Vol. 159, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1989 Pages 53-60

PHOSPHOLIPASE A2 INHIBITORY ACTIVITY IN THYMOCYTES OF DEXAMETHASONE-TREATED MICE – POSSIBLE IMPLICATION OF LIPOCORTINS

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Received November 18, 1988

Summary : The cellular phospholipase A2 activity of mouse thymocytes was estimated in vitro by the release of [3H]-Arachidonic acid from labeled and calcium ionophore A23187-stimulated cells. This activity was decreased in thymocytes from dexamethasone-treated mice. Thus, the presence of phospholipase A2 inhibitory proteins in mouse thymus was investigated. Three main proteins (36 kDa I, 36 kDa II, 73 kDa) were purified. These proteins were able to inhibit both phospholipase A2 in vitro, and the release of [3H]-Arachidonic acid from labeled and stimulated mouse thymocytes. Biochemical analysis revealed that the three proteins were lipocortin-like proteins. Our results show that in vivo dexamethasone treatment induces a phospholipase A2 inhibitory activity in mouse thymus, such an inhibition can be reproduced on isolated thymocytes by purified thymic lipocortins, known as glucocorticosteroid- inducible proteins.

Glucocorticosteroids are potent anti-inflammatory drugs. Their anti-inflammatory effect has been attributed partly to their ability to induce the synthesis and release of phospholipase A2 (PLA2) inhibitory proteins named lipocortin"s" (LC"s") (1). The inhibition of PLA2 activity led to the decrease of pro-inflammatory mediators such as prostaglandins (PGs), leukotrienes (LTs). This phenomenon was first demonstrated in rat macrophages (2), rabbit neutrophils (3), then in rat renomedullary interstitial cells (4), thymus (5) and human fibroblasts (6). The relationship between glucocorticosteroids and LCs was thought to involve a very simple

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ABBREVIATIONS: PLA2; phospholipase A2. AA; arachidonic acid. LC(s); lipocortin(s). EGTA; ethyleneglycol-bis (ß-aminoethylether)- N,N'-tetraacetic acid. FPLC; fast protein liquid chromatography. SDS; sodium dodecyl sulfate. PAGE; polyacrylamide gel electrophoresis. IEF; isoelectric focusing.EDTA; ethylenediamine-tetraacetic acid. Hepes; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. FAF- BSA; fatty acid-free bovine serum albumin. PMSF; phenylmethanesulfonyl fluoride. MEM; modified Eagle's medium. PBS; phosphate buffered saline. DMSO; dimethylsulfoxide.

0006-291X/89 $1.50

pattern : LCs were the antiphospholipase A2 second messengers of glucocorticosteroids, and the inhibition was thought to be due to a direct interaction between LC and the PLA2 enzyme (7). LCs were shown to inhibit PLA2 in vitro due to their property to bind to negatively charged phospholipids (8,9), questionning the specificity of their PLA2 inhibitory property. Recently, pharmacological studies were performed on LCs. The human recombinant LC I was shown to inhibit both, prostacylin production in human endothelial cells (10), and thromboxane A2 formation in perfused guinea-pig lungs (11). In addition, we reported similar data regarding the inhibition of cellular PLA2 activity on whole cells with mouse (12,13) and human LCs (14). The mechanism of cellular PLA2 inhibition by LCs is not known, but it argues for a link between glucocorticosteroids and LCs regarding their cellular PLA2-induced inhibition.

The aim of our study was to investigate whether PLA2 activity of isolated thymocytes was affected by in vivo dexamethasone treatment of mice, and to check whether purified PLA2-inhibitory proteins from mouse thymus were able to modulate PLA2 activity of isolated thymocytes.

MATERIALS AND METHODS

Preparation of mouse thymocytes: Mice received intravenously dexamethasone (Sigma, St Louis, Mo) (0.2 mg/kg) or its vehicle (ethanol, 0.01%) at the appropriate dilution. Two hours later, the animals were killed, their thymuses removed and placed in cold phosphate buffered saline (PBS). Thymuses were homogenized using a teflon Potter. The homogenate was placed for 10 min at 4°C to allow particulate material to deposit. The resulting supernatant was centrifuged for 15 min at 600 g, the supernatant was discarded and the cell pellet (thymocytes) resuspended in 30 ml MEM-Hepes pH 7.4 (10^7 cells/ml). This preparation allowed to obtain about $5x10^7$ cells per thymus.

