Sexual dimorphism in the hypothalamo-pituitary-adrenal (HPA) axis and TNFα responses to phospholipase A_2-related neurotoxin (from *crotalus durissus terrificus*) challenge

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ABSTRACT. Neuroendocrine-immune interactions are vital for the individual's survival in certain physiopathological conditions such as sepsis and tissular injury. It is known that several snake venoms (SV) are potent neurotoxic compounds and that their main component is a specific phospholipase type 2 (PLA_2). It has been recently described that the venom from *crotalus durissus terrificus* (SV) possesses a cytotoxic effect in different *in vitro* and *in vivo* animal models. In the present study we investigated whether SV is able to stimulate both TNFα and neuroendocrine functions in a sexual dimorphic fashion. For this purpose the modulatory role of endogenous sex steroids during neurotoxemia was evaluated. Our results indicate that SV (25 μg/animal) stimulates the hypothalamo-pituitary-adrenal axis and TNFα secretion when administered (ip) to adult male mice, such responses were characterized by a time-related enhance in plasma glucose, ACTH, corticosterone and TNFα levels. SV-stimulated glycemia, corticosteronemia and adrenal glucocorticoid were sexually dimorphic. Twenty-day gonadectomized mice showed a similar sexual dimorphism to that found in intact animals, however, they additionally showed a sexual dimorphic pattern in cytokine release in plasma 30 min post-SV. Estradiol (E_2) treatment, in gonadectomized mice, abolished some characteristics of the sexual dimorphism, such as hyperglycemia, hypercorticosteronemia and hypercytokinemia. Finally, *in vitro* experiments indicate that: a) gonadectomy increased spontaneous and SV-stimulated cytokine output by incubated peripheral mononuclear cells (PMNC), regardless of the sex; and b) despite E_2 treatment, in gonadectomized, did not modify the pattern of basal and SV-elicited TNFα secretion induced by orchidectomy, fully reversed the enhance in basal and SV-stimulated cytokine release found after ovariectomy alone. Our results further indicate that neurotoxemia, due to SV challenge, induces several symptoms common to those of inflammatory stress; they also strongly support that both gender and endogenous sex steroids are responsible for neuroendocrine-immunological sexual dimorphism.

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INTRODUCTION

A stimulatory effect of a phospholipase (PL) A_2-related neurotoxin on the hypothalamo-pituitary-adrenal (HPA) and immune axes function has recently been reported by our group (1). The main sites of action of this compound seem to be at the hypothalamic and corticotrope levels as well as on peripheral immune cells (1). PLA_2 is a lipolytic enzyme which hydrolyzes membrane phospholipids producing lysophosphatide and arachidonic acid (AA). It is known that AA cascade metabolites modulate HPA axis function, namely at the hypothalamic CRH-ergic system level (2). Immune system-derived substances, such as cytokines, stimulate PLA_2 activity (3) and, reciprocally, prostaglandins appear to be involved in cytokine-stimulated ACTH secretion (4); thus the reciprocal communication between the immune, HPA and PLA_2 systems seems to play an important role during the inflammatory response induced by PLA_2-related neurotoxins (5-7).

Key-words: PLA2, glucose, ACTH, glucocorticoid, TNFα, inflammatory stress.

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Among others, an important aspect of the HPA and immune axes responses to inflammatory *stimuli* is the fact that they develop in sexually dimorphic fashion (8, 9). A sexual dimorphic pattern of the immune response to different *stimuli* and an alteration of the immune response by gonadectomy have been reported earlier (8, 9). This characteristic sexual dimorphism of both HPA axis and immune functions seems to be dependent on both: gender and sex-steroid hormone basis (10-12). This evidence is strongly supported by the fact that specific receptors for sex steroid hormones are present in the adrenal gland (13) and in organs responsible for the immune response (10).

PLA_2 is an important component of several venoms and the venom from *crotalus durissus terrificus* origin (snake venom, SV) belongs to this category. It is known that this SV induces a local inflammation (7), and that it is able to stimulate HPA axis function in rats (14) and mice (1). Thus the aim of the present study was to determine whether the ablation of endogenous sex-steroids, from gonadal origin, and estradiol (E_2) treatment in gonadectomized animals play any modulatory role on the inflammatory response during neurotoxemia.

MATERIALS AND METHODS

Animals

Adult (8-10 weeks) male and random cycling female BALB/c mice were kept in standard conditions of light (on between 07:00 and 19:00 h) and temperature (22±2 C); they were fed with laboratory chow and tap water *ad libitum*. Animals were handled gently daily, for a week, in order to minimize stress conditions. Experiments were carried out during the circadian trough of the HPA axis (15), between 08:00 and 09:00 h. All procedures were done according to our institution's animal care rules.

