

Fasting Differentially Modulates the Immunological System: Its Mechanism and Sex Difference

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Summary The immunological properties and hormonal metabolism in rodents are affected by physical and psychological stress more strongly in males than in females. To elucidate the mechanism and physiological significance of the sex difference in the susceptibility of animal to stresses, changes in the immunological system in plasma and intestine and hormonal status in plasma were compared among 8-week-old male and female ICR mice before and after fasting. During the fasting of animals, the expression of immunoglobulin A in intestinal mucosa, and cortisol, interleukin-10 and interferon- γ in plasma increased. These changes occurred more apparently in males than in females. Under identical conditions, the plasma levels of testosterone decreased markedly with concomitant occurrence of apoptosis in the testis, while the plasma levels of estradiol decreased calmly, and no appreciable apoptosis occurred in the ovary. These results indicate that testosterone enhances the stress-induced modulation of the immune system by some mechanism that was antagonized by estradiol.

Key Words: fasting, sex difference, interleukin-10, interferon- γ , estradiol, testosterone

Introduction

Stress affects physiological and behavioral responses differently from one species to another [1–3] and also within the same species [4–7]. Sensitivity of animals to stress is linked to the development of a variety of physical and psychological disorders [8–10]. Therefore, understanding the mechanism of different responses of individuals to stress is important for the basic science of stress vulnerability as well as for effective treatment and prevention of stress-related illnesses.

Corticotropin-releasing hormone (CRH), a 41-amino acid peptide, plays a pivotal role in the coordination of the stress response and the regulation of immune/inflammatory reac-

tions [11–14]. CRH modulates the synthesis and release of adrenocorticotrophic hormone (ACTH) and mediates various hormonal, autonomic, behavioral, and immunological action to stress [15–17]. In fact, CRH inhibits the reproductive and growth axes, stimulates the sympathetic nervous system, causes enhanced arousal, and suppresses the immune system through the activation of hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system [12, 14, 18, 19].

Sex differences in the regulation of stress response, in general, and the HPA axis, in particular, as well as sexual dimorphism of immune/inflammatory reactions including susceptibility to autoimmune disease, have been described in various species [20–22]. These studies have suggested that gonadal steroids interact in a regulatory manner with central nervous system and peripheral systems including the HPA axis suggested to underlie the mechanism of the immune/inflammatory reactions. Although estrogens and androgens have been these differences, whether their effects

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occur directly or indirectly via estrogen- or androgen-dependent pathways remains unclear. The exact site(s) of the interaction and its mechanism also remain to be elucidated. The central role of HPA axis in these systems suggests that this pathway may be a common pathway for the regulation of stress-related immunomodulation.

The present work describes sex differences in the immunomodulation by stress elicited by fasting.

Materials and Methods

Animal experiments

Specific pathogen-free 8-week-old male and female ICR mice (SLC Hamamatsu, Japan) were subjected to experiments according to the animal care regulations of Osaka City University Medical School. The study protocol ran for 3 days and included the following groups, normal fed, 24, 48, and 72 h of fasting.

Ovariectomy and testosterone administration

For hormone replacement studies, female mice were ovariectomized (OVX) to remove the primary source of endogenous estrogen. One week after surgery, OVX and intact female mice were randomly assigned to either testosterone administrated group or placebo group. Approximately 10 μ g/day of testosterone (Katayama, Osaka, Japan) in polyethylene glycol 400 (PG; Wako, Osaka, Japan) was injected intraperitoneally into OVX and intact female mice throughout the experimental period. Only PG was injected into the female placebo groups.

Castration and 17 β -estradiol administration

For hormone replacement studies, male mice were castrated (CAX) to remove the primary source of endogenous testosterone. One week after surgery, CAX and intact male mice were randomly assigned to either 17 β -estradiol treated or placebo groups. Approximately 10 μ g/day of 17 β -estradiol (Sigma) in PG was injected intraperitoneally into CAX and intact male mice throughout the experimental period. Only PG was injected into the male placebo groups.

