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr. Rev.* 5:25-44.
5. Flower, R. J. 1984. Macroscortin and the antiphospholipase proteins. *Adv. Inflammation Res.* 8:1-34.
6. Heinrikson, R. L., E. T. Krueger, and P. S. Keim. 1977. Amino acid sequence of phospholipase A₂-alpha from the venom of *Crotalus adamanteus*. *J. Biol. Chem.* 252:4913-4921.
7. Vishwanath, B. S., A. A. Fawzy, and R. C. Franson. 1988. Edema-inducing activity of phospholipase A₂ purified from human synovial fluid and inhibition by aristolochic acid. *Inflammation*. 12:549-561.
8. Seilhamer, J. J., W. Pruzanski, P. Vadas, S. Plant, J. A. Miller, J. Kloss, and L. K. Johnson. 1989. Cloning and recombinant expression of phospholipase A₂ present in rheumatoid arthritic synovial fluid. *J. Biol. Chem.* 264:5335-5338.
9. Murakami, M., I. Kudo, H. Nakamura, Y. Yokoyama, H. Mori, and K. Inoue. 1990. Exacerbation of rat adjuvant arthritis by intradermal injection of purified mammalian 14-kDa group II phospholipase A₂. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 268:113-116.
10. Oka, S., and H. Arita. 1991. Inflammatory factors stimulate expression of group II phospholipase A₂ in rat cultured astrocytes. *J. Biol. Chem.* 266:9956-9960.
11. Nakano, T., O. Ohara, H. Teraoka, and H. Arita. 1990. Group II phospholipase A₂ mRNA synthesis is stimulated by two distinct mechanisms in rat vascular smooth muscle cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 261:171-174.
12. Davidson, F. F., E. A. Dennis, M. Powell, and J. R. Glenney, Jr. 1987. Inhibition of phospholipase A₂ by "lipocortins" and calpactins. *J. Biol. Chem.* 262:1698-1705.
13. Haigler, H. T., D. D. Schlaepfer, and W. H. Burges. 1987. Characterization of lipocortin I and an immunologically unrelated 33-kDa protein as epidermal growth factor receptor/kinase substrates and phospholipase A₂ inhibitors. *J. Biol. Chem.* 262:6921-6930.
14. Nakano, T., S. Ohara, H. Teraoka, and H. Arita. 1990. Glucocorticoids suppress group II phospholipase A₂ production by blocking mRNA synthesis and posttranscriptional expression. *J. Biol. Chem.* 265:12745-12748.
15. Nakano, T., and H. Arita. 1990. Enhanced expression of group II phospholipase A₂ gene in the tissues of endotoxin shock rats and its suppression by glucocorticoids. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 273:23-26.
16. Képinov, L. C. 1922. Surrénales et anaphylaxie. *Séances de la Société.* 87:327.
17. Flower, R. J., L. Parente, P. Persico, and J. A. Salmon. 1986. A comparison of the acute inflammatory response in adrenalectomized and shamoperated rats. *Br. J. Pharmacol.* 87:57-62.
18. Laue, L., S. Kawai, D. D. Brandon, D. Brightwell, K. Barnes, R. A. Knazek, D. L. Loriaux, and G. P. Chrousos. 1988. Receptor-mediated effects of glucocorticoids on inflammation: enhancement of the inflammatory response with a glucocorticoid antagonist. *J. Steroid Biochem.* 29:591-598.
19. Bertini, R., M. Bianchi, and P. Ghezzi. 1988. Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor. *J. Exp. Med.* 167:1708-1712.
20. Mugridge, K. G., M. Perretti, C. Becherucci, and L. Parente. 1991. Persistent effects of interleukin-1 on smooth muscle preparations from adrenalectomized rats: implications for increased phospholipase-A₂ activity via stimulation of 5-lipoxygenase. *J. Pharmacol. Exp. Ther.* 25:29-37.
21. Vishwanath, B. S., F. J. Frey, M. Bradbury, M. F. Dallman, and B. M. Frey. 1992. Adrenalectomy decreases lipocortin-I messenger ribonucleic acid and tissue protein content in rats. *Endocrinology*. 130:585-591.
22. Clemens, M. J. 1984. Purification of eukaryotic messenger RNA. In *Transcription and Translation. A Practical approach*. B. D. Hames and S. J. Higgins, editors. IRL Press Ltd., Oxford, England. pp. 211-230.
23. Ishizaki, J., O. Ohara, E. Nakamura, M. Tamaki, T. Ono, A. Kanda, N. Yoshida, H. Teraoka, H. Tojo, and M. Okamoto. 1989. cDNA cloning and sequence determination of rat membrane-associated phospholipase A₂. *Biochem. Biophys. Res. Commun.* 162:1030-1036.
24. Wang, A. M., M. V. Doyle, and D. F. Mark. 1989. Quantification of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA*. 86:9717-9721.