Experimental designs

In vivo experiments

Experiment 1. Several groups of male mice (n=9-11 mice per group) were ip injected with 50 µl of vehicle alone (sterile saline solution) or containing a PLA_2-related SV (from *crotalus durissus terrificus*, Sigma Chem. Co. V-7125, 25 µg per mouse) (1) and returned to their cages. Mice were then killed by decapitation at either 0.5, 1, 2 or 4 h after Veh (sample time zero) 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), TNFα and glucose (GLU; by the glucose-oxidase method from Wiener Argentina Laboratories) concentrations. Immediately after decapitation, brain tissues were quickly removed and the anterior pituitary (AP) gland and the adrenal glands (AG) were dissected as previously described (16) and transferred into Eppendorf tubes containing a small volume (500 µl and 100 µl for AP and AG, respectively) of acetic acid 0.1 N; tissues were then sonicated (20-30 sec) and centrifuged at 10,000xg, at 4 C, 3-4 min, and the supernatants kept frozen (-20 C) until the determination of AP ACTH and AG B contents.

Experiment 2. Mice of both sexes were either bilaterally orchidectomized (Odx), ovariectomized (Ovx) or sham-operated under light ether anesthesia. Animals were then divided by sex and surgery and maintained in plastic cages (9-11 mice per cage). On day 1 after surgery, gonadectomized mice were injected sc (09:00 h) with E_2 benzoate (Sigma Chem. Co., St. Louis, MO; 2 µg/50 µl oil) (12) (Odx+E_2 and Ovx+E_2). Sham-operated animals (Male and Female) as well as Odx and Ovx mice were injected with oil alone. These injections were repeated on alternate days. The groups of female animals were followed by checking vaginal smears daily from the 3rd day after surgery; Ovx mice attained almost a constant (during the last 15 days before killing them) vaginal smear similar to that observed at the normal diestrus; on the other hand, Ovx+E_2 mice attained a constant vaginal smear similar to a normal proestrus/estrus from 48 h after the beginning of sex steroid replacement treatment. Only 20 day-orchidectomized mice displaying plasma testosterone levels lower than 0.25 ng/ml were included in appropriate groups. On day 20 after surgery, different groups of animals (Male, Female, Odx, Ovx, Odx+E_2 and Ovx+E_2) were injected (08:00-09:00 h) ip with either 50 µl of sterile saline solution alone or containing 25 µg of SV. Thirty minutes after treatment mice were decapitated and trunk blood was collected. Plasma samples were frozen (-20 C) until the determination of GLU, ACTH, B and TNFα concentrations.

In vitro experiments

Incubation of peripheral mononuclear cells (PMNC). This method is similar to the one previously described with minor modifications (1). Heparinized blood was collected by right jugular vein puncture from mice (untreated Male, Female, Odx, Ovx, Odx+E_2 and Ovx+E_2) 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 700xg, for

30 min, at room temperature. PMNC were washed twice with sterile saline solution and the final pellet was re-suspended with an appropriate volume of RPMI-1640 (HEPES 25 mM, antibiotics, 10% fetal calf serum, pH 7.3) in order 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 containing (final concentration) SV (0.1 µg/ml). In parallel, 0.1 ml of medium alone or 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 6-9 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 concentrations of ACTH were determined by a previously described immunoradiometric assay (17). Plasma and medium concentrations of B were measured by a specific radioimmunoassay earlier reported in detail (17). The intra-assay coefficients of variation were 2-3 and 4-6%, for ACTH and B, respectively; and, the inter-assay coefficients of variation were 6-8 and 8-10%, for ACTH and B, respectively. The assay of the cytokine consisted in the determination of the cytolytic effect of TNFα on L929 cells (from mouse fibrosarcoma) as previously described (18). Briefly, TNFα used as standard was purchased from Genzyme Lab. (82437). Cells were maintained in MEM containing 10% (v/v) of fetal calf serum, glutamine and antibiotics (pH 7.4). Ninety-six-well microtiter trays were seeded at 6x104 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 of 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% w/v in methanol:water, 1:5) was added and allowed for 30 min incubation at 37 C. Plates were rinsed with water and dried, then 100 µl per well of 33% acetic acid was added. Plates were shacked 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 inter-assay coefficients of variation ranged between 7-9 and 9-11%, respectively.

Analysis of data

Results are expressed as the mean±SE. Data were analyzed by one-factor analysis of variance followed by Fisher's test for comparison of different mean values (19).

