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A Phospholipase A_2-Related Snake Venom (from *Crotalus durissus terrificus*) Stimulates Neuroendocrine and Immune Functions: Determination of Different Sites of Action*

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ABSTRACT

Immune neuroendocrine interactions are vital for the individual's survival in certain physiopathological conditions, such as sepsis and tissular injury. It is known that several animal venoms, such as those from different snakes, are potent neurotoxic compounds and that their main component is a specific phospholipase A type 2 (PLA_2). It has been described recently that the venom from *Crotalus durissus terrificus* [snake venom (SV), in the present study] possesses some cytotoxic effect in different *in vitro* and *in vivo* animal models. In the present study, we investigated whether SV and its main component, PLA_2 (obtained from the same source), are able to stimulate both immune and neuroendocrine functions in mice, thus characterizing this type of neurotoxic shock. For this purpose, several *in vivo* and *in vitro* designs were used to further determine the sites of action of SV-PLA_2 on the hypothalamo-pituitary-adrenal (HPA) axis function and on the release of the pathognomonic cytokine, tumor necrosis factor α (TNFα), of different types of inflammatory stress. Our results indicate that SV (25 μg/animal) and PLA_2 (5 μg/animal), from the same origin, stimulate the HPA and immune axes when administered (ip) to adult mice; both preparations were able to enhance plasma glucose, ACTH, corticosterone (B), and TNFα plasma levels in a time-related fashion. SV was found to activate CRH- and arginine vasopressin-ergic functions *in vivo* and, *in vitro*, SV and PLA_2 induced a concentration-related (0.05–10 μg/ml) effect on the release of both neuropeptides. SV also was effective in changing anterior pituitary ACTH and adrenal B contents, also in a time-dependent fashion. Direct effects of SV and PLA_2 on anterior pituitary ACTH secretion also were found to function in a concentration-related fashion (0.001–1 μg/ml), and the direct corticotropin-releasing activity of PLA_2 was additive to those of CRH and arginine vasopressin; the corticotropin-releasing activity of both SV and PLA_2 were partially reversed by the specific PLA_2 inhibitor, manoalide. On the other hand, neither preparation was able to directly modify spontaneous and ACTH-stimulated adrenal B output. The stimulatory effect of SV and PLA_2 on *in vivo* TNFα release was confirmed by *in vitro* experiments on peripheral mononuclear cells; in fact, both PLA2 (0.001–1 μg/ml) and SV (0.1–10 μg/ml), as well as concavalin A (1–100 μg/ml), were able to stimulate TNFα output in the incubation medium.

Our results clearly indicate that PLA_2-dependent mechanisms are responsible for several symptoms of inflammatory stress induced during neurotoxemia. In fact, we found that this particular PLA_2-related SV is able to stimulate both HPA axis and immune functions during the acute phase response of the inflammatory processes. (*Endocrinology* **139:** 617–625, 1998)

PHOSPHOLIPASE A_2 (PLA_2) is a lipolytic enzyme that hydrolyses the fatty acyl ester at the sn-2 position of membrane phospholipids producing equimolar amounts of lisophosphatide and FFA, mainly arachidonic acid (AA); these products then become available for conversion to potent proinflammatory mediators, such as platelet-activating factor (1) and eicosanoids (2), respectively. Further AA metabolism is initiated by the three key enzymes known as cyclooxygenase, lipoxygenase, and epoxygenase (3). It is accepted that AA cascade metabolites modulate the hypothalamo-pituitary-adrenal (HPA) axis function by controlling CRH release (4). Because immune cells-derived cytokines have been described as stimulators of PLA_2 and cyclooxygenase activities (5, 6) and because prostaglandins play a role in the interleukin (IL)-1-stimulated ACTH output *in vivo* (7, 8), the importance of neurotoxins, with intrinsic PLA_2 activity, on the stimulation of the HPA and immune axes remains an interesting open field of research.

Bidirectional communication between the immune and HPA axes is already well accepted. High levels of PLA_2 activity have been reported during several inflammatory diseases (9, 10) and, as mentioned above, the integrity of the HPA axis function protects the organism after injury or tissue damage (11). PLA_2 has been shown to induce pituitary ACTH and β-endorphin secretion (12), and this enzyme is an important component of several snake (among other species) venoms with intrinsic presynaptic neurotoxin activity (13).

The venom from *Crotalus durissus terrificus* origin [snake venom (SV)] belongs to this category; it is known that this SV induces a local inflammatory process, characterized by vascular injury and the release of several mediators of inflammation (14), and that it stimulates HPA axis function when

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administered *in vivo* to rats (15). It has been suggested that the neurotoxic effect of several PLA_2-related snake venoms is caused by the hydrolysis of cell membrane phospholipids (16), and that there is a dissociation between neurotoxicity and enzymatic activity (17). For instance, β-bungarotoxin binds to and blocks a subtype of voltage-gated K^+ channels by a mechanism independent of its PLA_2 activity (17, 18). In addition, this SV has been described to cause a cytotoxic effect *in vivo* on mouse Lewis lung carcinoma (19), and *in vitro* on murine erythroleukemia cells (20).

Thus, the aims of the present study were: 1) to elucidate whether SV-induced neurotoxic shock is mediated by increased HPA and immune functions in mice; and 2) to determine whether the main component of this SV, PLA_2, is responsible for some of the SV effects. For these purposes, several experiments were performed using both *in vivo* and *in vitro* paradigms.

Materials and Methods

Animals

Adult (9–11 weeks old) random cycling female BALB/c mice were used in all experiments, they were kept in standard conditions of light (on between 0700 to 1900 h) and temperature (22 ± 2 C) and fed with laboratory chow and tap water *ad libitum*. The animals to be used for *in vivo* studies were handled gently daily, for a week, to minimize stress conditions. Experiments were carried out during the circadian trough of the HPA axis (21), between 0800 and 0900 h. All procedures were done according to our institution's animal care rules.

In vivo experiments

Several groups of mice were ip injected with 50 μl of vehicle alone (Veh: sterile saline solution; n = 4–5 mice per time-point) or vehicle containing snake venom (SV: from *Crotalus durissus terrificus*, Sigma Chemical Co. V-7125, 25 μg per mouse; n = 9–11 mice per time-point) and returned to their cages. In preliminary experiments, we found that this dose of SV was lethal for 30% of the animals at 4 h after treatment. Thereafter, experiments were carried out by evaluating metabolic changes occurred up to 2 h after treatment; for this purpose mice were killed by decapitation at either 0.5, 1, or 2 h after Veh or SV treatment. Trunk blood was collected in plastic tubes containing EDTA and plasma samples kept frozen (−20 C) until further determinations of ACTH, corticosterone (B), tumor necrosis factor α (TNFα), and glucose (GLU; by the GLU-oxidase method from Wiener Argentina Laboratories) concentrations. Immediately after decapitation, brain tissues were quickly removed; and the hypothalamus (HT) [containing the median eminence (ME), limits: anterior, border of the optic chiasm; posterior, border of the mammillary bodies; and lateral, HT border, approximately 2–3 mm deep], the anterior pituitary (AP) gland, the neurointermediate lobe of the pituitary gland (NIL), and the adrenal glands (AG) were dissected, as previously described (22), and transferred into Eppendorf tubes containing a small vol (300 μl, 500 μl, 100 μl, and 100 μl for HT, AP, NIL, and AG, respectively) of acetic acid 0.1 N; tissues were then sonicated (20–30 sec) and centrifuged at 10,000 × *g* at 4 C, 3–4 min, and the supernatants kept frozen (−20 C) until the determination of tissue hormone content (HT CRH and vasopressin; AP ACTH, NIL vasopressin, and AG glucocorticoid). Additional mice groups (8–10 mice per time-point) were ip injected with 5 μg PLA_2 (from the same snake venom source, Sigma P-5910), returned to their home cages, and killed at similar times to those described above; plasma samples were kept frozen (−20 C) until determination of GLU, ACTH, B, and TNFα concentrations.

In vitro experiments

Adult female mice were decapitated, under minimal stress condition, and brain tissues quickly removed. Immediately thereafter, HTs and APs were dissected. Additional groups of mice also were killed, as described above, for further dissection of the ME (22), the APs, and the AGs. Tissues were then used in the experiments described below.

