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Enhanced Proinflammatory Cytokine Response to Bacterial Lipopolysaccharide in the Adult Male Rat after either Neonatal or Prepubertal Ablation of Biological Testosterone Activity

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Key Words
Lipopolysaccharide · Tumor necrosis factor alpha · Glucocorticoid · Androgen · Leptin · Flutamide · Orchidectomy

Abstract
A sex steroid-dependent modulation of the immune function in mammals is accepted, and evidence suggests that while estrogens enhance, androgens inhibit the immune response. The aim of this study was to explore in the adult male rat the effect of either neonatal flutamide (FTM) treatment or prepubertal orchidectomy (ODX) on endocrine markers in the basal condition and peripheral tumor necrosis factor alpha (TNFα) levels during inflammatory stress. For these purposes, (1) 5-day-old male rats were subcutaneously injected with either sterile vehicle alone or containing 1.75 mg FTM, and (2) 25-day-old male rats were sham operated or had ODX. Rats were sacrificed (at 100 days of age) in the basal condition for determination of peripheral metabolite levels. Additional rats were intravenously injected with bacterial lipopolysaccharide (LPS; 25 μg/kg body weight, i.v.) and bled for up to 4 h. Data indicate that (1) ODX increased peripheral glucocorticoid levels and reduced those of testosterone, whereas FTM-treated rats displayed low circulating leptin concentrations, and (2) LPS-induced TNFα secretion in plasma was significantly enhanced in the FTM and ODX groups. Our study supports that neonatal FTM treatment affected adiposity function, and adds data maintaining that androgens have a suppressive role in proinflammatory cytokine release in plasma during inflammation.

Introduction

The discovery of a feedback loop between the immune system and the brain [1–3] resulted in one of the most exciting advances in the field of neuroimmunomodulation. It is openly accepted that immune cells, once stimulated by microorganism-derived toxins, secrete cytokines [4]. In turn, cytokines induce many host responses associated with endotoxemia [5, 6]. Tumor necrosis factor alpha (TNFα) [7] is a key mediator in the development of several symptoms characterizing the acute phase response of inflammation.

Among different types of bidirectional communication between the immune and endocrine systems, the immune and the hypothalamo-pituitary-gonadal (HPG) [8] axes have been thoroughly investigated. Once injured, immune cells secrete TNFα, a substance able to impact on the hypothalamic paraventricualr nucleus enhancing

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corticotropin-releasing hormone neuron functionality [9]. The enhanced corticotropin-releasing hormone neuron activity induces anorexia [10], stimulation of glucocorticoid secretion [11] and inhibition of HPG axis function [12].

Regarding the end products of the HPG axis, it is accepted that sex steroid hormones modulate the immune function, both the humoral and cell-mediated one [13]. Moreover, data support the fact that while estrogens enhance immune function [14], androgens inhibit [15] it in mammals, and it has been reported that gonadectomy is able to alter the immune function as well [16, 17]. The regulatory activity of sex steroid hormones on immune cell function is supported by studies revealing the expression of specific receptors for these molecules in organs responsible for the immune response [13]. In addition to data on the sex steroid effect on hypothalamo-pituitary-adrenal axis function [18, 19], the well-known anti-inflammatory and immunosuppressive roles of glucocorticoids [20, 21] support the establishment of a 3-level compartment vital for survival: the cytokine-glucocorticoid-sex steroid cross talk.

The anorectic adipokine leptin [10], due to its high affinity to cytokine class I receptors [22], modulates immune function [23], and is released in blood during endotoxemia [24]. In turn, leptin modulates HPG [12] and hypothalamo-pituitary-adrenal [25] axes during inflammation. Leptin-circulating levels are dependent not only on individual body fat mass [26] but on gender [27] as well, the latter being a sex steroid-dependent characteristic [28]. Thus, leptin became another participant in the interrelationship between the immune and the endocrine systems.

The aim of the present study was to explore in the adult male rat the effect of an earlier reduction in endogenous androgen activity, due to either the neonatal treatment with flutamide [FTM, an androgen receptor (AR) blocker] or the prepubertal bilateral orchidectomy (ODX), on (1) the peripheral levels of glucocorticoid, testosterone and leptin in basal conditions and (2) the pattern of circulating TNFα concentrations during the acute phase response of inflammation.