Quantification of hormones and cytokines by enzyme-linked immunosorbent assay (ELISA)

Blood samples were taken from the heart from each study group, and the plasma was obtained. Plasma levels of ACTH and cortisol were determined using a commercial ELISA kit (ACTH; Phenix Pharmaceuticals Inc, CA; Cortisol; Oxford Biochemical Research Inc, MI) according to the manufacturer's instructions. Plasma estradiol and testosterone contents were determined using a commercial ELISA kit (Cayman Chemical, MI). Interleukin-10 (IL-10) and interferon- γ (IFN- γ) were determined using a commercial ELISA kit (Endogen, Rockford, IL).

Preparation and staining of ovary, testis, and intestine

The ovary, testis, and intestine (colon) were fixed in phosphate-buffered paraformaldehyde (4%), embedded in frozen Tissue Tek, OCT Compound, and cut into 5 μ m thick sections. In the ovary and testis, apoptosis was analyzed using an *in situ* apoptosis detection kit (Takara Biomedicals, Tokyo, Japan). The expression of immunoglobulin A (IgA) was evaluated immunohistochemically under a fluorescent microscope as described previously [23].

Statistical analysis

Six mice were used for each assay group within an experiment, and the results are expressed as the mean \pm SD. The significance of differences was analyzed by either Student's *t* test or ANOVA using computer software. Differences were considered to be significant when $p < 0.05$.

Results

Effect of fasting on plasma cortisol and ACTH

The plasma levels of cortisol and ACTH significantly increased after fasting (Fig. 1 and 2). In response to fasting, the males showed more remarkable increase than the female. In the CAX group receiving 17 β -estradiol, the plasma levels of cortisol and ACTH significantly decreased as compared with control group. In the testosterone-injected female group and OVX group, plasma levels of cortisol and ACTH significantly increased as compared with control group. However, the concentration of plasma cortisol and ACTH in the 17 β -estradiol-injected male group and CAX group remained unchanged.

Effect of fasting on plasma IL-10

The neuro-immune network system is activated by a

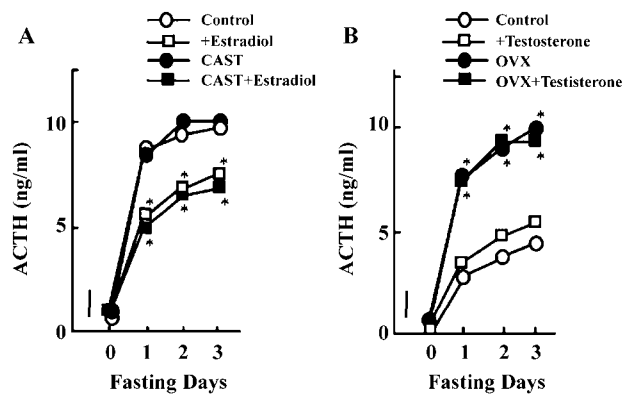


Fig. 1. Effect of fasting on plasma ACTH. At the indicated times of fasting, plasma levels of males (A), females (B), were measured as described in the text. The statistical difference between control and experimental groups was analyzed by Student's *t* test. Bar is pooled SEM. * $p < 0.05$.

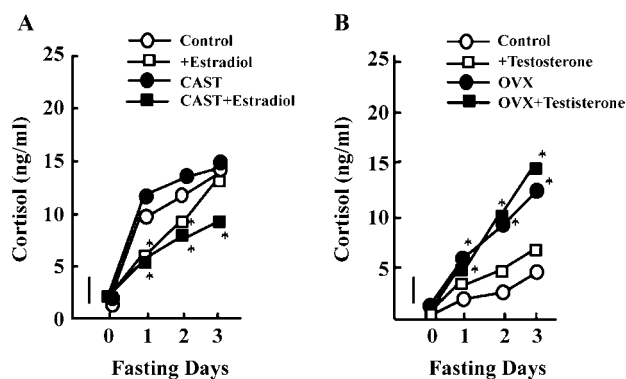


Fig. 2. Effect of fasting on plasma cortisol. At the indicated times of fasting, plasma levels of males (A) and females (B), were measured as described in the text. The statistical difference between control and experimental groups was analyzed by Student's *t* test. Bar is pooled SEM. **p*<0.05.