RESULTS

Phospholipase A_2-related SV-induced HPA axis and TNFα responses

The results of experiment 1 are summarized in Figure 1. This figure shows the results of several metabolites in plasma samples before (sample time zero) and several times after ip injection of male mice with SV (25 µg per animal). It must be pointed out that ip administration of vehicle alone did not significantly vary plasma and tissue metabolite concentrations at all time-points studied; thereafter, all

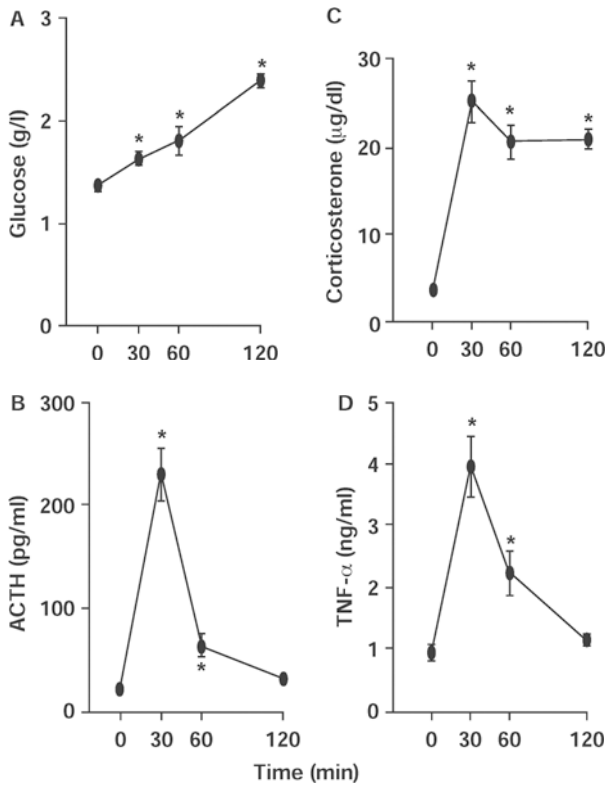

Fig. 1 - *Time-course of plasma glucose (A), ACTH (B), corticosterone (C) and TNF-α (D) before (sample time zero) and several times after ip administration of SV (25 µg per animal) in intact male mice. Values are the mean±SE (n=9-11 mice per time per group). *P<0.05 vs the respective sample time-zero values.*

time values were pooled and they represent sample time-zero values. Figure 1 (panel A): 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 (panel B): 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 ($p<0.05$) higher than time-zero values; they reached the baseline at 120 min after SV injection. Figure 1 (panel C): plasma B levels before (sample time-0) 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* sample time-0 values); then values remained at the maximal level up to 120 min after SV administration. Figure 1 (panel D): 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-0) 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.

Effects of gonadectomy and E_2 treatment on glycemia, HPA axis function and circulating TNFα during neurotoxemia

Since a maximal immune-neuroendocrine response was found 30 min post-SV, we explored gender-dependent characteristics of the GLU, HPA axis and immune system responses on this time.

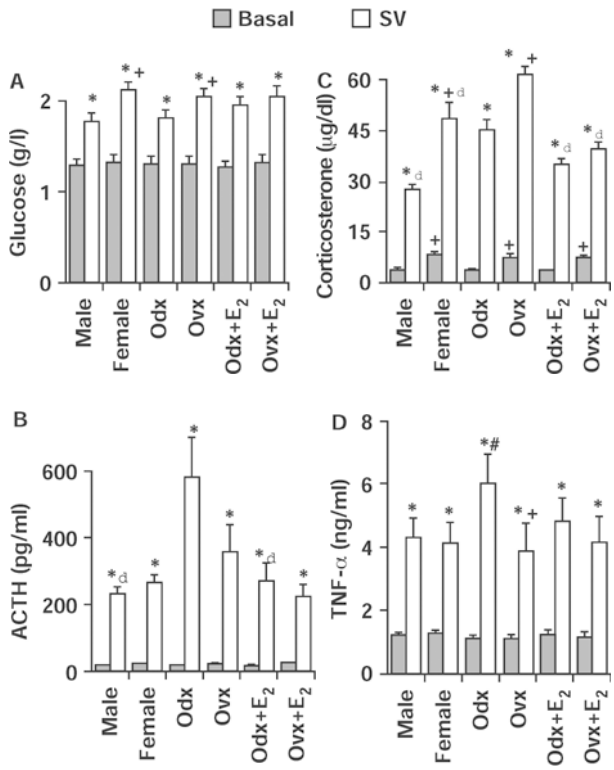

Fig. 2 - *Plasma glucose (A), ACTH (B), corticosterone (C) and TNF-α (D) levels before (basal) and 30 min after ip administration of SV (25 μg per animal) in several groups of mice. Values are the mean±SE (n=9-11 mice per group).*
P<0.05* vs *the respective basal values;* #p*<0.05* vs *SV values in male mice;* +P*<0.05 between sexes in similar conditions;* •p*<0.05 or less* vs *values in Gnx mice of similar sex and in similar condition.*

Glucose. Figure 2 (panel A) shows the results of plasma GLU concentrations in basal and 30 min after SV administration conditions. Basal GLU concentrations were significantly ($p<0.05$) enhanced, over the respective baseline, 30 min after SV injection, regardless of the group. The results indicate that in intact mice such an increase was induced in a dimorphic fashion, with higher ($p<0.05$) values in Female than in Male animals. Those characteristics were maintained in gonadectomized mice of both sexes (Odx and Ovx) and E_2 treatment, although did not influence SV-stimulated GLU secretion, abolished the characteristic sexual dimorphism found in intact and gonadectomized mice; in fact, plasma GLU values were statistically similar in Odx+E_2 and Ovx+E_2 groups.