Incubation of mouse HT. This method is similar to the one previously described (23), with few modifications. Briefly, HTs were placed in Earle's balanced salt solution (Grand Island Biological Corp., Grand Island, NY) containing BSA (0.2%, wt/vol), $NaCO_3H$ (1 g/liter), GLU (1 g/liter), ascorbic acid (20 mg/liter), Trasylol (100 IU/ml; Aprotinin, Mobay Chemical Corp., New York, NY), and antibiotics, pH 7.4 (incubation medium). Each HT was transferred into a plastic flask containing 1 ml of fresh incubation medium and washed by shaking for 20 min at 37 C in a 95% O_2-5% CO_2 atmosphere. At least six HTs per control or test group were used in each experiment. After the wash, media were discarded, and the HTs were resuspended in 1 ml of fresh incubation medium and incubated for 40 min as described above. The HTs were incubated for a second 40-min period in 1 ml of fresh medium alone (control) or medium containing SV (0.1, 1, and 10 μg/ml) or PLA_2 (0.01, 0.1, and 1 μg/ml), and at the end of the incubation, media were decanted and frozen (−20 C) until CRH and arginine vasopressin (AVP) measurement by specific assays.

Superfusion of mouse ME fragments. This method also is similar to the one previously described (23). ME fragments (16 per experiment) were packed in a polystyrene syringe (Terumo Europe NV, Belgium; 2.5 cm^3) and allowed to stabilize by superfusing them with presaged (with 95% O_2-5% CO_2) incubation medium (0.25 ml/min) for 20 min. Then, ME fragments were superfused with medium alone (basal condition) or medium containing either KCl (48 mM; 8 min) or PLA_2 (0.05, 0.5, and 5 μg/ml; 12 min) or SV (0.1, 1 and 10 μg/ml; 12 min). Medium CRH and AVP concentrations were determined in the 8-min fractions collected.

Superfusion of mouse AP-dispersed cells. This methodology has been described in detail in a previous article (24). Briefly, APs were enzymatically dispersed, packed in a column (6,000,000 AP cells/column, approximately), and superfused (0.35 ml/min) with medium only (basal) or medium containing PLA_2 (0.001, 0.01, and 0.1 μg/ml), SV (0.01, 0.1, and 1 μg/ml), CRH (Sigma; 1 ng/ml), AVP (Sigma; 100 ng/ml), or different combinations (3-min pulses). Medium ACTH concentration was measured in the 3-min fractions collected.

Incubation of isolated AP cells. This method is similar to the one earlier described, with minor modifications (25). Mouse-dispersed AP cells were obtained, as described above, and resuspended in 10–15 ml of incubation medium; they were preincubated at 37 C by shaking for 30 min in a 95% O_2-5% CO_2 atmosphere. Cells were then centrifuged (10 min at 100 × *g*, at room temperature) and resuspended in an appropriate volume of incubation medium to obtain a final concentration of 80,000 cells/0.9 ml of medium; this volume was then distributed into 12 × 75-mm polystyrene tubes and incubated with (0.1 ml) medium alone (basal) or medium containing PLA_2 (0.01 μg/ml), SV (0.1 μg/ml), and manoalide (MLD, Calbiochem-Novabiochem Corp., La Jolla, CA; 0.5 μg/ml). When MLD was included in a particular assay, it was added in a 10-μl vol. Tubes were then incubated by shaking for 2 h at 37 C in similar conditions to those described above. In each experiment, at least 8 tubes were used for each control or test substance. At the end of incubation, the tubes were centrifuged for 10 min at room temperature, and the supernatant was separated from the cell pellet for ACTH measurement.

Incubation of dispersed AG cells. This method has been previously described (26). Briefly, AGs (dissected free of adipose tissue) were enzymatically dispersed and resuspended in 15–20 ml of incubation medium and preincubated at 37 C by shaking for 30 min in a 95% O_2-5% CO_2 atmosphere. Cells were then centrifuged (10 min at 100 × *g*, at room temperature) and resuspended in an appropriate volume of incubation medium to obtain a final concentration of 150,000 cells/0.8 ml of medium; this volume was then distributed into 12 × 75-mm polystyrene tubes and incubated with 0.2 ml medium alone (basal), or medium containing PLA_2 (0.005, 0.05, and 0.5 μg/ml), or SV (0.01, 0.1, and 1 μg/ml), or ACTH (Calbiochem-Novabiochem Corp.; 1 ng/ml), or different combinations. In each experiment, at least 10 tubes were used for each control or test substance. After 2 h incubation of the tubes, in similar conditions to those described above, they were centrifuged at 100 × *g* for 10 min at room temperature, and supernatants were frozen (−20 C) until the measurement of B concentrations.

Incubation of peripheral mononuclear cells (PMNC). This method is similar to the one previously described, with minor modifications (27). Heparinized blood was collected by right jugular vein puncture from mice under light ether anesthesia. The PMNC were isolated by density (1.077 g/ml)-gradient in the Ficoll solution (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). Blood sample:Ficoll solution (1:1) were centrifuged at 700 × *g* for 30 min at room temperature. PMNC were washed twice with sterile saline solution, and the final pellet was resuspended with an appropriate volume of RPMI-1640 (HEPES 25 mM, antibiotics, 10% FCS, pH 7.3) to obtain 100,000 PMNC per 0.1 ml of medium; this volume of PMNC suspension was distributed into 96-well flat bottom microtiter trays and cultured for 48 h inside the humidified chamber of a 5% CO_2 incubator with 0.1 ml of medium alone (control) or medium containing (final concentration) Concavalin A (Con A, Sigma, C-2010; 1, 10, and 100 μg/ml), PLA_2 (0.001, 0.01, 0.1 and, 1 μg/ml), or SV (0.01, 0.1, 1, and 10 μg/ml). In parallel, 0.1 ml of medium alone or medium containing different test substances (at similar concentrations, as described above) were incubated, in similar conditions, in 96-well trays containing 0.1 ml of medium without PMNC. At least 5–6 wells were run for each control, or test substance, in each experiment. At the end of culture, 0.1-ml aliquots were separated and kept frozen (−20 C) until assayed for TNFα concentrations, as described below.

Hormones and cytokine measurements

Plasma and medium concentrations of ACTH were determined by a previously described immunoradiometric assay (28), and those of B by a specific RIA earlier reported in detail (22). The intraassay coefficients of variation were 2–3 and 4–6%, for ACTH and B, respectively; and, the interassay coefficients of variation were 6–8 and 8–10%, for ACTH and B, respectively. AVP sample concentration was determined by a specific RIA previously reported (22); the intra- and interassay coefficients of variation were 5–8 and 10–12%, respectively. Medium CRH concentration was measured by a specific immunoradiometric assay similar to one described before (29), developed in our laboratory. Briefly, standard (h, r CRH from Calbiochem, Switzerland; range 1,250–5 pg/ml) and samples (200 μl) were incubated (16 h at 4 C) in assay buffer with 50 μl of sheep anti-CRH (developed against the C-terminal portion) and 50 μl of ^{125}I-labeled rabbit anti-CRH (developed against the N-terminal portion); CRH bound ^{125}I-labeled rabbit anti-CRH IgG was separated from free by incubation (3 h at 4 C) in the presence of 50 μl of donkey antisheep IgG (1:8) followed by the addition of 1 ml of 3% wt/vol polyethylene glycol solution and centrifuged 30 min at 4,000 rpm at 4 C. After a second wash with 1 ml of 3% polyethylene glycol, supernatants were aspirated to waste before radioactivity was counted; the intra- and interassay coefficients of variation ranged between 2–3 and 6–8%, respectively. Standard curves, in different assays, were run in parallel in the presence of various concentrations of either SV or PLA_2, and they did not show any interference, regardless of the assay.