Labeling of mouse thymocytes with [^{3}H]-Arachidonic acid: Five µCi [^{3}H]-Arachidonic acid (AA) (Amersham International, Amersham, UK) were added to each cell suspension prepared from both vehicle- and dexamethasone-treated mice. The cells were incubated for 30 min at 37 °C, then washed twice with PBS containing 0.5 % FAF-BSA in order to eliminate unbound [^{3}H]-AA from the incubation medium. Thymocytes from vehicle- and dexamethasone- treated mice were adjusted to a same concentration (25,000-30,000 cpm/4-5 $x10^6$ cells/0.45 ml) in MEM-Hepes before their exposure for 10 min at 37 °C to the calcium ionophore A23187 at a known concentration (50µl), or to its vehicle DMSO (0.01%). The reaction was terminated by the addition of 500 µl of cold 10mM EGTA containing 0.5% FAF-BSA, pH 7.4. Samples were centrifuged for 5 min at 10,000 g, aliquots of the supernatants were counted by liquid scintillation spectrometry to determine the released radioactivity.

Purification of PLA2 inhibitory proteins: Mouse thymuses were removed in 10mM Hepes, 0.15M NaCl, 5mM EDTA, 0.2mM PMSF, 200 U/ml aprotinin, pH 7.4, and homogenized using a teflon Potter. Proteins were purified using the methods described for purification of mouse lung LCs (12). The PLA2 inhibitory activity of purified proteins was checked as described before (6), by inhibition of purified porcine pancreatic PLA2 with [3H]-oleic acid labeled E. Coli membranes as a substrate.

Western blot analysis: Western blot analysis were performed after a classical SDS-PAGE according to the procedure of Laemmli (15). The protein transfer to nitrocellulose sheet was performed according to the procedure described by Towbin et al.(16) in 20mM Tris, 150mM glycine and 20% methanol buffer in an EMBL transfer-blot apparatus (Heidelberg, RFG) following the manufacturer's instructions. The nitrocellulose paper was incubated for 16 hr in PBS containing 5% powdered skim milk (17), then washed

five times with PBS and incubated for 1 hr at 37°C in a PBS containing an appropriate dilution of the required antiserum. The nitrocellulose paper was then washed five times with PBS and incubated 1 hr at 37°C with [^{125}I]-labeled protein A (0.1 μCi/ml). After this last incubation, the nitrocellulose paper was washed five times with PBS, dried and exposed for 24 hr to a Fuji X-ray film.

Amino acid analysis: Amino acid analysis was performed on a Biotronik amino acid analyzer LC 5001 using a single-column procedure (18). Samples containing 30 μg of protein were hydrolyzed in vacuo in 0.1 ml of 6 N HCl for 20 hr at 110 °C (19). Methionine was determined as methionine sulfone after performic acid oxidation according to Hirs (20). Half-cystine was estimated as cysteic acid after performic acid oxidation.

Statistical analysis: Results are expressed as mean ± S.E. Means are compared using Student's t-test, in our experimental conditions, differences were considered as non-significant when p values were above 0.05.

RESULTS

PLA2 activity of thymocytes derived from dexamethasone-treated mice

Thymocytes prepared from vehicle- and dexamethasone-treated mice were labeled with [^{3}H]-AA as described in methods. Cells were then stimulated with increasing concentrations of the calcium ionophore A23187. In both groups of thymocytes, the calcium ionophore induced a concentration-related release of [^{3}H]-AA (Figure 1). As shown on the figure, thymocytes isolated from dexamethasone-treated mice released significantly less radioactivity in response to the calcium ionophore. [^{3}H]-AA release was assessed as a measure of global cellular PLA2 activity.