Materials and Methods

Animals, Treatments and Surgeries

Adult male and female Sprague-Dawley rats were allowed to mate in colony cages in a light- (lights on from 07:00 to 19:00 h) and temperature- (22°C) controlled room. Rat chow and water were available ad libitum. Pregnant rats were transferred to individual cages. On day 5 after parturition, male pups were injected subcutaneously with either 50 μl sterile corn oil alone (CT) or containing FTM (1.75 mg/pup; Lab. Bioprofarma SA, Argentina). Rats were left undisturbed until weaned on day 21 of age, and then they were individually housed until the experiments were conducted. A second set of male animals (individually caged after weaning) was submitted, on day 25 of age and under light ether anesthesia, to bilateral ODX or sham operation. They were left undisturbed until the experiments were done.

Rats were submitted to experimentation and sacrificed by following protocols for animal use in agreement with NIH Guidelines for care and use of experimental animals. All experiments received approval from our Institutional Animal Care Committees.

Experimental Designs

Because we established in preliminary experiments (data not shown) that the peripheral levels of several metabolites in different experimental conditions were similar in CT and sham-operated 100-day-old rats (n = 5 rats/group), data obtained from CT and sham-ODX rats constituted a combined group (CT/S). These values were then contrasted with those from both FTM and ODX animals. Animals were used for experimentation at 100 days of age.

Experiment 1: Studies Performed in Basal Conditions. Rats from different groups (CT/S, FTM and ODX) were sacrificed (at 08:00–09:00 h) in basal conditions. Blood samples were collected into plastic centrifuge tubes containing 0.5 ml EDTA 10%. Plasma samples were kept frozen until assayed for corticosterone, testosterone and leptin concentrations.

Experiment 2: Intravenous Lipopolysaccharide-Induced Endotoxic Shock. Rats from different groups (CT/S, FTM and ODX) were implanted, under light ketamine anesthesia (between 08:00 and 09:00 h), with intravenous (i.v.) catheters and bled before (sample time 0) and 1, 2, 3 and 4 h after i.v. injection of bacterial lipopolysaccharide (LPS; 25 μg/kg) [24]. After each blood sample withdrawn, a similar volume of fresh red blood cells resuspended in artificial plasma was injected i.v. into animals. Plasma samples were kept frozen (–80°C) until assayed for quantification of TNFα concentrations.

Steroids and Adipocytokine Measurements

Plasmatic concentrations of corticosterone [29], testosterone [30] and leptin [31] were determined by previously described specific radioimmunoassays. The standard curve for B assay ranged between 1 and 250 μg/dl and the intra- and interassay coefficients of variation (CVs) were 4–6 and 8–10%, respectively. The standard curve for testosterone assay ranged between 0.01 and 4 ng/ml and intra- and interassay CVs of 4–7 and 9–13%, respectively. The standard curve for leptin assay ranged between 0.04 and 12.5 ng/ml with intra- and interassay CVs of 5–8 and 10–13%, respectively. Plasma TNFα concentrations were determined with a commercial kit (rat TNF ELISA Set; and BD Biosciences Pharmingen, San Diego, Calif., USA; Cat. No. 558535) and by following the recommendations of the manufacturer; the standard curve ranged between 1 and 1,000 pg/ml.

Statistics

Data are expressed as means ± SEM. Mean plasma TNFα values were analyzed by two-way (time-treatment) ANOVA with re-

Table 1. Body weight values (in g) and basal circulating levels of leptin (in ng/ml per 100 g body weight) in 100-day-old CT/S, neonatal FTM and ODX rats

	CT/S	FTM	ODX
Body weight	284.41 ± 6.73	262.73 ± 5.89*	256.85 ± 4.22*
Leptin	1.44 ± 0.19	0.61 ± 0.09*, +	1.35 ± 0.27

Values are means ± SEM (n = 8–9 rats/group).
* $p < 0.05$ vs. CT/S values; + $p < 0.05$ vs. ODX values.

peated measures, followed by Student-Newman-Keul's test for a comparison of the different mean values. The results of basal plasma metabolites concentrations and the area under the curve of TNFα levels were also analyzed by ANOVA [32].

Results

Peripheral Hormone Levels and Body Weights

Figure 1 shows the results of the circulating levels of several hormones in animals studied in basal conditions. As depicted, plasma corticosterone levels (fig. 1a) were somewhat higher, albeit not significantly so, in neonatal FTM-treated than in normal CT/S rats. Conversely, the circulating levels of glucocorticoid were significantly ($p < 0.05$) higher in prepubertal ODX than in normal CT/S animals. Additionally, plasma testosterone concentrations (fig. 1b), although not modified by neonatal FTM treatment, were drastically ($p < 0.05$ vs. CT/S rats; $p = 0.09$ vs. ODX rats) reduced by prepubertal ODX (ODX group). Finally, neonatal FTM-treated rats displayed significantly ($p < 0.05$) reduced plasma leptin concentrations (fig. 1c) when compared with both normal and prepubertal ODX group values.