The assay of the cytokine consisted in the determination of the cytolytic effect of TNFα on L929 cells (from mouse fibrosarcoma), as previously described (30). TNFα, used as standard, was purchased from Genzyme Lab. (82437). Cells were maintained in MEM containing 10% (vol/vol) of FCS, glutamine, and antibiotics (pH 7.4). Ninety six-well microtiter trays were seeded at 6×10^4 L929 cells per well in 100 μl of culture medium and incubated 24 h in 5% CO_2 atmosphere at 37 C. On the following day, 100 μl TNFα standard solution (range 20–12,000 pg/ml) and unknowns (plasma, run at 1:4, 1:8, and 1:16 dilutions; and medium samples) were added in the presence of actinomycin D (1 μg/ml) (in quadruplicate). Plates were similarly incubated for 24 h, and 50 μl crystal violet (0.05% wt/vol in methanol:water, 1:5) was added and incubated for 30 min at 37 C. Plates were rinsed with water and dried, then 100 μl per well of 33% acetic acid was added. Plates were shaken twice; and absorbance, at 595 nm, was measured in a 7530 Multiplate Reader, Cambridge Technology. The reader was blanked with a plate having more than 95% cell destruction, and absorbance was inversely proportional to TNFα bioactivity. The intra- and interassay coefficients of variation ranged between 7–9 and 9–11%, respectively.

Analysis of data

Results are expressed as the mean ± SEM. Data were analyzed by multifactorial ANOVA, followed by Fisher's test for comparison of different mean values (31).

Results

Characterization of SV-induced HPA and immune axes activation

Figure 1 shows the results of several metabolites in plasma samples before (sample time zero) and at several times after ip injection of mice with SV (25 μg/animal). It must be pointed out that ip administration of Veh did not significantly vary plasma and tissue metabolite concentrations at all time-points studied; thereafter, all time values were pooled, and they represent the sample time-zero values. Figure 1 (*upper left panel*) shows that SV administration induced a significant ($P < 0.05$) increase, *vs.* basal values, in plasma GLU levels at all time-points studied after treatment. Figure 1 (*lower left panel*) shows that plasma ACTH levels in animals under neurotoxic shock were characterized by a peak value of ACTH in plasma at 30 min after SV; then values declined at 60 min after treatment, although they were significantly

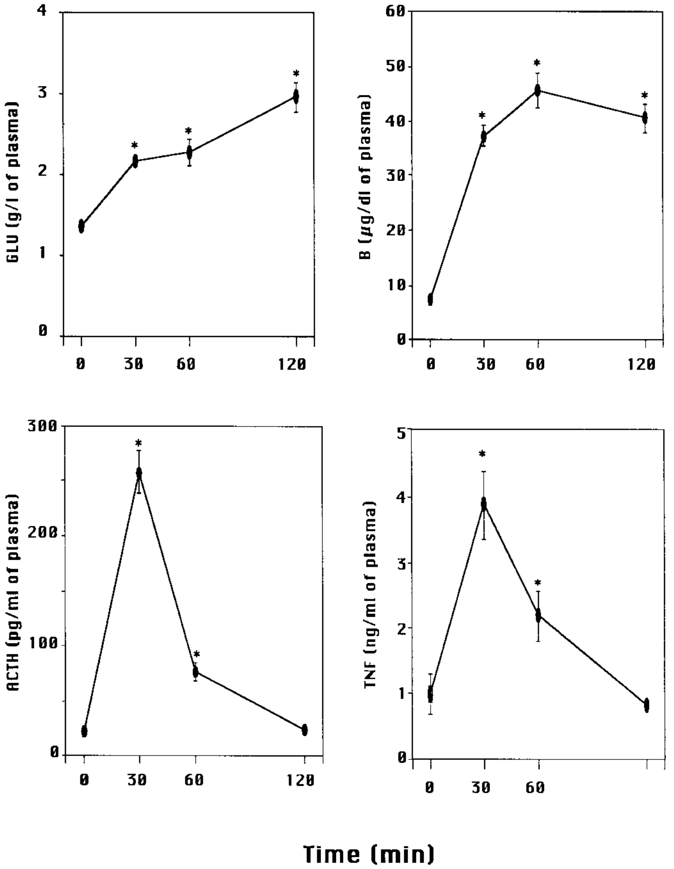

FIG. 1. Effects of *in vivo* administration of snake venom on plasma metabolites levels. Time-course of plasma glucose (*upper left panel*), ACTH (*lower left panel*), corticosterone (*upper right panel*), and TNFα (*lower right panel*) before (sample time zero) and several times after ip administration of SV (25 μg per animal) in female mice. Values are the mean ± SEM (n = 9–11 mice per time-point). *, $P < 0.05$ or less *vs.* sample time-zero values.

($P < 0.05$) higher than time-zero values. They reached the baseline at 120 min after SV injection. Figure 1 (*upper right panel*) shows plasma B levels before (sample time zero) and several times after SV injection. As depicted, at 30 min after SV administration, plasma B levels peaked, reaching maximal adrenal response ($P < 0.02$ or less *vs.* the respective sample time zero); then values remained at the maximal level up to 120 min after SV administration. Figure 1 (*lower right panel*) shows plasma TNFα before and several times after SV administration. The administration of SV (ip) was able to enhance plasma cytokine levels several fold ($P < 0.05$) over the baseline value (sample time zero) as early as 30 min after treatment; then values declined at 60 min (still significantly higher, $P < 0.05$, than the baseline), and they returned to basal plasma TNFα levels by 120 min after SV injection.

Figure 2 (*upper left panel*) shows HT CRH before and at several times after SV injection. Basal HT CRH decreased significantly ($P < 0.05$) 30 min after treatment and remained at a similar level up to 60 min after injection; thereafter, 120 min after SV, it slightly increased (*vs.* 60-min sample values), although it still remained significantly ($P < 0.05$) lower than basal values. Figure 2 (*lower left panel*) shows HT AVP in mice before and several times after SV administration. HT AVP decreased, although not significantly *vs.* sample time-zero values, at 30 min after treatment; however, the decrease in this parameter (*vs.* sample time-zero values) was significant ($P < 0.05$) at 60 min after SV administration and remained low up to 120 min after treatment. Figure 2 (*upper right panel*) shows AP ACTH before and during neurotoxic shock. AP ACTH decreased significantly ($P < 0.05$ *vs.* the respective sample time-zero values) 30 min after treatment, returning to basal levels after 60 min of shock; thereafter, AP ACTH increased several fold ($P < 0.05$) over time-zero values at 2 h after SV treatment. Figure 2 (*lower right panel*) shows AG B in mice before and several times after SV injection. The time-course of the variation was as follows: it increased significantly ($P < 0.05$ *vs.* sample time zero) 30 min after injection and reached a maximal AG B 60 min after shock; thereafter, AG B decreased toward basal values (although still significantly higher, $P < 0.05$, than basal values) 120 min after shock. Finally (see Table 1), NIL AVP did not vary throughout the entire experiment.

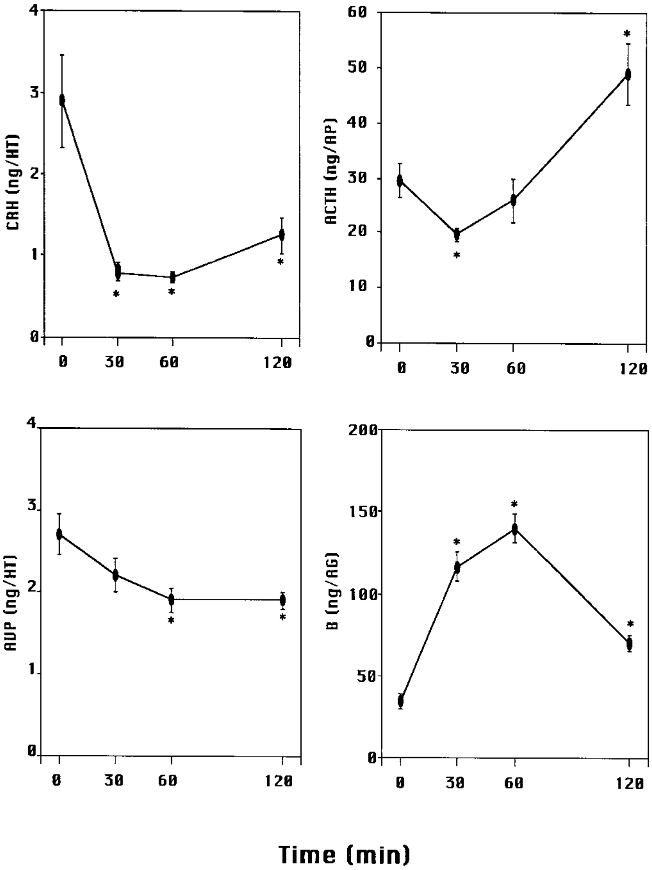

FIG. 2. Snake venom-induced changes at different levels of the HPA axis. HT CRH (*upper left panel*), AVP (*lower left panel*), AP ACTH (*upper right panel*), and AG corticosterone (*lower right panel*) contents before and several times after ip administration of SV (25 μg per animal) in female mice. Values are the mean ± SEM (n = 9–11 mice per time-point). *, $P < 0.05$ or less *vs.* sample time-zero values.