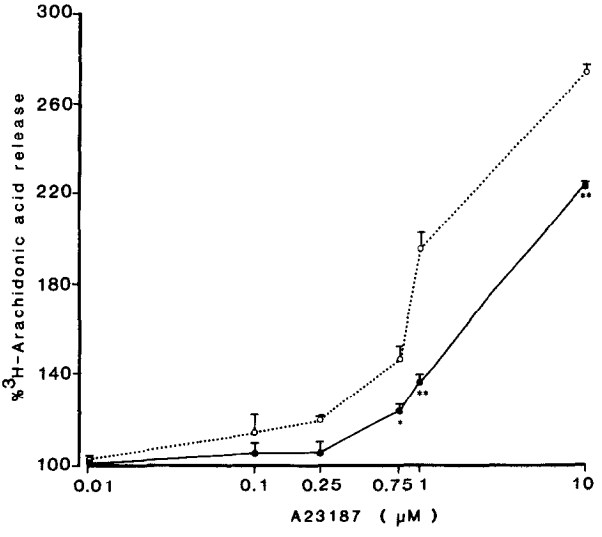

Figure 1
Decrease of cellular PLA2 activity in thymocytes from dexamethasone-treated mice. Thymocytes were isolated from vehicle (-----) and dexamethasone-treated mice (—).The radioactivity released from A23187-treated cells was expressed as a per cent of the radioactivity released from DMSO-treated cells. Data of a typical experiment performed in triplicates are shown. Results are expressed as mean ± S.E. Experiments were performed three times. *: p<0.025, **: p<0.01.

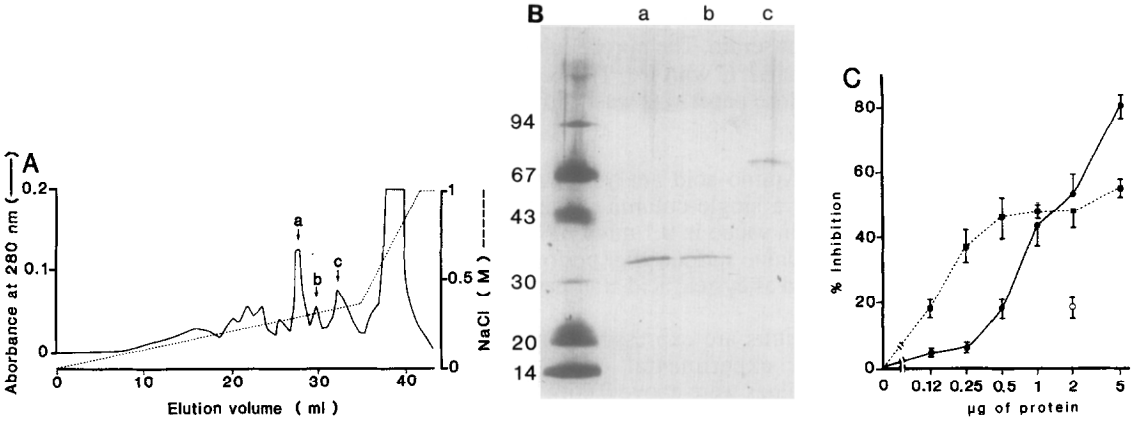

Figure 2
A) FPLC separation on a mono Q column of proteins extracted from mouse thymus. The protein extract was subjected to a mono Q column (FPLC system). After elution of unabsorbed material, a NaCl gradient (-----) was generated by buffer B from 0 to 0.35 M over 35 min and from 0.35 to 1 M over 7 min at a flow rate of 1 ml/min. Absorbance (——) was monitored at 280 nm. Peaks a, b, and c correspond to the main purified proteins.
B) SDS-PAGE of purified mouse proteins: The purified proteins from mouse thymus were analysed in SDS-PAGE (10-15 % polyacrylamide). Molecular weights of standard proteins (phosphorylase b: 94 kDa; albumin: 67 kDa; ovalbumin: 43 kDa; carbonic anhydrase: 30 kDa; trypsin inhibitor: 20 kDa; alpha-lactalbumin: 14 kDa) are indicated.
C) Dose-response curve of mouse purified proteins on PLA2 activity. Different amounts of the purified proteins were incubated with 60 ng of porcine pancreatic PLA2 using [^{3}H]-oleic acid labeled E. Coli. membranes (25,000 cpm) as a substrate. Results of a typical experiment are expressed as mean % inhibition ± S.E. of PLA2 activity. (-●-): 36 kDa; (-■-): 73 kDa.; (o): 2 µg of boiled 36 kDa I protein. Experiments were performed at least three times.