Although there were no group differences in starting body weights (data not shown) at an adult age, rats from the androgen activity-deprived groups reached significantly ($p < 0.05$) lower body weight values than those from the normal group (table 1). The differences among groups already seen in plasma leptin levels (fig. 1c) remained after expressing those levels by their respective individual body weight values (table 1). Interestingly, a significant positive correlation ($r = 0.8391$; $p < 0.05$) was found between body weight and leptinemia group values with the following group progression: FTM < ODX < CT/S.

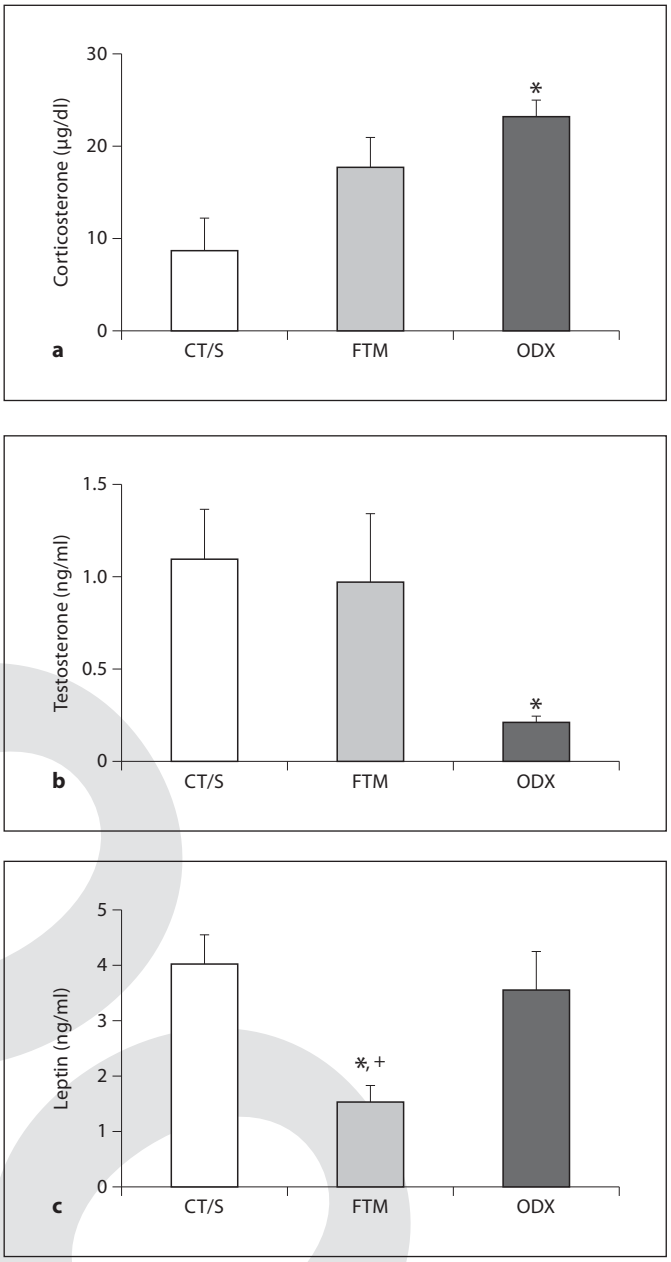

Fig. 1. Basal plasma concentrations of corticosterone (**a**), testosterone (**b**) and leptin (**c**) in 100-day-old male rats from different groups. Values are means ± SEM, n = 6–7 rats/group. * $p < 0.05$ vs. CT/S values; + $p < 0.05$ vs. ODX values.