Effects of PLA_2 in vivo administration on HPA and immune axes function

Figure 3 shows the results of several parameters in plasma samples before (sample time zero) and after ip administration of PLA_2 (5 μg per mouse). Figure 3 (*upper left panel*) shows basal plasma GLU levels were significantly ($P < 0.05$) higher than the baseline (sample time-zero) 30 min after treatment; thereafter, values remained higher ($P < 0.05$) than the baseline up to 120 min after injection. Figure 3 (*lower left panel*) shows plasma ACTH levels in female mice in basal and post-PLA_2 administration conditions. A peak value of ACTH in plasma was induced 30 min after enzyme administration, then values returned to the baseline by 60 min and more after treatment. Figure 3 (*upper right panel*) shows plasma B levels before (sample time zero) and several times after PLA_2 injection. Basal plasma B levels were significantly ($P < 0.01$) enhanced over the baseline 30 min after treatment, and this maximal plasma B response remained stable up to 120 min after PLA_2 injection. Figure 3 (*lower right panel*) shows plasma TNFα before and several times after PLA_2 administration. Injection of the enzyme enhanced plasma TNFα levels ($P < 0.05$) over baseline values 30 min after treatment, then values declined by 60 min (still significantly higher, $P < 0.05$, than baseline values) and returned to basal plasma TNFα levels by 120 min after PLA_2 injection.

Effects of SV and PLA_2 on CRH and AVP release from incubated HTs

The effects of SV (0.1, 1, and 10 μg/ml) and PLA_2 (0.05, 0.5 and 5 μg/ml) on CRH output by incubated HT fragments are

TABLE 1. Neurointermediate lobe (NIL) arginine-vasopressin (AVP) content before (time zero) and several times after SV (25 μg per mouse, ip) administration

Time (min)	AVP (μg/NIL)
0	0.63 ± 0.02
30	0.61 ± 0.01
60	0.58 ± 0.02
120	0.55 ± 0.03

Results are the mean ± SEM of 9–11 mice per time-point.

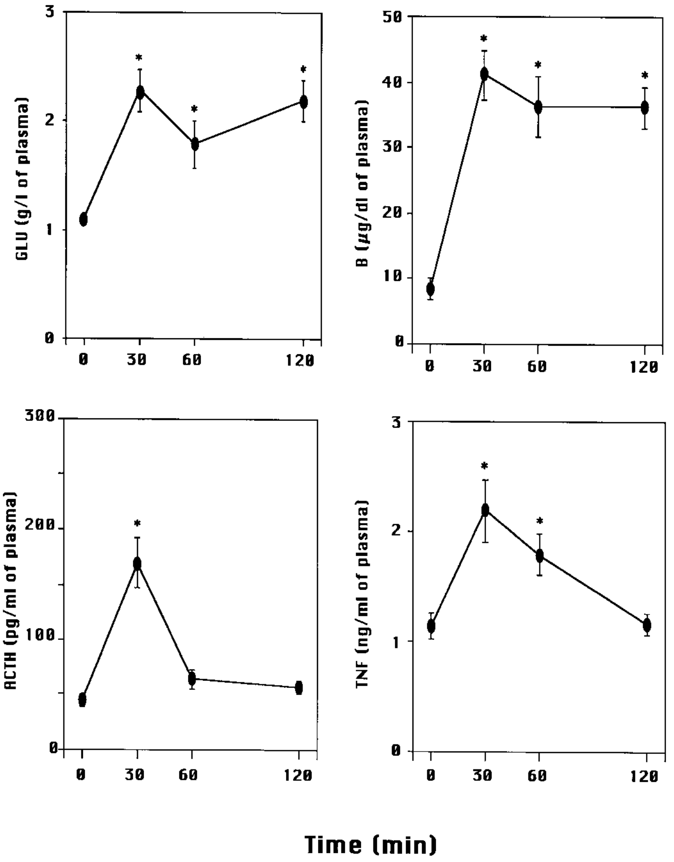

FIG. 3. Effects of *in vivo* administration of PLA_2 on plasma metabolites levels. Plasma glucose (*upper left panel*), ACTH (*lower left panel*), corticosterone (*upper right panel*), and TNFα (*lower right panel*) before (sample time zero) and several times after ip injection of PLA_2 (5 µg per mouse) in female mice. Values are the mean ± SEM (n = 6–8 mice per time-point). *, $P < 0.05$ or less *vs.* sample time-zero values.

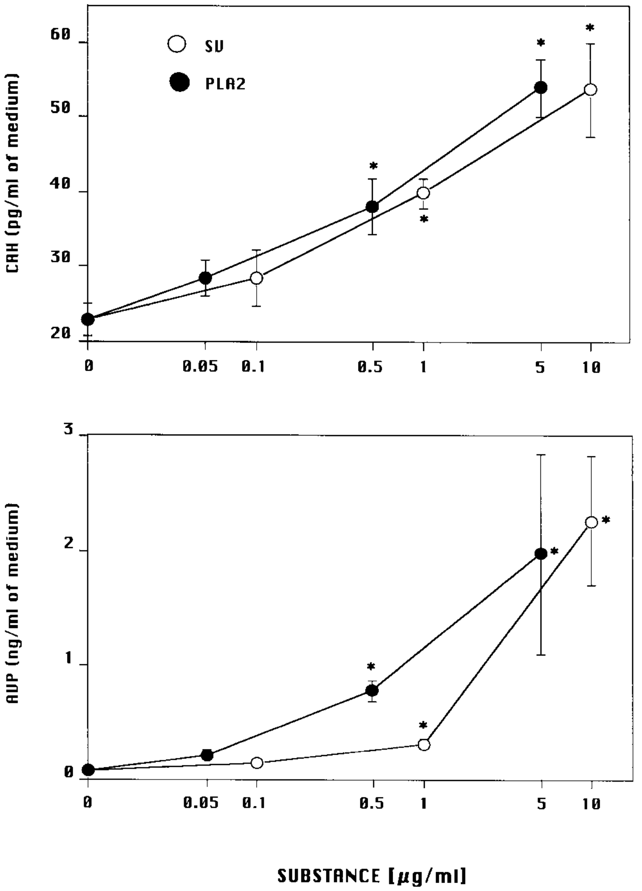

FIG. 4. HT effects of snake venom and PLA_2. CRH (*upper panel*) and AVP (*lower panel*) concentration-responses of HTs, from female mice, incubated *in vitro* with SV and PLA_2 at several concentrations. Results are the mean ± SEM of three different experiments with at least six tubes per point per experiment. *, $P < 0.05$ *vs.* baseline values (substance concentration zero).

shown in Fig. 4 (*upper panel*). A clear, concentration-related, effect of SV and PLA_2 on CRH secretion was found. However, only the intermediate (1 and 0.5 µg/ml for SV and PLA_2, respectively) and the highest concentrations of both products tested were able to significantly ($P < 0.05$ or less) enhance HT CRH release over basal values (concentration zero).

Figure 4 (*lower panel*) shows HT AVP secretion into the medium in basal condition (concentration zero) and after incubation with several concentrations of SV or PLA_2. As depicted, only the highest SV concentration (10 µg/ml) was able to significantly ($P < 0.05$) enhance HT AVP release over basal values. As for the effect of PLA_2, both the intermediate and the highest concentrations (0.5 and 5 µg/ml) were effective in significantly ($P < 0.05$) increasing HT AVP output over basal values.