Purification of PLA2 inhibitory proteins from mouse thymus

Purification of the protein extract from mouse thymuses revealed the presence of 3 main proteins (Figure 2A). When analysed in SDS-PAGE, peak a, as peak b gave a single protein band at 36 kDa, peak c contained a single protein at about 73 kDa (Figure 2B). As assessed by native IEF, the pI of the two 36 kDa proteins were indistinguishable (4.7-4.8), and the pI of the 73 kDa protein was of 4.9 (not shown). In the following studies, proteins of peak a and peak b are referred to as 36 kDa I and 36 kDa II respectively.

The PLA2 inhibitory activity of the proteins was assessed as described in methods (Figure 2C, only 36 kDa I and 73 kDa are given). The proteins are inactive after boiling (Figure 2C shows only the result for 2 µg 36 kDa I protein).

Immunological characterization of mouse PLA2 inhibitory proteins

An antiserum against the mouse 36 kDa I was developped. Its specificity was assessed by Western blotting. This antiserum was screened on the purified mouse proteins and a group of other lipocortin-related proteins: a 32 kDa protein purified from human blood mononuclear cells (14) and calpactins (I and II) from bovine intestine (Kindly provided by Dr. Glenney) (21). Figure 3A, shows the transferred proteins onto the nitrocellulose sheet, screened with the 36 kDa I antiserum. Purified mouse 36 kDa I and II (Figure 3A, lane 1 and 2) and human 32 kDa (Figure 3A, lane 6) proteins were recognized by the antiserum, whereas the mouse

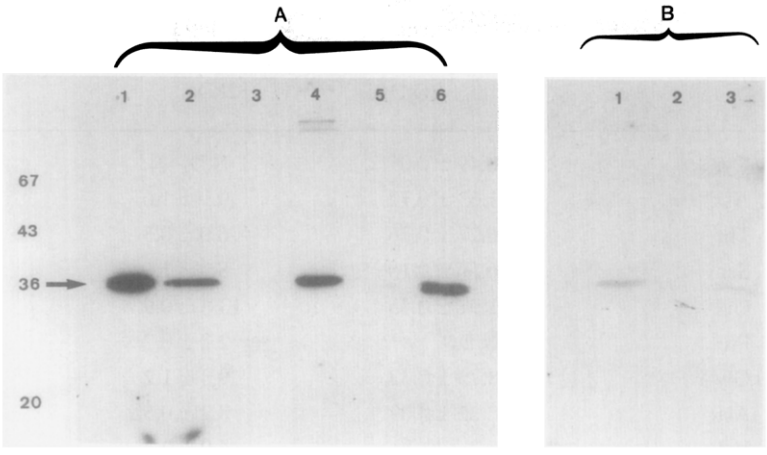

Figure 3
SDS-PAGE and Western blot analysis of the PLA2 inhibitory proteins:
A) The protein extract and the purified proteins from mouse thymus and the human 32 kDa LC were analysed in SDS-PAGE as indicated in methods. Proteins were transferred onto a nitrocellulose sheet, and visualized by 3% TCA-0.2% Ponceau S. Then, they were screened with 36 kDa I antiserum (1:750). Lanes; 1: 36 kDa I; 2: 36 kDa II; 3: 73 kDa; 4: enriched thymus protein extract; 5: total thymus homogenate; 6: human 32 kDa lipocortin. Bands correspond to the cross-reacting proteins.
B) Mouse 36 kDa I (lane 1) and 73 kDa (lane 2) proteins, and the human 32 kDa lipocortin (lane 3) transferred onto a nitrocellulose sheet were screened with a monoclonal antibody (BF26) raised against a rat lipocortin. Molecular weights are indicated on the left.