Effect of the Lack of Androgen Activity on LPS-Induced TNFα Release in the Circulation

Figure 2 shows circulating TNFα levels before and several times after i.v. LPS (25 μg/kg) administration in different groups. No differences among groups were found in the basal values of TNFα concentrations. However, a

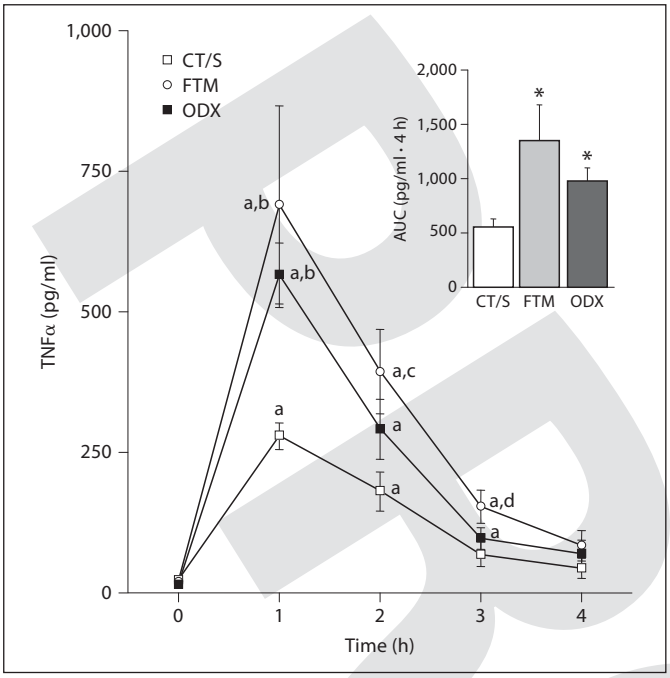

Fig. 2. TNFα concentrations in plasma samples taken before (time 0) and several hours after i.v. administration of 25 μg/kg of LPS in normal (CT/S), neonatal FTM and prepubertal ODX rats studied at day 100 of age. The area under the curve (AUC) of peripheral TNFα concentrations throughout the LPS test is also depicted (**inset**). Data are means ± SEM, n = 6–7 rats/group. [a] $p < 0.05$ vs. basal values of the same group; [b] $p < 0.05$ vs. 1-hour values in the CT/S group; [c] $p < 0.05$ vs. 2-hour values in the CT/S group; [d] $p < 0.05$ vs. 3-hour values in the CT/S group; * $p < 0.05$ vs. CT/S values.

time-related increase over the baseline in plasma TNFα concentrations was found after LPS i.v. administration, regardless of the group examined. Moreover, when plasma cytokine concentrations were analyzed within a group: (1) the values were significantly higher ($p < 0.05$) than the baseline at 1 and 2 h after LPS and they were back (at 3 and 4 h) to basal values in CT/S rats, and (2) the values were significantly higher ($p < 0.05$) than the respective baseline at 1, 2 and 3 h after LPS, then (at 4 h) they recovered to the corresponding basal values in both FTM and ODX rats.

When values were compared among groups, data indicated that the circulating TNFα levels were significantly ($p < 0.05$) higher in FTM and ODX rats than in CT/S animals at 1 h after LPS. On the other hand, at 2 h and more after LPS, the circulating levels of TNFα were similar in ODX and CT/S rats. Conversely, at 2 and 3 h after LPS, peripheral cytokine concentrations remained significantly ($p < 0.05$) higher in FTM than in CT/S rats. Finally, no differences among plasma TNFα concentrations were noticed when comparing values from FTM and ODX rats, regardless of the time of the examination.

The areas under the curve (fig. 2, inset) of circulating proinflammatory cytokine levels were significantly ($p < 0.05$) higher in FTM and ODX rats than in CT/S animals, whereas no statistical difference was found among values from FTM and ODX F rats.

Discussion

Our study supports a suppressive role of endogenous androgen on proinflammatory cytokine secretion during endotoxemia. Moreover, a sex steroid basis-dependent mechanism seems to be mediating such an effect. Although there has been previous evidence for this action [13, 15–17], to our knowledge, this is the first report indicating that a single neonatal FTM treatment in male pups deeply disrupted the organism's defense mechanism during inflammation in adulthood. Interestingly, the effect of neonatal FTM treatment was mimicked, at least in part, by prepubertal ablation of testicular function. These data strongly support that early in life, both a normal function of ARs and the pubertal/postpubertal priming with endogenous androgen are required for the development of the neuroimmunomodulatory effect of testosterone [13].

The neonatal androgenization of the female rat did alter metabolic [33] and immune [34] programming, thus indicating a long-lasting effect of androgens on rat developmental functions. FTM is a nonsteroidal pure anti-AR [35], successfully used in therapeutic treatment of androgen-dependent prostate cancer [36]. It has been reported that both pre- and neonatal FTM treatments in male rats are able to abolish sexual dimorphic characteristics of hypothalamic nuclei [37]. The latter report and data from other authors are in support of an AR-dependent sexual dimorphic pattern in developing rat brain structures, and clearly indicate that a sex steroid-related mechanism is involved in this AR-related effect [37, 38].