Determination of an ME site of action of SV and PLA_2 on neuropeptide secretion

To determine whether SV and PLA_2 are able to stimulate ME CRH and AVP secretion, ME fragments (from female mice) were superfused (12 min) with several concentrations of these substances or with 48 mM KCl (8 min). CRH secretion above baseline (8.67 ± 1.94 pg of CRH/ml of medium per 8-min fraction, n = 3 different experiments with 21 tubes per experiment) was 107 ± 30 pg CRH (mean ± SEM, n = 3 different experiments) after stimulation with 48 mM KCl (2 stimulations per experiment). Superfusion with either PLA_2 (0.05, 0.5, and 5 µg/ml) or SV (0.1, 1, and µg/ml) solutions significantly ($P < 0.05$) enhanced ME CRH release over the baseline in a concentration-dependent fashion (Fig. 5, *upper panel*). Similarly, KCl and test substances also were able to significantly ($P < 0.05$) increase ME AVP output over the baseline (131 ± 22 pg AVP/ml of medium per 8-min fraction, n = 3 different experiments with 21 tubes per experiment) although maximal AVP release was induced by the 2 highest concentrations of PLA_2 and by the highest concentration of SV (Fig. 5, *lower panel*).

Effects of SV and PLA_2 on AP ACTH secretion

To establish whether SV and its component, PLA_2, are able to stimulate ACTH secretion from AP-dispersed cells, packed cells (6,000,000 approximately) were superfused (3 min) with CRH (1 ng/ml), AVP (100 ng/ml), PLA_2 (0.001, 0.01, and 0.1 µg/ml) or SV (0.01, 0.1, and 1 µg/ml); and the ACTH released in response to these stimuli was expressed as

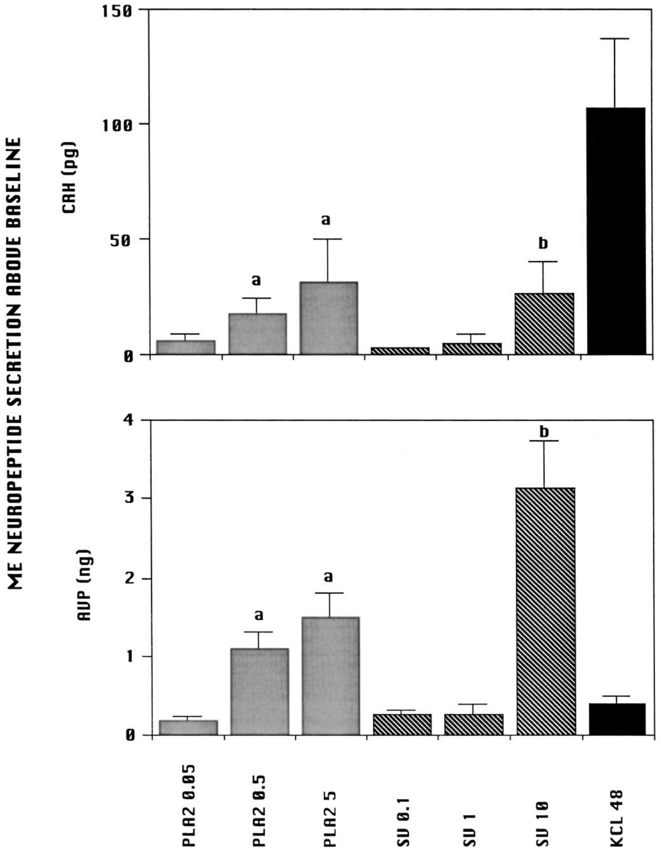

FIG. 5. Effects of snake venom and PLA_2 on neuropeptide secretion by ME nerve terminals. CRH (*upper panel*) and AVP (*lower panel*) concentration-responses of ME nerve terminals, from female mice, superfused with PLA_2 (0.05, 0.5, and 5 μg/ml; 12 min) or SV (0.1, 1, and 10 μg/ml; 12 min); additionally MEs were superfused with 48 mM KCl (8 min). *Bars* represent the mean ± SEM (n = 3 different experiments) of net neuropeptide release (total output minus the baseline). All values, except CRH secretion induced by SV 0.1 μg/ml, are significantly ($P < 0.05$) greater than the baseline. a, $P < 0.05$ *vs.* PLA_2 0.05 μg/ml values; b, $P < 0.05$ *vs.* SV 0.1 μg/ml values.

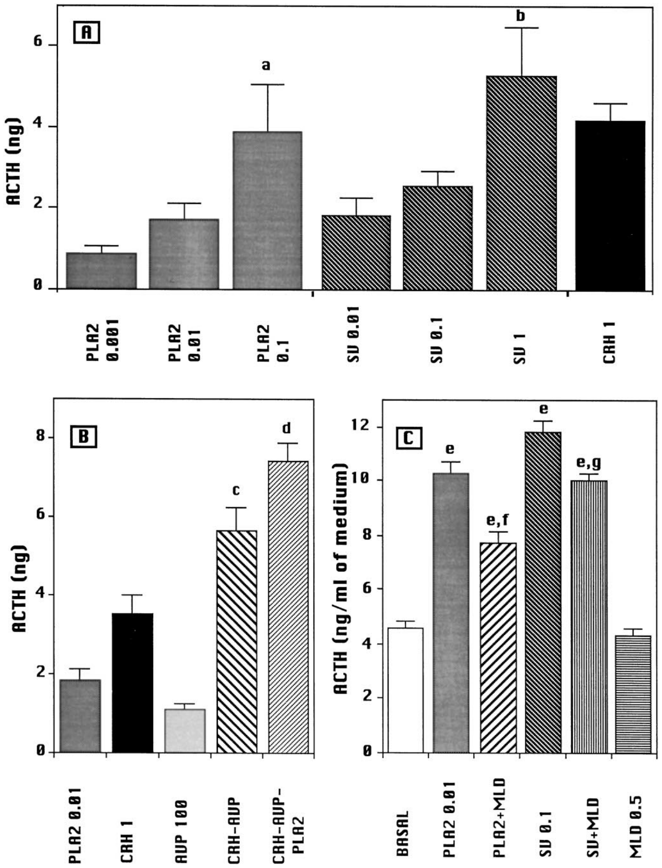

FIG. 6. Corticotropin-releasing activity (CRA) of snake venom and PLA_2 on mice AP isolated cells. A, CRA (expressed as total ACTH secretion minus the baseline) of PLA_2 (μg/ml), SV (μg/ml), and CRH (ng/ml) (3-min pulses) by superfused isolated AP cells from female mice (*bars* are the mean ± SEM of three different experiments); B, CRA of PLA_2 (μg/ml), CRH (ng/ml), AVP (ng/ml), and different combinations (3-min pulses) by superfused isolated AP cells from female mice (*bars* are the mean ± SEM of three different experiments); C, spontaneous (basal) and PLA_2 (0.01 μg/ml)- and SV (0.1 μg/ml)-induced ACTH release by incubated dispersed AP cells, from female mice, and the effect of MLD (0.5 μg/ml) on PLA_2- and SV-stimulated ACTH output (*bars* are the mean ± SEM of three different experiments with eight tubes per test-substance per experiment); a, $P < 0.05$ *vs.* PLA_2 0.001 μg/ml values; b, $P < 0.05$ *vs.* SV 0.01 μg/ml values; c, $P < 0.05$ *vs.* the addition of individual CRH and AVP values; d, additive effect of PLA_2 values to those of the CRH and AVP combination; e, $P < 0.05$ or less *vs.* basal values; f, $P < 0.05$ *vs.* PLA_2 0.01 μg/ml values; g, $P < 0.05$ *vs.* SV 0.1 μg/ml values.

net ACTH released (total release minus baseline, n = 6 different experiments with 30–36 tubes per experiment, 0.76 ± 0.06 ng/ml of medium per 3-min fraction). Figure 6A shows that superfused AP cells released a significant ($P < 0.05$) amount of ACTH over the baseline after stimulation with either (CRH, PLA_2, and SV) stimulus. Both PLA_2 and SV test substances were able to enhance ACTH release over the baseline in a concentration-related fashion; for comparison purposes, the ACTH-releasing activity of 1 ng/ml CRH (3 min) is also shown. Figure 6B shows ACTH-releasing activity of the intermediate concentration of PLA_2 (0.01 μg/ml), CRH (1 ng/ml), and AVP (100 ng/ml), and that of different combinations. In our experiments, AVP (100 ng/ml) induced lower ($P < 0.05$) ACTH secretion than that of CRH. When combined, CRH and AVP potentiate their effects; and the PLA_2 effect was additional to that of the CRH-AVP combination. Similarly, the ACTH-releasing activity of PLA_2 was additive to those of CRH and AVP individually (data not shown). Finally, to determine whether the ACTH-releasing activity of SV is mediated by specific PLA_2 binding sites in AP cells, isolated AP cells (80,000 cells per ml, approximately) were incubated with medium alone (basal) and medium containing either SV (0.1 μg/ml) or PLA_2 (0.01 μg/ml). Figure 6C shows that SV and PLA_2 significantly ($P < 0.05$) increased ACTH output over the baseline after 2 h of incubation. Incubation of cells with either PLA_2 or SV in the presence of MLD (0.5 μg/ml), a PLA_2 inhibitor, significantly ($P < 0.05$) reduced the ACTH-releasing activity of PLA_2 and SV (Fig. 6, *lower panel*). Finally, MLD (by itself) did not modify spontaneous ACTH secretion (also see Fig. 6C).