25. Sarkar, G., and S. S. Sommer. 1990. Shedding light on PCR contamination. *Nature (Lond.)*. 343:27.
26. Ohara, O., M. Tamaki, E. Nakamura, Y. Tsuruta, Y. Fujii, M. Shin, H. Teraoka, and M. Okamoto. 1986. Dog and rat pancreatic phospholipases A₂: Complete amino acid sequences deduced from complementary DNAs. *J. Biochem.* 99:733-739.
27. Märki, F., and R. Franson. 1986. Endogenous suppression of neutral-actin and calcium-dependent phospholipase A₂ in human polymorphonuclear leukocytes. *Biochim. Biophys. Acta*. 879:149-156.
28. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. SDS-polyacrylamide gel electrophoresis of proteins. In *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 18.47-18.59.
29. Rothut, B., F. Russo-Marie, J. Wood, M. Di Rosa, and R. J. Flower. 1983. Further characterization of the glucocorticoid-induced antiphospholipase protein "Renocortin". *Biochem. Biophys. Res. Commun.* 117:878-884.
30. Beutler, B., N. Krochin, I. W. Milsark, C. Luedke, and A. Cerami. 1986. Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. *Science (Wash. DC)*. 232:977-980.
31. Knudsen, P. J., C. A. Dinarello, and T. B. Strom. 1987. Glucocorticoids inhibit transcriptional and posttranscriptional expression of interleukin 1 in U937 cells. *J. Immunol.* 139:4129-4134.
32. Kramer, R. M., C. Hession, B. Johanson, G. Hayes, P. McGray, E. P. Chow, R. Tizard, and R. B. Pepinsky. 1989. Structure and properties of a human non-pancreatic phospholipase A₂. *J. Biol. Chem.* 264:5768-5775.
33. Wallner, B. P., R. J. Mattaliano, C. Hession, R. L. Cate, R. Tizard, L. K. Sinclair, C. Foeller, E. P. Chow, J. L. Browning, K. L. Ramachandran, and R. B. Pepinsky. 1986. Cloning and expression of human lipocortin, a phospholipase A₂ inhibitor with potential anti-inflammatory activity. *Nature (Lond.)*. 320:77-81.
34. Pepinsky, R. B., L. K. Sinclair, J. L. Browning, R. J. Mattaliano, J. E. Smart, E. P. Chow, T. Falbel, A. Ribolini, J. L. Garwin, and B. P. Wallner. 1986. Purification and partial sequence analysis of a 37-kDa protein that inhibits phospholipase A₂ activity from rat peritoneal exudates. *J. Biol. Chem.* 261:4239-4246.
35. Goulding, N. J., and P. M. Guyre. 1993. Glucocorticoids, lipocortins and the immune response. *Curr. Opin. Immunol.* 5:108-113.
36. Fava, R. A., J. McKanna, and S. Cohen. 1989. Lipocortin I (p35) is abundant in a restricted number of differentiated cell types in adult organs. *J. Cell. Physiol.* 141:284-293.
37. Browning, J. L., M. P. Ward, B. P. Wallner, and R. B. Pepinsky. 1990. Studies on the structural properties of lipocortin-I and the regulation of its synthesis by steroids. *Prog. Clin. Biol. Res.* 349:27-45.
38. Ambrose, M. P., C. L. C. Bahns, and G. W. Hunninghake. 1992. Lipocortin-I production by human alveolar macrophages. *Am. J. Resp. Cell Mol. Biol.* 6:17-21.
39. Wong, W. T., S. C. Frost, and H. S. Nick. 1991. Protein-synthesis-dependent induction of Annexin I by glucocorticoid. *Biochem. J.* 275:313-319.
40. Solito, E., G. Raugi, M. Melli, and L. Parente. 1991. Dexamethasone induces the expression of the mRNA of lipocortin-1 and 2 and the release of lipocortin-1 and 5 in differentiated, but not undifferentiated U-937 cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 291:238-244.
41. Goulding, N. J., J. L. Godolphin, P. R. Sharland, S. H. Peers, M. Sampson, P. J. Maddison, and R. J. Flower. 1990. Anti-inflammatory lipocortin-1 production by peripheral blood leucocytes in response to hydrocortisone. *Lancet*. 335:1416-1418.
42. Pilch, A., L. Sun, R. A. Fava, and J. Hayashi. 1989. Lipocortin-independent effect of dexamethasone on phospholipase activity in a thymic epithelial cell line. *Biochem. J.* 261:395-400.
43. Gebicke-Haerter, P. J., A. Schobert, P. Dieter, P. Honegger, and G. Hertting. 1991. Regulation and glucocorticoid-independent induction of lipocortin I in cultured Astrocytes. *J. Neurochem.* 57:175-183.
44. Cirino, G., S. H. Peers, R. J. Flower, J. L. Browning, and R. B. Pepinsky. 1989. Human recombinant lipocortin-1 has acute local antiinflammatory properties in the rat paw edema test. *Proc. Natl. Acad. Sci. USA*. 86:3428-3432.