ACTH. Figure 2 (panel B) shows the results of plasma ACTH concentrations in basal and 30 min after SV administration conditions. As depicted, resting plasma ACTH levels were not influenced by the sex nor gonadectomy alone or combined with E_2 treatment. Although SV injection was able to enhance plasma ACTH levels 30 min after injection in all groups studied, this effect was not in a sexual dimorphic fashion, regardless of the pairs compared (intact, gonadectomized and E_2-treated gonadectomized mice). However, Odx was able to significantly ($p<0.05$ *vs* SV-injected Male values) enhance SV-elicited ACTH release, and E_2 treatment (Odx+E_2) fully reversed such an effect. Conversely, SV-stimulated ACTH secretion in plasma was similar in all groups of females, regardless of surgery and E_2 treatment.

Glucocorticoid. Figure 2 (panel C) shows the results of plasma B levels in basal and 30 min after SV administration conditions. Resting plasma B concentrations were found in a sexual dimorphic fashion in all pair-groups examined, with significantly higher ($p<0.05$) levels in mice of female sex than of male

sex. SV administration significantly ($p<0.05$ *vs* the respective baseline) increased B output in plasma, regardless of the group. Interestingly, SV-stimulated B secretion in plasma was significantly ($p<0.05$ *vs values* found in intact mice of the respective sex) enhanced by gonadectomy, regardless of the sex. The effect induced by gonadectomy was fully reversed by estradiol benzoate treatment in gonadectomized mice of both sexes.

Cytokine. Figure 2 (panel D) shows the results of plasma TNFα levels in basal and 30 min after SV administration conditions. Basal plasma TNFα concentrations were similar in all groups studied. SV-induced TNFα secretion in plasma was several fold higher ($p<0.05$) than the respective baseline. In intact animals, there was no sexual dimorphism in neurotoxin-stimulated cytokine output. Although Ovx alone and combined with E_2 treatment (Ovx+E_2) did not modify SV-induced TNFα release, Odx was able to significantly ($p<0.05$) enhance SV-elicited TNFα output, thus rendering a sexual dimorphism of such response in gonadectomized mice. The effect of Odx on TNFα response during the neurotoxic shock was prevented by E_2 treatment (Odx+E_2).

Effects of gonadectomy and E_2 treatment on tissue hormone content during neurotoxemia

Figure 3 (upper panel): AP ACTH concentrations before and 30 min after SV administration. Basal AP ACTH, when expressed by mg of tissue protein, was found of a similar fashion in mice, regardless of the group examined. SV injection significantly ($p<0.05$ *vs* the respective basal) reduced AP ACTH in intact, gonadectomized and E_2-treated gonadectomized mice, despite no significant differences between groups.

Figure 3 (lower panel): AG B in mice before and 30 min after SV injection. Basal AG B was found in a dimorphic fashion in all pair-groups studied, being values in mice of the female sex significantly ($p<0.05$) higher than the respective in animals of the male sex, regardless surgery and steroid treatment. SV administration significantly ($p<0.05$ *vs* the respective baseline) enhanced AG B in all groups studied without any significant difference between groups.

Effects of gonadectomy alone and followed by E_2 treatment on spontaneous and SV-induced TNFα secretion by incubated PMNC

When PMNC (from different donors) were incubated (48 h) with medium alone (basal), a detectable amount of TNFα was found in the medium, regardless of the group (Fig. 4). Although no significant differences in spontaneous TNFα output were found between Male and Female groups, gonadectomy enhanced basal cytokine release in a significant ($p<0.05$ *vs* Male values) fashion only when performed in males (Odx group). E_2 treatment, in gonadectomized mice, whereas did not vary spontaneous cytokine output in males (Odx+E_2), significantly reduced ($p<0.05$ *vs* Ovx values) basal TNFα release in ovariectomized mice (Ovx+E_2). Figure 4 shows also the results of SV (0.1 μg/ml)-stimulated cytokine output by PMNC from different donors. SV was able to significantly ($p<0.05$) increase TNFα release over the respective