Effects of SV and PLA_2 on adrenal glucocorticoid secretion

To evaluate whether PLA_2 and SV could directly modify B release when incubated with isolated total AG cells, these substances were added to the incubates at several concentrations: 0.005, 0.05, and 0.5 μg/ml for PLA_2 and 0.01, 0.1, and 1 μg/ml for SV. See Table 2 for results (mean ± SEM, n = 2 different experiments, with 8–10 flasks per point per experiment). Neither PLA_2 nor SV (at several concentrations) was able to modify basal B secretion from incubated dispersed adrenal cells. Table 2 shows that ACTH (220 pM) was effective in increasing B output several fold ($P < 0.0001$) over the baseline, whereas coincubation of PLA_2 (0.05 μg/ml) with ACTH (220 pM) did not alter ACTH-stimulated B secretion.

TNFα-releasing activity of PLA_2 and SV on incubated PMNC

When PMNC (100,000 cells per well, approximately) were incubated (48 h) with medium alone (control), a detectable amount of TNFα was found in the medium; see Fig. 7 for these results (mean ± SEM, n = 3 different experiments, 5–8 wells per point per experiment). Incubation of PMNC with Con A (1, 10, and 100 μg/ml) induced a significant ($P < 0.05$) output of TNFα above control values. When PMNC were incubated with PLA_2 (0.001, 0.01, 0.1, and 1 μg/ml), TNFα secretion was significantly ($P < 0.05$) increased above control values. Similarly, PMNC incubated in the presence of SV (0.1, 1, and 10 μg/ml) released a significantly ($P < 0.05$) higher amount of TNFα into the medium above control values. Finally, control or test substances incubated with 0.1 ml of medium alone (instead of PMNC) did not induce any cytolytic effect on L929 cells, thus indicating a specific effect of the substances on TNFα output by PMNC (data not shown).

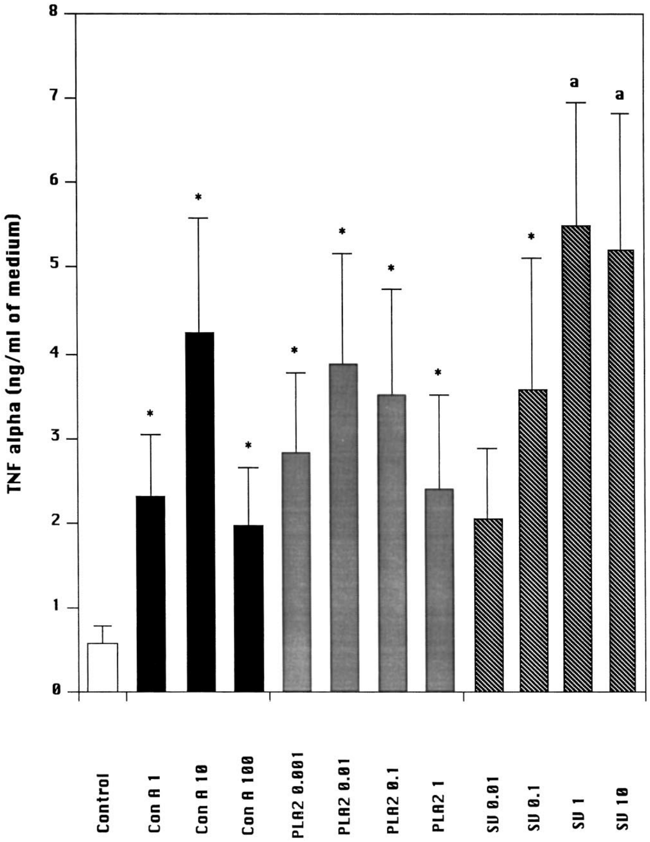

FIG. 7. *In vitro* stimulatory effects of snake venom and PLA_2 on immune function. Effects of medium alone (control) and medium containing several concentrations (μg/ml) of either Con A, PLA_2, or SV on TNFα release by incubated PMNC from female mice. *Bars* are the mean ± SEM of three different experiments (with 5–8 tubes per test-substance per experiment). *, $P < 0.05$ or less *vs.* control values; a, $P < 0.05$ or less *vs.* control and SV 0.01 values.

Discussion

In the present study, we have demonstrated that both SV and its main component, PLA_2, are able to stimulate *in vivo* HPA and immune axes function in mice. The *in vivo* neurotoxic shock was characterized by a time-related: 1) hyperglycemic effect; 2) increase in the release of ACTH, glucocorticoid, and TNFα in plasma; and 3) change in HT ACTH-releasing neuropeptides (CRH and AVP), AP ACTH, and AG glucocorticoid. During this type of shock, we found that the activation of the HPA and immune axes function did not involve changes in magnocellular AVP production. It is important to stress that the activation of the HPA axis by either SV or PLA_2 was mainly caused by a stimulatory effect of either substance on HT neuropeptide (CRH and AVP) release, acting at both the entire neuronal systems (HT) and on ME nerve terminals. Because of these effects, increased secretion of CRH and AVP, in turn, stimulate AP ACTH synthesis and release. We also have found that SV and PLA_2 directly stimulate AP ACTH output and that the ACTH-releasing activity of PLA_2 is additive to that exerted by CRH plus AVP. We also determined that the effect of this particular SV at pituitary level, at least in part, is caused by the activation of specific PLA_2 binding sites in isolated AP cells, because MLD (a specific PLA_2 inhibitor) was able to significantly reduce SV- and PLA_2-induced ACTH secretion. Conversely, none of these products was effective in modifying either magnocellular AVP metabolism or spontaneous and ACTH-stimulated AG glucocorticoid output, thus indicating the level-specificity of such events on the stimulation of HPA axis function. Regarding the effect of PLA_2 and SV on the immune system, our results indicate that PLA_2-related events are responsible for activation of the immune function, because the increase in TNFα output in plasma during neurotoxemia could very well be caused by an effect, at least in part, on peripheral immune cells; in fact, we found that SV and PLA_2, from the same source, have stimulatory activity on the release of TNFα by PMNC. We earlier described that *in vivo* single administration of the same SV used in the present study induced, in both LEW/N and F344/N rats, an increase in plasma ACTH levels, over the baseline, 1 h after admin-

TABLE 2. Effects of PLA_2 and SV on corticosterone (B) output by isolated total adrenal gland cells incubated *in vitro*

	B secretion (ng/ml of medium)
BASAL	9.36 ± 2.11
PLA_2 (0.005 μg/ml)	7.35 ± 1.25
PLA_2 (0.05 μg/ml)	6.54 ± 1.01
PLA_2 (0.5 μg/ml)	9.27 ± 3.15
SV (0.01 μg/ml)	7.18 ± 2.01
SV (0.1 μg/ml)	6.68 ± 1.23
SV (1 μg/ml)	6.29 ± 1.37
ACTH (220 pM)	519.71 ± 36.81[a]
ACTH 220 pM + PLA_2 0.05 μg/ml	483.92 ± 63.22[a]

[a] $P < 0.001$ *vs.* basal values.