45. Sautebin, L., R. Carnuccio, A. Ialenti, and M. Di Rosa. 1992. Lipocortin and Vasocortin-2 species of antiinflammatory proteins mimicking the effect of glucocorticoids. *Pharmacol. Res.* 25:1-12.
46. Camussi, G., C. Tetta, E. Turello, and C. Baglioni. 1990. Antiinflammatory peptides inhibit synthesis of platelet activating factor. *Proc. Clin. Biol. Res.* 349:69-80.
47. Carey, F., R. Forder, M. D. Edge, A. R. Greene, M. A. Horan, P. J. L. M.

- Stribos, and N. J. Rothwell. 1990. Lipocortin-1 modifies pyrogenic actions of cytokines in the rat. *Am. J. Physiol.* 259:266–269.
48. Relton, J. K., P. J. L. M. Stribos, C. T. O'Shaughnessy, F. Carey, R. A. Forder, F. J. H. Tilders, and N. J. Rothwell. 1991. Lipocortin-1 is an endogenous inhibitor of ischemic damage in the rat brain. *J. Exp. Med.* 174:305–310.
49. Hirata, F., K. Matsuda, Y. Notsu, T. Hattori, and R. D. Carmine. 1984. Phosphorylation at a tyrosine residue of lipomodulin in mitogen-stimulated murine thymocytes. *Proc. Natl. Acad. Sci. USA.* 81:4717–4721.
50. Hirata, F. 1981. The regulation of lipomodulin, a phospholipase inhibitory protein, in rabbit neutrophils by phosphorylation. *J. Biol. Chem.* 256:7730–7733.
51. Frey, B. M., F. J. Frey, V. R. Lingappa, and H. Trachsel. 1991. Expression of human recombinant lipocortin I in a Wheat-germ cell-free system and *Xenopus* oocytes. *Biochem. J.* 275:219–225.
52. Ghiara, P., R. Meli, L. Parente, and P. Persico. 1984. Distinct inhibition of membrane-bound and lysosomal phospholipase A₂ by glucocorticoid-induced proteins. *Biochem. Pharmacol.* 33:1445–1450.
53. Pierik, A., J. G. Nijssen, A. J. Aarsman, and H. Van den Bosch. 1988. Calcium-independent phospholipase A₂ in rat tissue cytosols. *Biochim. Biophys. Acta.* 962:345–353.
54. Hazen, S. L., D. A. Ford, and R. W. Gross. 1991. Activation of a membrane associated phospholipase A₂ during myocardial ischemia which is highly selective for plasmalogen substrate. *J. Biol. Chem.* 266:5629–5633.
55. Hazen, S. L., and R. W. Gross. 1992. Identification and characterization of human myocardial phospholipase A₂ from transplant recipients suffering from end-stage ischemic heart disease. *Circ. Res.* 70:486–495.
56. Gronich, J. H., J. V. Bonventre, and R. A. Nemenoff. 1990. Purification of a high molecular-mass form of phospholipase A₂ from rat kidney activated at physiological calcium concentrations. *Biochem. J.* 271:37–43.
57. Clark, J. D., L. L. Lin, R. W. Kriz, C. S. Ramesha, L. A. Sultzman, A. Y. Lin, N. Milona, and J. L. Knopf. 1991. A novel arachidonic acid-selective cytosolic PLA₂ contains a Ca²⁺-dependent translocation domain with homology to PKC and GAP. *Cell.* 65:1043–1051.
58. Bonventre, J. V., and R. Nemenoff. 1991. Renal tubular arachidonic acid metabolism. *Kidney Int.* 39:438–449.
59. Machoczek, K., M. Fisher, and H-D. Söling. 1989. Lipocortin I and lipocortin II inhibit phosphoinositide- and polyphosphoinositide-specific phospholipase C. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 251:207–212.
60. Kobayashi, M., V. S. Bansal, I. Singh, and J. N. Kanfer. 1988. Dexamethasone-induced reduction of phospholipase D activity in the rat. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 236:380–382.
61. Green, M., and K. H. Lim. 1971. Bronchial asthma with Addison's disease. *Lancet. i:* 1159–1165.
62. Carryer, H. M., D. W. Sherrick, and C. F. Gastineau. 1960. Occurrence of allergic diseases in patients with adrenal cortical hypofunction. *JAMA (J. Am. Med. Assoc.).* 172:1356.
63. Frey, F. J., B. Trost, and A. Zimmermann. 1991. Autoimmune adrenalitis, asthma and membranoproliferative glomerulonephritis. *Am. J. Nephrol.* 11:341–342.
64. Sternberg, E. M., J. M. Hill, G. P. Chrousos, T. Kamilaris, S. J. Listwak, P. W. Gold, and R. L. Wilder. 1989. Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. *Proc. Natl. Acad. Sci. USA.* 86:2374–2378.