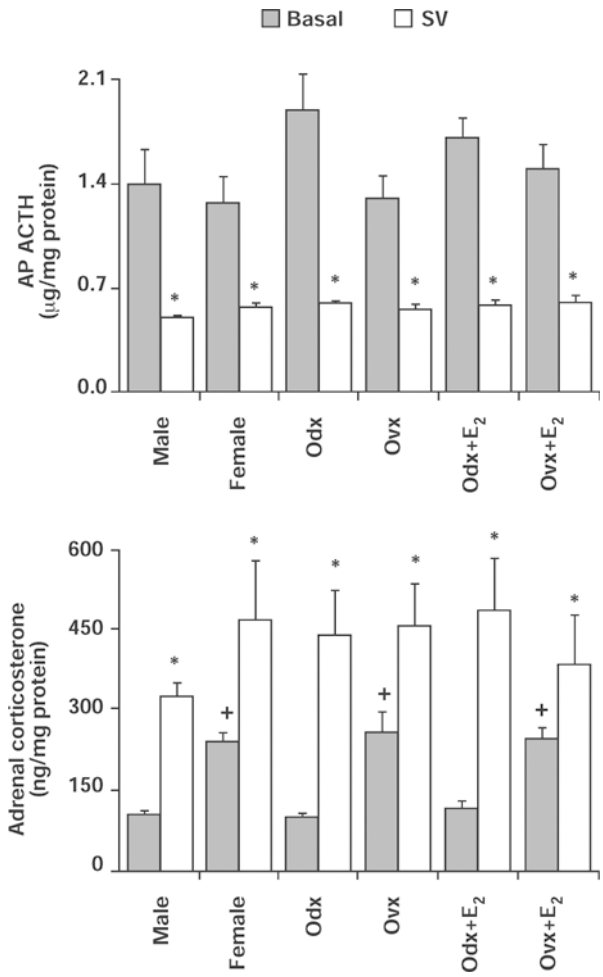

Fig. 3 - *Anterior pituitary (AP) ACTH (upper panel) and adrenal corticosterone (lower panel) contents before (basal) and 30 minutes after ip administration of SV (25 μg per animal) in several groups of mice. Values are the mean±SE (n=9-11 mice per group). *p<0.05 vs the respective basal values; +p<0.05 between sexes in similar condition.*

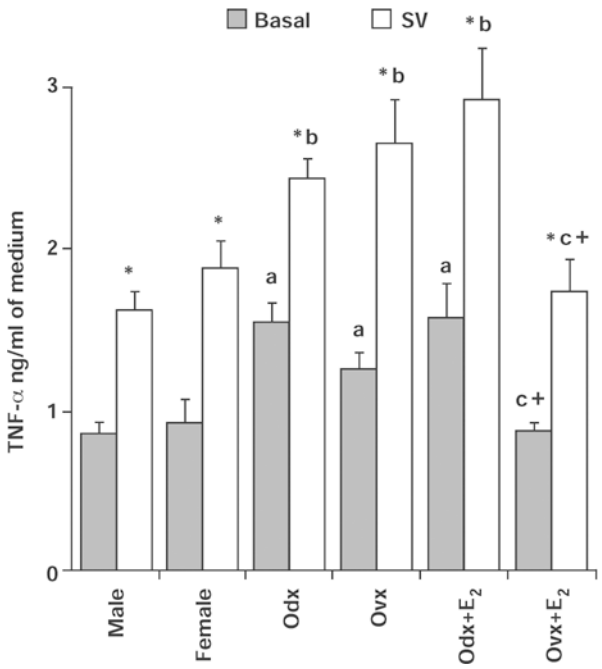

Fig. 4 - Ex vivo *immune function in mice. TNF-α release by PMNC, from different donors, incubated with medium alone (basal) or containing SV (0.1 µg/ml). Values are the mean of 2 different experiments (n=5-6 wells per experiment).* [a]p<*0.05* vs *basal values in intact mice of the same sex;* [b]p<*0.05* vs *SV values in intact mice of the same sex;* [c]p<*0.05* vs *Ovx values similar condition;* [+]p<*0.05 vs between sexes in similar conditions.*

baseline, regardless of the group. Gonadectomy, in both genders, significantly ($p<0.05$) enhanced SV-stimulated TNFα release *vs* the respective, non-gonadectomized, gender values. Finally, although E_2 treatment did not influence the effect of Odx on SV-elicited cytokine output, it fully reversed ($p<0.05$ *vs* Ovx values) the enhance in PMNC response to SV in Ovx mice (Fig. 4).

DISCUSSION

A neuroendocrine-immune response to PLA_2-related neurotoxin administration was found in intact male mice. This response was characterized by: 1) persistent hyperglycemia; 2) transient increases in plasma levels of ACTH and TNFα and 3) maximal adrenal B production. The transient increase in corticotrope and immune cell activities, which finally decline towards their respective baselines, are probably due to the rapid negative feedback effect of high glucocorticoid levels on both cell populations (20). It must be pointed out that this PLA_2-related SV possesses a direct stimulatory effect on TNFα output from PMNC [(1) and these results] and has no effect on adrenal gland glucocorticoid production (1).