istration (15). In that study, we proposed the possibility of a stimulatory effect by the PLA_2-related venom on immune cells that would increase peripheral plasma cytokines levels. This issue has now been confirmed by the findings that at least TNFα release in plasma is enhanced by SV and PLA_2 and that both products are effective in stimulating the secretion of this cytokine when incubated with PMNC. It is well known that lipoxygenase-formed AA metabolites are rate-limited by the effect of phospholipases, including that of PLA_2 (12). The lipoxygenase-formed AA metabolites already have been implicated in stimulus-secretion coupling events in various endocrine organs (32), including AP cells (12, 33–35). In addition, 12(S)-HETE has been shown to mediate some of the effects of lipoxygenase-formed AA metabolites on stimulated ACTH secretion *in vitro*, although the magnitude of the effect mediated by this mechanism seems to be independent of the amount of arachidonate released from the cell membrane (36). In addition, on different levels of the HPA axis, AA metabolites have been found to positively modulate CRH output by incubated HT fragments (4). A reciprocal interaction between the HPA axis and the immune system is now well known (11), and AA metabolites have been described to mediate both central (7, 37, 38) and peripheral (39, 40) effects of cytokines on HPA axis activation. However, in the present study, we found that stimulation of AA metabolites production by PLA_2 not only enhances HPA axis function but also stimulates immune system activity by increasing (probably among other cytokines) TNFα output in plasma. This effect was corroborated in our *in vitro* design of PMNC. Although AA metabolites have been described to act as mediators in the paracrine stimulatory effect of IL-1 on nerve growth factor secretion (41), to our knowledge, this is the first time that PLA_2-related events are described as being directly involved in the mechanisms of TNFα production.

PLA_2 recently has been described as an enzyme turned hormone (42). It is known that the major toxic component from the venom of *Crotalus durissus terrificus* is a potent β-neurotoxin with intrinsic PLA_2 activity and that it exerts its lethal action by blocking neuromuscular transmission, primarily at the presynaptic level (43). This SV has been named crotoxin (C) and is a heterodimer composed of a basic and weakly toxic PLA_2 CB subunit and by an acidic nontoxic and nonenzymatic CA subunit, which is homologous to a 3-fragment-less, posttranslationally removed PLA_2 (see 43 for references); CA and CB form a complex and act synergistically to exert the toxic effect of this crotoxin (43). In the present study, we have demonstrated that the effects of SV on both HPA and immune axes are mimicked by its PLA_2 component and that the potent antiinflammatory sesterpenoid (MLD), which is known to inhibit irreversible PLA_2 activity (44), was able to significantly decrease SV/PLA_2-stimulated ACTH secretion by isolated AP cells and that SV/PLA_2 did not directly modify adrenal glucocorticoid release. These observations clearly support the level-specificity of some effects of this enzyme on HPA axis function.

The relationship between cytokines and PLA_2 activity has been investigated by others, some of whom have reported that the multifunctional cytokines, IL-1 (α and β) and TNFα, are able to stimulate and that TGF-β1 decreases PLA_2 secretion from rat calvarial cells (45); these results suggest one of the directions by which these two systems (immune and PLA_2) communicate, but as described in the present study, we found a reciprocal way of communication between them, because a stimulatory effect of PLA_2 on cytokine (TNFα) production seems to play an important regulatory role during SV-, PLA_2-related, induced neurotoxic shock. Regarding the mechanism of action of several snake venoms with intrinsic neurotoxic effect, it still remains unclear whether the PLA_2 component is essential for presynaptic neurotoxicity. It is known that inhibition of Na^+/K^+ adenosine triphosphatase (ATPase) results in enhanced transmitter release (46), whereas stimulation of this enzyme blocks that effect (47). Some snake venoms are able to depolarize synaptosomes (48) and to inhibit Na^+/K^+ ATPase activity (49); however, it has been found that rat synaptosome membrane depolarization is directly caused by PLA_2 enzymatic activity and production of FFA (50). In addition, studies of the contractural effect on skeletal muscle of these snake venoms indicate, at this level, modification of sarcoplasmic reticulum Ca^{2+} release, whereas red blood cells hemolysis seemed instead to be related to long-term effects on lipid metabolism (51).

Briefly, our results demonstrate that this PLA_2-related SV is able to induce a well-characterized stress, similar to that described after other inflammatory stresses (52), by direct stimulation of both neuroendocrine (HPA axis) and immune (TNFα output) functions and by a hyperglycemic effect to protect the organism immediately after injury. Regarding the hyperglycemic effect of SV/PLA_2, such a mechanism could probably be initiated by a toxic action of increased lisophosphatides on red cell membrane integrity (51). Peripheral carbohydrate metabolism is controlled, at least partially, by the central nervous system (CNS); therefore, we must not rule out the possibility that such an increase in plasma glucose levels could be caused by an effect of PLA_2 on the CNS. In turn, stimulation of the CNS increases sympathetic nerve activity to the pancreas (53) or the AG (54) to stimulate the release of glucagon or epinephrine, causing peripheral hyperglycemia. The present data also indicate that some of the cytotoxic effects claimed for this PLA_2-related snake venom (19, 20), at least in part, could be caused by a direct effect on TNFα release by toxenzyme-activated immune cells.

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References

1. **Dennis EA** 1978 Phospholipase A_2 mechanism inhibition and role in arachidonic acid release. Drug Dev Res 10:205–220
2. **Chang J, Musser JH, Mc Gregor H** 1987 Phospholipase A_2 function and pharmacological regulation. Biochem Pharmacol 36:2429–2436
3. **Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkovith JB** 1986 Arachidonic acid metabolism. Annu Rev Biochem 55:69–102
4. **Bernardini R, Chiarenza A, Calogero AE, Gold PW, Chrousos GP** 1989 Arachidonic acid metabolites modulate rat hypothalamic corticotropin-releasing hormone secretion *in vitro*. Neuroendocrinology 50:708–715
5. **Burch RM, Connor JR, Axelrod J** 1988 Interleukin 1 amplifies receptor-mediated activation of phospholipase A_2 in 3T3 fibroblasts. Proc Natl Acad Sci USA 85:6306–6309
6. **Pruzanski W, Vadas P** 1991 Phospholipase A_2-a mediator between proximal and distal effectors of inflammation. Immunol Today 12:143–146