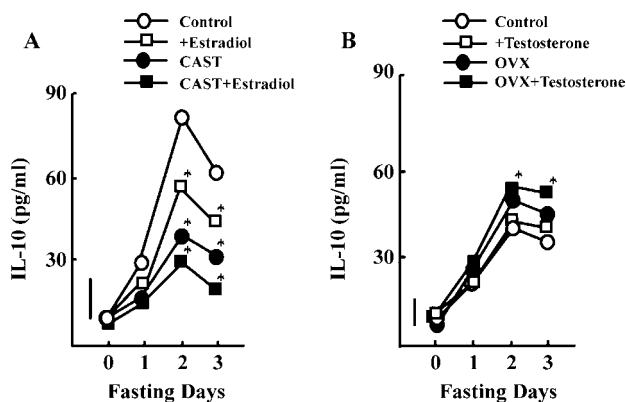


Fig. 3. Effect of fasting on plasma IL-10. At the indicated times of fasting, plasma levels of males (A) and females (B), were measured as described in the text. The statistical difference between control and experimental groups was analyzed by Student's *t* test. Bar is pooled SEM. **p*<0.05.

sequence of stress reactions to stimulate Th2-dependent immune system. To classify the mechanism for the sex different reactions, we analyzed plasma levels of IL-10 (Fig. 3) and IgA in intestinal mucosa (Fig. 5) as markers for Th2-type immune reaction during the fasting.

Plasma levels of IL-10 significantly increased after fasting and reached to a maximum level on day 2 after fasting. The male animals showed more remarkable increase than the female in response to fasting. In the 17 β -estradiol-injected male group and the 17 β -estradiol-injected group after CAX, plasma levels of IL-10 significantly decreased as compared to that in the control male mice. Plasma levels of IL-10 increased slightly in the OVX group.

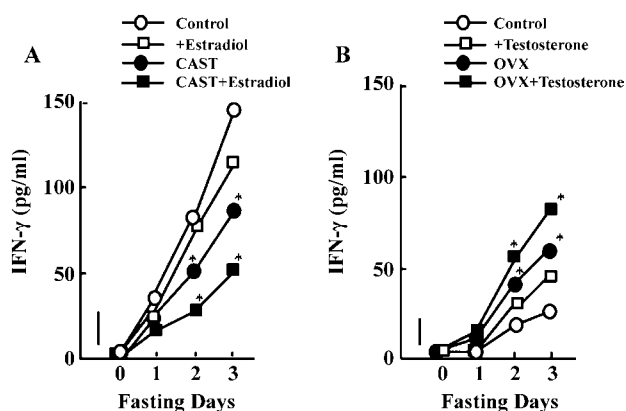


Fig. 4. Effect of fasting on plasma IFN- γ . At the indicated times of fasting, plasma levels of males (A) and females (B), were measured as described in the text. The statistical difference between control and experimental groups was analyzed by Student's *t* test. Bar is pooled SEM. **p*<0.05.

Effect of fasting on plasma IFN- γ

Plasma levels of IFN- γ increased after fasting while it was suppressed in females for the first and two days (Fig. 4). In the 17 β -estradiol-injected male group and the 17 β -estradiol-injected group after CAX, plasma levels of IFN- γ significantly decreased as compared with the control male mice and the OVX group. Plasma levels of IFN- γ significantly increased as compared with the control female mice.

Effect of fasting on mucosal IgA

Clonal expression of IgA increased markedly from day one after fasting in males not in females (Fig. 5). In the 17 β -estradiol-injected group after CAX, no such increase in the expression of IgA was observed. In contrast, the expression of IgA was apparent in OVX group.