We found gender-related differences in basal circulating B and AG B; these differences were characterized by higher values in females than in males. These findings agree with previous results in different animal models (11, 12, 21, 22). We also found a sexual dimorphic pattern in both the hyperglycemic effect and the adrenal response elicited by SV; these responses were higher in females than in males. Although the sexual dimorphism in the adrenal response has been reported by using different inflammatory challenges (11, 12, 15, 21, 23), to our knowledge, no gender-dependent differences in neurotoxin-induced hyperglycemia have been previously described. It is important to emphasize that higher GLU levels, post-SV, in females than in males could contribute as a significant body defence mechanism. In fact, higher mortality was found in male (70%) than in female (30%) mice 4 h post-SV. Regarding the mechanisms involved in the hyperglycemic effect of SV, there are at least two possibilities to consider: 1) it is known that PLA_2 activity induces the removal of fatty acid from the 2 positions of phosphoglycerides thus increasing lysophosphatides, these compounds in high concentrations are toxic and injurious to cell membranes (24); and 2) neurotoxin could affect the central nervous system level, which is known to control carbohydrate metabolism, thus inducing an enhancement in sympathetic nerve activity to the pancreas (25) or the adrenals (26) to stimulate glucagon or epinephrine, thus causing peripheral hyperglycemia. However, whether there exists a gender- or a sex steroid-dependent regulation on the exact mechanisms involved in SV-induced hyperglycemia, still remains unknown.

SV-induced ACTH release in plasma was similar in intact animals of both sexes (even when examined before and after 30 min, data not shown). Although in different animal models, inflammatory stress-induced ACTH release has been found in a sexual dimorphic fashion (21, 23); the discrepancy with the present finding could be due to differences in the nature of the challenge employed (LPS *vs* SV). SV-stimulated TNFα secretion was similar in intact mice of both sexes; we have previously reported that LPS ip administration, in intact mice, did not induce any sexual dimorphic pattern in the release of TNFα 2 h post-injection (12). When Gnx was performed, our results indicate that the sexual dimorphism found in intact animals persisted (GLU and B plasma levels), and, although Gnx did not induce differences in GLU levels between Gnx and intact mice of both sexes, a higher adrenal response was found in Gnx

than in intact mice of the respective sex. As earlier reported (although using LPS) (27), Odx, but not Ovx, enhanced corticotrope response to SV treatment. In that report, it was also found that testosterone (T) treatment only partially restored LPS-stimulated ACTH secretion to values attained in intact males (27). Interestingly, a clear sexual dimorphism in SV-elicited TNFα secretion was found in Gnx mice, in fact, TNFα levels were significantly higher in Odx than in Ovx mice. The results on the effects of Gnx on TNFα response to inflammatory challenge agree with those found in Gnx mice after LPS (12). We found (12) that LPS-treated Gnx mice, of both sexes, released more cytokine than intact mice of similar gender. It could be further suggested that the mechanisms involved in the modulation of the immune response by sex-steroids seem to be dependent also on the inflammatory stimulus employed. It is important to stress that this sex-related difference was due to the higher cytokine secretion in Odx than in intact males, without changes in the immune responses between intact female and Ovx mice. Importantly, the immunological sexual dimorphism that we now describe was not only found *in vivo* but also after the examination of PMNC function *ex vivo*. Spontaneous TNFα output, although not influenced by gender in intact mice, was enhanced 20 days after Odx but not after Ovx. Additionally, SV-stimulated TNFα secretion by PMNC was also enhanced by Gnx in animals of both genders. Our results fully agree with previous reports from other groups. For instance, skin allograft rejection time has been reported to be longer in male than in female mice and, interestingly, Odx was effective in reducing this rejection time (28). Similarly, NZB/NZW mice of the male gender are more resistant than females to develop autoimmune lupus, however, they will die after orchidectomy (29). The sexual dimorphism found in basal adrenal B content, with values significantly higher in females than in males, was not affected by Gnx followed or not by E_2 treatment. The sexual dimorphism in basal adrenal function was correlated with B peripheral levels. Conversely, AP ACTH, basal and post-SV treatment, was independent of both gender and Gnx, either alone or followed by E_2 therapy.