73 kDa protein (Figure 3A, lane 3) was not. From the enriched protein extract of mouse thymus (Figure 3A, lane 4) and the total thymus homogenate (Figure 3A, lane 5), a single band was found, it migrated at the same position. The same proteins as described on figure 3A were screened with a specific antiserum (1:750) raised against the human 32 kDa LC. Results of the Western blot analysis were strikingly similar to those reported with the antiserum raised against the mouse 36 kDa I (Figure 3A). Therefore, this result is not presented. Neither the purified mouse proteins, nor the human 32 kDa LC cross-reacted with any of the calpactin's antisera (data not shown). Figure 3B shows the cross-reactivity of the monoclonal antibody BF26 (purified IgG1, final concentration 80 µg/ml) raised against a rat LC (22) with the mouse 36 kDa I (Figure 3B, lane 1) and the human 32 kDa LC (Figure 3B, lane 3), but not with the mouse 73 kDa (Figure 3B, lane 2).

Amino acid analysis of the 36 kDa I

The amino acid composition of the mouse 36 kDa I was performed as indicated in methods (Table 1). The results show that the amino acid composition of the mouse 36 kDa I and the human 32 kDa LC is highly similar. As a whole, western blotting and amino acid analysis show that mouse 36 kDa and human 32 kDa LCs are homologous proteins.

Effect of the mouse 36 kDa I protein on [^{3}H]-AA labeled thymocytes

Thymocytes were isolated from untreated mice, and labeled with [^{3}H]-AA as indicated in methods. [^{3}H]-AA labeled thymocytes (25,000-30,000 cpm/4-5x10^6 cells / 0.40 ml) were

Table 1
Amino acid composition of the mouse 36 kDa I protein

	36kDa I	32kDa
Cys	0.49 ± 0.01	N.D
Asx	11.63 ± 0.72	9.9 ± 1.0
Thr	6.27 ± 0.26	6.0 ± 0.3
Ser	6.56 ± 0.18	8.3 ± 1.6
Glx	11.91 ± 0.45	13.8 ± 0.9
Pro	N.D	2.2 ± 0.2
Gly	8.75 ± 0.33	9.3 ± 1.2
Ala	8.22 ± 0.09	8.2 ± 0.5
Val	5.7 ± 0.20	4.0 ± 0.2
Met	2.09 ± 0.07	1.2 ± 0.6
Ile	5.43 ± 0.34	4.5 ± 0.1
Leu	10.55 ± 0.43	10.7 ± 0.9
Tyr	N.D	3.8 ± 0.6
Phe	4.09 ± 0.08	4.0 ± 0.4
His	1.99 (n=1)	1.5 ± 0.4
Lys	6.67 ± 0.63	7.6 ± 1.1
Arg	5.94 ± 0.14	5.7 ± 0.7
Trp	N.D	N.D

Comparison with the human 32 kDa lipocortin purified from human blood mononuclear cells. Data are expressed as mol % ± S.E. (S.D. for the human protein) of 3 preparations (n). N.D: Not done.

preincubated for 20 min at 37 °C with a known amount of the 36 kDa I protein (50 µl). Cells were then exposed to the calcium ionophore A23187. The latter induced a dose-related increase of [^{3}H]-AA release from the cells (figure 4A). The protein (2µg)-treated cells released significantly less radioactivity in the medium than control cells. This protein-induced inhibition of [3H]-AA release from labeled thymocytes was reproduced in a concentration-dependent manner (Figure 4B). Mouse 36 kDa II and 73 kDa protein were also able to inhibit similarly the release of [^{3}H]-AA from prelabeled and stimulated thymocytes (data not shown).