7. **Katsuura G, Gottschall PE, Dahl RR, Arimura A** 1988 Adrenocorticotropin release induced by intracerebroventricular injection of recombinant interleukin-1 in rats: possible involvement of prostaglandin. Endocrinology 122:1773–1779
8. **Watanabe T, Morimoto A, Sakata Y, Murakami N** 1990 ACTH response induced by interleukin-1 is mediated by CRF secretion stimulated by hypothalamic PGE. Experientia 46:481–484
9. **Pruzanski W, Vadas P, Kim J, Jacobs H, Stefanski E** 1988 Phospholipase activity associated with synovial fluid cells. J Rheumatol 15:791–794
10. **Forster S, Iderton E, Norris J, Summerly R, Yardley H** 1985 Characterization and activity of phospholipase A_2 in normal human epidermis and in lesion-free epidermis of patients with psoriasis or eczema. Br J Dermatol 112:135–147
11. **Bateman A, Singh A, Kral T, Solomon S** 1989 The immune-hypothalamic-pituitary-adrenal axis. Endocr Rev 10:92–112
12. **Knepel W, Meyen G** 1986 Effects of various blockers of arachidonic acid metabolism on release of beta-endorphin- and adrenocorticotropin-like immunoreactivity induced by phospholipase A_2 from rat adenohypophysis *in vitro*. Neuroendocrinology 43:44–48
13. **Verheij HN, Boffa MC, Rothen C, Bryckaert MC, Verger R, De Hass G** 1981 Correlation of enzymatic activity and anticoagulant properties of phospholipase A_2. Eur J Biochem 147:66–71
14. **Rubsamen K, Brehithaupt H, Haberman H** 1971 Biochemistry and pharmacology of the crotoxin complex. I. Subfractionation and recombination of the crotoxin complex. Arch Exp Pathol Pharmacol 270:274–279
15. **Spinedi E, Salas M, Chisari A, Perone M, Carino M, Gaillard RC** 1994 Sex differences in the hypothalamo-pituitary-adrenal axis response to inflammatory and neuroendocrine stressors. Neuroendocrinology 60:609–617
16. **Halliwell JV, Dolly JO** 1982 Preferential action of β-Bu TX at nerve terminal regions in the hippocampus. Neurosci Lett 30:321–327
17. **Benishin CG** 1990 Potassium channel blockade by B subunit of β-bungarotoxin. Mol Pharmacol 38:164–169
18. **Awan KA, Dolly JO** 1991 K^+ channel sub-types in rat brain: characteristic locations revealed using β-bungarotoxin, α- and δ-dendrotoxins. Neuroscience 40:29–39
19. **Newman RA, Vidal JC, Viskatis LJ, Johnson J, Etcheverry MA** 1993 VRCTC-310 a novel compound of purified animal toxins separates antitumor efficacy from neurotoxicity. Invest New Drugs 11:151–159
20. **Corin RE, Viskatis LJ, Vidal JC, Etcheverry MA** 1993 Cytotoxicity of crotoxin on murine erythroleukemia cells *in vitro*. Invest New Drugs 11:11–15
21. **Kaneko M, Hiroshige T, Shinsako J, Dallman MF** 1980 Diurnal changes in amplification of hormone rhythms in the adrenocortical system. Am J Physiol 239:R309–R316
22. **Spinedi E, Giacomini M, Jacquier M-C, Gaillard RC** 1991 Changes in the hypothalamo-corticotrope axis after bilateral adrenalectomy: evidence for a median eminence site of glucocorticoid action. Neuroendocrinology 53:160–170
23. **Spinedi E, Hadid R, Daneva T, Gaillard RC** 1992 Cytokines stimulate the CRH but not the vasopressin neuronal system: evidence for a median eminence site of interleukin-6 action. Neuroendocrinology 56:46–53
24. **Gaillard RC, Grossman A, Gillies G, Rees LH, Besser GM** 1981 Angiotensin II stimulates the release of ACTH from dispersed rat anterior pituitary cells. Clin Endocrinol (Oxf) 15:573–578
25. **Spinedi E, Rodriguez G** 1986 Angiotensin II and adrenocorticotropin release: mediation by endogenous corticotropin-releasing factor. Endocrinology 119:1397–1402
26. **Spinedi E, Aguado L, Basilotta G, Carrizo D** 1989 Angiotensin II and glucocorticoid release: direct effect at the adrenal level and modulation of the adrenocorticotropin-induced glucocorticoid release. J Endocrinol Invest 12:321–327
27. **Singh VK** 1989 Stimulatory effect of corticotropin-releasing neurohormone on human lymphocyte proliferation and interleukin-2 receptor expression. J Neuroimmunol 23:257–262
28. **Hodgkinson SC, Allolio B, Landon J, Lowry PJ** 1984 Development of non-extracted two-site immunoradiometric assay for corticotropin utilizing extreme amino- and carboxy-terminally directed antibodies. Biochem J 218:703–711
29. **Corder R, Lowry PJ** 1985 An immunoradiometric assay for the measurement of neuropeptide Y in plasma. Peptides 6:1195–1200
30. **Flick DA, Gifford GE** 1984 Comparison of *in vitro* cell cytotoxic assay for tumor necrosis factor. J Immunol Methods 68:167–175
31. **Zar JH** 1974 Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, NJ
32. **Metz S, Van Rollins M, Strife R, Fujimoto W, Robertson RP** 1983 Lipoxygenase pathway in islet endocrine cells. J Clin Invest 71:1191–1205
33. **Canonico PL, Valdenegro CA, Judd AM, MacLeod RM** 1984 Arachidonic acid metabolism and thyrotropin secretion *in vitro*. Eur J Pharmacol 98:45–52
34. **Grandison L** 1984 Stimulation of anterior pituitary prolactin release by melittin, an activation of phospholipase A_2. Endocrinology 114:1–7
35. **Snyder GD, Capdevila J, Chacos N, Manna S, Falck JR** 1983 Action of luteinizing hormone-releasing hormone: involvement of novel arachidonic acid metabolites. Proc Natl Acad Sci USA 80:3504–3507
36. **Won JGS, Orth DN** 1994 Role of lipoxygenase metabolites of arachidonic acid in the regulation of adrenocorticotropin secretion by perifused rat anterior pituitary cells. Endocrinology 135:1496–1503
37. **Navarra P, Tsagarakis S, Faria MS, Rees LH, Besser GM, Grossman AB** 1990 Interleukins-1 and -6 stimulate the release of corticotropin-releasing hormone-41 from rat hypothalamus *in vitro* via the eicosanoid cyclooxygenase pathway. Endocrinology 128:37–44
38. **Lyson K, McCann SM** 1992 Involvement of arachidonic acid cascade pathways in interleukin-6-stimulated corticotropin-releasing factor release *in vitro*. Neuroendocrinology 55:708–713
39. **Rivier C, Vale W** 1991 Stimulatory effect of interleukin-1 on adrenocorticotropin secretion in the rat: is it modulated by prostaglandins? Endocrinology 129:384–388
40. **Tominaga T, Fukata J, Naito Y, Usui T, Murakami N, Fukushima M, Nakai Y, Hirai Y, Imura H** 1991 Prostaglandin-dependent *in vitro* stimulation of adrenocortical steroidogenesis by interleukins. Endocrinology 128:528–531
41. **Carman-Krzan M, Wise BC** 1993 Arachidonic acid lipoxygenation may mediate interleukin-1 stimulation of nerve growth factor secretion in astroglial cultures. J Neurosci Res 34:225–232
42. **Glaser KB** 1995 Phospholipase A_2: a 1995 perspective. Program of the 77th Annual Meeting of The Endocrine Society, Washington DC, p 39 (Abstract)
43. **Perales J, Villela C, Domont GB, Choumet V, Saliou B, Moussatche H, Bon C, Faure G** 1995 Molecular structure and mechanism of action of crotoxin inhibitor from *crotalus durissus terrificus* serum. Eur J Biochem 227:19–26
44. **Bennet CF, Mong S, Clarke MA, Kruse LI, Crooke ST** 1987 Differential effects of manoalide on secreted and intracellular phospholipases. Biochem Pharmacol 36:733–740
45. **Ellies LG, Gupta AK, Aubin JE** 1992 Differential regulation of phospholipase A_2 by cytokines inhibiting bone formation and mineralization. Biochem Biophys Res Commun 188:1047–1053
46. **Meyer EM, Cooper JR** 1981 Correlations between Na^+-K^+ ATPase activity and acetylcholine release in rat cortical synaptosomes. J Neurochem 36:467–475
47. **Vizi ES** 1977 Termination of transmitter release by stimulation of sodium-potassium activated ATPase. J Physiol (Lond) 267:261–280
48. **Nichols D, Snelling R, Dolly O** 1985 Bionergetic actions of β-bungarotoxin, dendrotoxin, and bee venom phospholipase A_2 on guinea pig synaptosomes. Biochem J 229:653–662
49. **Bougis PE, Khelif A, Rochat H** 1989 On the inhibition of Na^+/K^+-ATPases by the components of *Naja mossambica mossambica* venom: evidence for two distinct rat brain Na^+/K^+-ATPase activities. Biochemistry 28:3037–3043
50. **Yates SL, Rosenberg P** 1991 Comparative effects of phospholipase A_2 neurotoxins and enzymes on membrane potential and Na^+/K^+ ATPase activity of rat brain synaptosomes. Toxicol Appl Pharmacol 109:207–218
51. **Fletcher JE, Jiang M-S, Gong Q-H, Yudkowsky ML, Wieland SJ** 1991 Effects of cardiotoxin from *Naja Naja kaouthia* venom on skeletal muscle: involvement of calcium-induced calcium release, sodium ion currents and phospholipases A_2 and C. Toxicon 29:1489–1500
52. **Spinedi E, Suescun MO, Hadid R, Daneva T, Gaillard RC** 1992 Effects of gonadectomy and sex hormone therapy on the endotoxin-stimulated hypothalamo-pituitary-adrenal axis: evidence for a neuroendocrine-immune sexual dimorphism. Endocrinology 131:2430–2436
53. **Frohman LA, Bernardis LL** 1971 Effect of hypothalamic stimulation on plasma glucose, insulin, and glucagon levels. Am J Physiol 221:1596–1603
54. **Van Loon GR, Appel NM** 1981 β-Endorphin-induced hyperglycemia is mediated by increased central sympathetic outflow to adrenal medulla. Brain Res 204:236–241