Our results clearly indicate that the removal of endogenous androgens induces differences between several parameters (when compared Odx *vs* both Ovx and intact male mice); thus a relevant inhibitory role of T on immune-neuroendocrine function could be inferred (8, 9, 12, 30, 31). When the E_2 effect in Gnx animals was examined, our results indicate that the sexual dimorphism in basal B levels and adrenal B was still present. However, the sexual dimorphic characteristic was not longer present in several other metabolic parameters; in fact, E_2 therapy in SV-treated Gnx mice: 1) abolished the sex-dependent hyperglycemia found in intact mice; 2) reversed the enhance in ACTH and TNFα output post-Odx; and 3) restored increase in B release induced by gonadectomy. To our knowledge, there are no previous reports on the effect of sex steroid on stimulus-induced hyperglycemia, however, in our design, E_2 seems to be stimulatory of SV-induced GLU output. E_2 treatment in Gnx mice inhibited the enhancement in both SV-stimulated ACTH secretion in Odx mice, and in SV-elicited adrenal responses in animals of both sexes. It has been demonstrated that E_2-treated, 20-day Gnx, mice developed a lower HPA axis response to LPS than Gnx counterparts, regardless of gender; in addition, the responses were similar to those found in intact mice of the respective sex (12). The last mentioned results, taken together with the present data, suggest that E_2, although less effective than T, plays an inhibitory role on stimulus-induced inflammation. This *in vivo* observation was confirmed by our *in vitro* experiments on PMNC function. In fact, E_2 treatment, in Gnx mice, was able to fully reverse the enhance in SV-stimulated TNFα output induced after Ovx alone. The possibility that sex steroids may affect the release of other cytokines after SV stimulation should not be ruled out; it is known that these substances, in turn, stimulate HPA axis activity (32-34).

Acute inflammatory process represents a threat to the integrity of the organism that requires metabolic changes (35, 36) for survival after injury, our study reinforces (12) the concept that sex hormones blunt the effect of inflammation.

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REFERENCES

1. Chisari A., Spinedi E., Voirol M.-J., Giovambattista A., Gaillard R.C.
A phospholipase A_2-related snake venom (from *Crotalus Durissus Terrificus*) stimulates neuroendocrine and immune functions: determination of different sites of action.
Endocrinology 1998, *139:* 617-625.

2. Bernardini R., Chiarenza A., Calogero A.E., Gold P.W., Chrousos G.P.
Arachidonic acid metabolites modulate rat hypothalamic corticotropin-releasing hormone secretion *in vitro*.
Neuroendocrinology 1989, *50:* 708-715.
3. Burch R.M., Connor J.R., Axelrod J.
Interleukin 1 amplifies receptor-mediated activation of phospholipase A_2 in 3T3 fibroblasts.
Proc. Soc. Natl. Acad. Sci. USA 1988, *85:* 6306-6309.
4. Katsuura G., Gottschall P.E., Dahl R.R., Arimura A.
Adrenocorticotropin release induced by intracerebroventricular injection of recombinant interleukin-1 in rats: possible involvement of prostaglandin.
Endocrinology 1988, *122:* 1773-1779.
5. Pruzanski W., Vadas P., Kim J., Jacobs H., Stefanski E.
Phospholipase A_2 activity associated with synovial fluid cells.
J. Rheumatol. 1988, *15:* 791-794.
6. Forster S., Iderton E., Norris J., Summerly R., Yardley H.
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. 1985, *112:* 135-147.
7. Rubsamen K., Brehithaupt H., Haberman H.
Biochemistry and pharmacology of the crotoxin complex. I. Subfractionation and recombination of the crotoxin complex.
Arch. Exp. Pathol. Pharmacol. 1971, *270:* 274-288.
8. Grossman C.J.
Interactions between the gonadal steroids and the immune system.
Science 1985, *227:* 257-261.
9. Grossman C.J.
Are there underlying immune-neuroendocrine interactions responsible for immunological sexual dimorphism?
Progr. Neuroendocrinoimmunol. 1990, *2:* 75-82.
10. Grossman C.J.
Regulation of the immune system by sex steroids.
Endocrinol. Rev. 1984, *5:* 435-455.
11. Chisari A, Carino M, Perone M, Gaillard RC, Spinedi E.
Sex and strain variability in the rat hypothalamo-pituitary-adrenal (HPA) axis function.
J. Endocrinol. Invest. 1995, *18:* 25-33.
12. Spinedi E., Suescun M.O., Hadid R., Daneva T., Gaillard R.C.
Effects of gonadectomy and sex hormone therapy on the endotoxin-stimulated hypothalamo-pituitary-adrenal axis: evidence for a neuroendocrine-immunological sexual dimorphism.
Endocrinology 1992, *131:* 2430-2436.
13. Calandra R.S., Purvis K., Naess O., Attramadal A., Djoseland O., Hansson V.
Androgen receptors in the rat adrenal gland.
J. Steroid Biochem. 1978, *9:* 1009-1015.
14. Spinedi E., Salas M., Chisari A., Perone M., Carino M., Gaillard R.C.
Sex differences in the hypothalamo-pituitary-adrenal axis response to inflammatory and neuroendocrine stressors.
Neuroendocrinology 1994, *60:* 609-617.
15. Kaneko M., Hiroshige T., Shinsako J., Dallman M.F.
Diurnal changes in amplification of hormone rhythms in the adrenocortical system.
Am. J. Physiol. 1980, *239:* R309-R316.
16. Spinedi E., Giacomini M., Jacquier M.-C., Gaillard R.C.
Changes in the hypothalamo-corticotrope axis after bilateral adrenalectomy: evidence for a median eminence site of glucocorticoid action.
Neuroendocrinology 1991, *53:* 160-170.
17. Hodgkinson S.C., Allolio B., Landon J., Lowry P.J.
Development of non-extracted two-site immunoradiometric assay for corticotropin utilizing extreme amino- and carboxy-terminally directed antibodies.
Biochem. J. 1984, *218:* 703-711.
18. Flick D.A., Gifford G.E.
Comparison of *in vitro* cell cytotoxic assay for tumor necrosis factor.
J. Immunol. Methods 1984, *68:* 167-175.
19. Zar J.H.
Biostatistical Analysis.
Prentice-Hall, Englewood Cliffs, New Jersey, 1974.
20. Bateman A., Singh A., Kral T., Solomon S.
The immune-hypothalamic-pituitary-adrenal axis.
Endocr. Rev. 1989, *10:* 92-112.
21. Rivier C.
Stimulatory effect of interleukin-1β on the hypothalamic-pituitary-adrenal axis of the rat: influence of age, gender and circulating sex steroids.
J. Endocrinol. 1994, *140:* 365-372.
22. Atkinson H.C., Waddell B.J.
Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle.
Endocrinology 1997, *138:* 3842-3848.
23. Daneva T., Spinedi E., Hadid R., Jacquier M.-C., Giacomini M., Gaillard R.C.
Transient sex-related changes in the mice hypothalamo-pituitary-adrenal (HPA) axis during the acute phase of inflammatory process.
Mediators of Inflammation 1993, *2:* 123-127.
24. Fletcher J.E., Jiang M.-S., Gong Q.-H., Yudkowsky M.L., Wieland S.J.
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 1991, *29:* 1489-1500.
25. Frohman L.A., Bernardis L.L.
Effect of hypothalamic stimulation on plasma glucose, insulin, and glucagon levels.
Am. J. Physiol. 1971, *221:* 1596-1603.