We have previously found a sexual dimorphic pattern in the LPS-induced neuroendocrine-immune response of rodents. Indeed, in a pioneer study from our laboratories [15], we demonstrated that LPS-stimulated TNFα secretion in plasma was drastically enhanced in gonadectomized, postpubertal mice of both sexes. Moreover, we established that testosterone replacement therapy did abolish such a difference in gonadectomized mice [15].

Observations clearly indicate that testosterone did suppress proinflammatory cytokine secretion during the acute phase response of inflammation in postpubertal rodents [15]. However, the enhanced TNFα response to either LPS or other immune stimuli [39, 40] in postpubertal ODX mice cannot be reversed by estradiol replacement therapy. Moreover, the inhibitory effect of physiological endogenous androgen levels on the cytokine response has also been observed when using ovariectomized mice receiving testosterone replacement therapy at the normal male levels [40]. Therefore, this is in support of a clear sex steroid hormone basis for the sexual dimorphism observed during the acute phase response of the inflammatory process. In the present study, and by using different experimental approaches, we extended our findings on a suppressive role of endogenous androgen activity on LPS-induced TNFα secretion in the blood. In fact, we studied this mechanism in two ways: the early ablation of the physiological androgen milieu due to the neonatal blockage of AR functionality and the prepubertal ODX. Interestingly, with both approaches we were able to demonstrate that a clear distorted pattern of TNFα secretion occurred when animals reached adult life. These data are supported by the fact that the neonatal FTM treatment is able to modify the testosterone-mediated programming of the immunological activity [41]. Besides data on the immunological sexual dimorphism developed in mammals and the precise role played by sex steroid hormones [13], recent data indicate that endogenous androgen deprivation in men with prostatic cancer did result in reactivation of the immune system function [42]. Another study [43] demonstrated that ODX in rodents, a condition characterized by the absence of any residual adrenal-derived dihydrotestosterone activity [44], reduced the abundance of ARs in the thymus gland. Moreover, Ahmad and Haldar [43] found that ODX enhanced in vitro thymocyte proliferation and IL-6 secretion, and that these effects of ODX were partly/fully reversed by testosterone replacement treatment.

In the present work, we found that either neonatal FTM treatment or prepubertal ODX did result in a common effect, an enhancement in TNFα secretion in plasma during the acute phase response of inflammatory stress. This effect has taken place in an endogenous environment characterized by (1) normal (FTM rats) or reduced (ODX rats) circulating testosterone levels, (2) slightly (FTM animals) or significantly higher (ODX rats) peripheral glucocorticoid levels, and (3) diminished (FTM animals) or unmodified (ODX) leptinemia. These data are, at least in part, in line with a previous report, indicating significantly higher and lower plasma corticosterone and testosterone levels, respectively, in FTM-treated than in normal rats [45]. Our results, however, indicate that an early lack of androgen activity, due to neonatal FTM treatment, resulted in a cytokine over-response during the acute phase of inflammatory stress. Similarly, the lack of androgen activity was also evident by testes removal before puberty. The effects of sex steroids on the immune system functionality of mammals have been extensively revisited [13]. However, other studies established that very early ODX in rats induced changes in the adult T cell functionality [46] and that peripubertal ODX postponed thymic atrophy and immune dysfunction [47].

Regarding the low leptin levels found in FTM rats, it has been reported that in the adult neonatally androgenized female rat [48] and in the normal male rat over-development [49], the increase in plasma leptin levels seems to be related, at least in part, to an androgen effect. It has been claimed that there is a proinflammatory role of leptin [23], which could contradict our data on LPS-stimulated TNFα secretion in FTM rats. However, our findings are in keeping with published data, showing a normal LPS-elicited proinflammatory cytokine release in blood in rats treated with leptin [50] and in genetically leptin-deficient mice [51]. Whether the lack of a full inhibitory action of endogenous androgens on LPS-stimulated TNFα release [15] could be overlapping any leptin effect [23, 52] remains open for research.

In conclusion, our study suggests that early in life, changes in androgen bioactivity severely impact physiological programming [33, 41]. Thus, an AR-dependent inhibitory effect of testosterone on neuroendocrine-immune function is strongly supported.

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