DISCUSSION

In this report, we have investigated the cellular PLA2 activity of mouse thymocytes, after an in vivo dexamethasone treatment of the animals. Our data show that the intravenous administration of dexamethasone to mice has induced an inhibition of the cellular PLA2 activity in their thymocytes. This effect was estimated by the decrease of [^{3}H]-AA release from labeled and stimulated thymocytes. The release of [^{3}H]-AA is supposed to derive essentially from cellular PLA2 activation. This result supports the hypothesis of

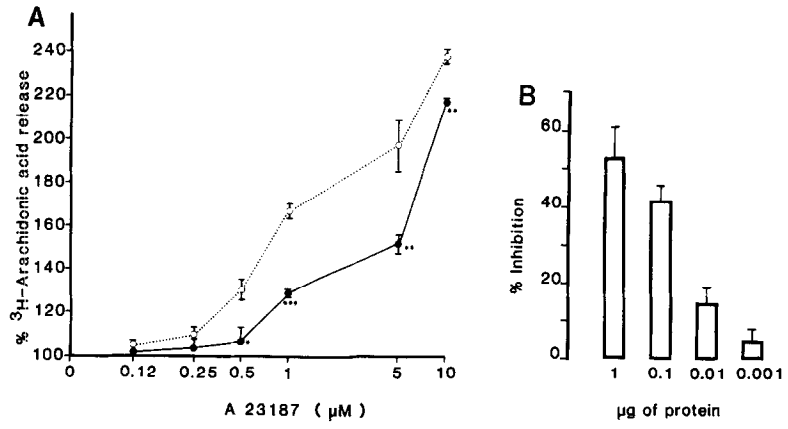

Figure 4
Effect of mouse 36 kDa I protein on [^{3}H]-Arachidonic acid release from A23187-stimulated thymocytes.
A) [^{3}H]AA-labeled thymocytes (25,000-30,000 cpm /4-5 x 10^6 / 0.4 ml) were preincubated for 20 min at 37 °C with 2 µg (50 µl) of mouse 36 kDa I protein (——) or with saline (- - -).The radioactivity released from A23187-treated cells was calculated as a per cent of the radioactivity released from DMSO-treated cells. The experiment was performed at least three times. The results of a typical experiment performed in triplicates are given as mean ± S.E. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.
B) Dose-response curve of mouse 36kDa I protein on [^{3}H]-Arachidonate release from prelabeled thymocytes. [^{3}H]-AA-labeled thymocytes were preincubated with increasing amounts of mouse 36 kDa I protein or with its vehicle for 20 min at 37 °C. Cells were then stimulated. The experiment was performed at least three times. Results of a typical experiment performed in triplicates are given as a % inhibition of the radioactivity released from 36 kDa I-treated cells versus vehicle-treated cells. Data are expressed as mean ± S.E.

corticosteroid-induced PLA2 inhibitory effect (1). Furthermore, we have purified three PLA2 inhibitory proteins from mouse thymus. The amino acid composition of the mouse 36 kDa I protein and the human 32 kDa lipocortin (14) was highly similar. Indeed, both mouse 36 kDa I protein and human 32 kDa lipocortin cross-reacted with their antisera, as well as they cross-reacted with the monoclonal antibody BF26 raised against rat LC. These data show that the two mouse 36 kDa proteins belong to the family of lipocortins but are different from the calpactins (i.e. LC I and LC II). The mouse 73 kDa protein did not cross-react with the monoclonal antibody BF26, despite its PLA2 inhibitory activity. Further investigations will permit to establish the precise relationship of these purified mouse thymus PLA2 inhibitory proteins with the recently described lipocortin family (23), and to clarify the mechanism of inhibition of cellular PLA2 activity induced by these proteins. Nevertheless, the three purified mouse PLA2 inhibitory proteins (36 kDa I and II, 73 kDa) have reproduced the dexamethasone-induced PLA2 inhibitory effect on mouse thymocytes, although, dexamethasone was given in vivo. Therefore, our results support the recent data regarding the ability of lipocortins to act on isolated cells when added exogenously (10-14). Further investigations are required to establish whether these proteins are directly involved in the PLA2 inhibitory effect induced by dexamethasone treatment of mice in vivo.

ACKNOWLEDGMENT: We thank Anne Marie Gilles for performing the amino acid analysis.

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