26. Van Loon G.R., Appel N.M.
β-Endorphin-induced hyperglycemia is mediated by increased central sympathetic outflow to adrenal medulla.
Brain Res. 1981, *204:* 236-241.

27. Hadid R., Spinedi E., Daneva T., Grau G., Gaillard R.C.
Repeated endotoxin treatment decreases immune and hypothalamo-pituitary-adrenal axis responses: effects of orchidectomy and testosterone therapy.
Neuroimmunomodulation 1995, *62:* 348-355.

28. Graff R.J., Lappe M.A., Snell G.D.
The influence of the gonads and adrenal glands on the immune response to skin grafts.
Transplant 1969, *7:* 105-109.

29. Roubinian J.R., Talal N., Siiteri P.K., Sadakin J.A.
Sex hormone modulation of autoimmunity in NZB/NZW mice.
Arthritis Rheum. 1979, *22:* 1162-1168.

30. Sthoeger Z.M., Chiorazzi N., Lahita R.G.
Regulation of the immune response by sex hormones. I. *In vivo* effects of estradiol and testosterone on pokeweed mitogen-induced human β-cell differentiation.
J. Immunol. 1988, *141:* 91-97.

31. Bingaman E.W., Magnuson D.J., Gray T.S., Handa R.J.
Androgen inhibits the increases in hypothalamic corticotropin-releasing hormone (CRH) and CRH-immunoreactivity following gonadectomy.
Neuroendocrinology 1994, *59:* 228-234.

32. Sapolsky R., Rivier C., Yamamoto G., Plotsky P., Vale W.
Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor.
Science 1987, *238:* 522-524.

33. Berkenbosch F., van Oers J., Del Rey A., Tilders F., Besedovsky H.
Corticotropin-releasing factor-producing neurons in the rat activated by interleukin-1.
Science 1987, *238:* 524-526.

34. Naitoh Y., Fukata J., Tominaga T., Nakai Y., Tamai S., Mori K., Imura H.
Interleukin-6 stimulates the secretion of adrenocorticotropic hormone in conscious, freely-moving, rats.
Biochem. Biophys. Res. Commun. 1988, *155:* 1459-1463.

35. Michie H.R., Springgs D.R., Manogue K.R., Sherman M.L., Revhaug A., O'Dwyer S., Arthur K., Dinarello C.A., Ceramy A., Wolff S.M., Kufe D.W., Wilmore D.W.
Tumor necrosis factor and endotoxin induce similar metabolic responses in human beings.
Surgery 1988, *104:* 280-286.

36. Munck A., Guyre P.M., Holbrook N.J.
Physiological functions of glucocorticoids in stress and their relation to pharmacological actions.
Endocr. Rev. 1984, *5:* 25-44.