Effect of fasting on reproductive organs

After fasting, apoptotic cells developed in the testis one day after fasting while they become apparent in the ovary only after 3 days of fasting (Fig. 6). The plasma levels of testosterone decreased with the fasting period while plasma levels of estradiol remained unchanged.

Discussion

The present work demonstrates that marked sex differences occur in the reaction of immune system during fasting; the extent of reaction was smaller with females than with males. Kinetic analysis revealed that estradiol was principally responsible for the observed sex differences.

The major response to stress is the activation of the HPA axis [24]. This system involves the production and release of CRH followed by the production and secretion of pro-

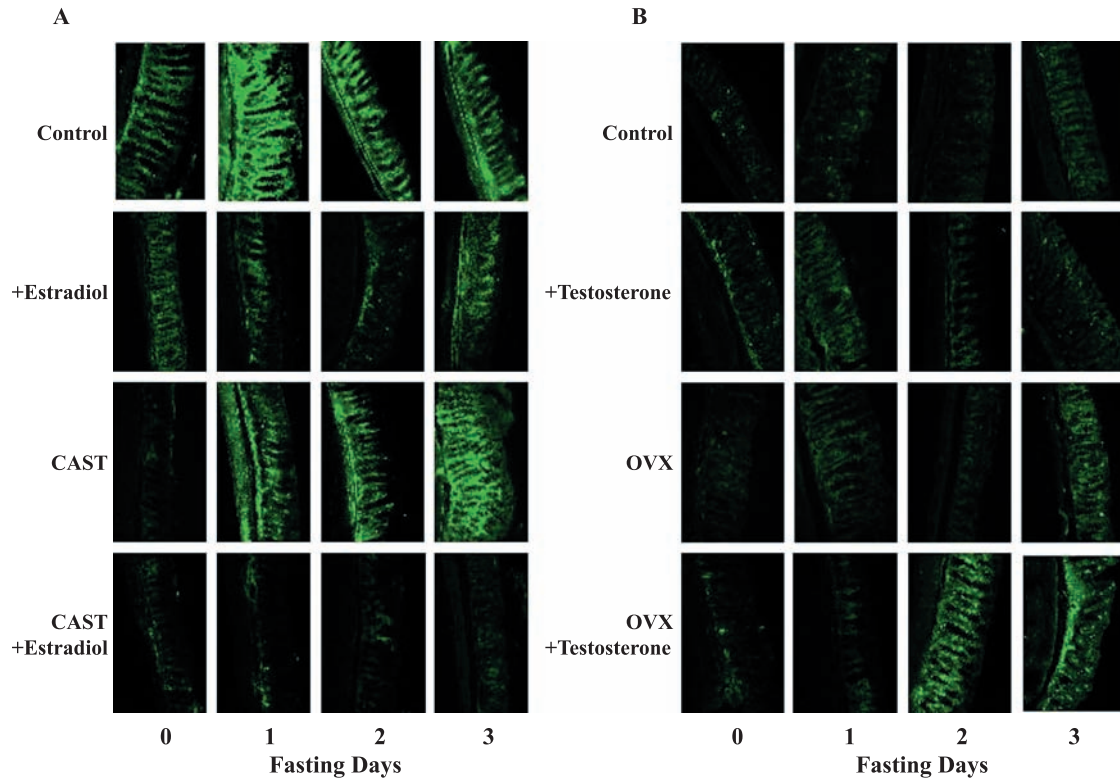


Fig. 5. Histological examination of the expression of IgA in the colon. At the indicated times of fasting, colon specimens were frozen, cut into thin sections, treated with anti-IgA antibody and then stained with FITC-conjugated second antibody. The expression of IgA was determined in the male mice (A) or female mice (B). Data show one typical result out of 3 representative experiments. Scale bar = 50 μ m.

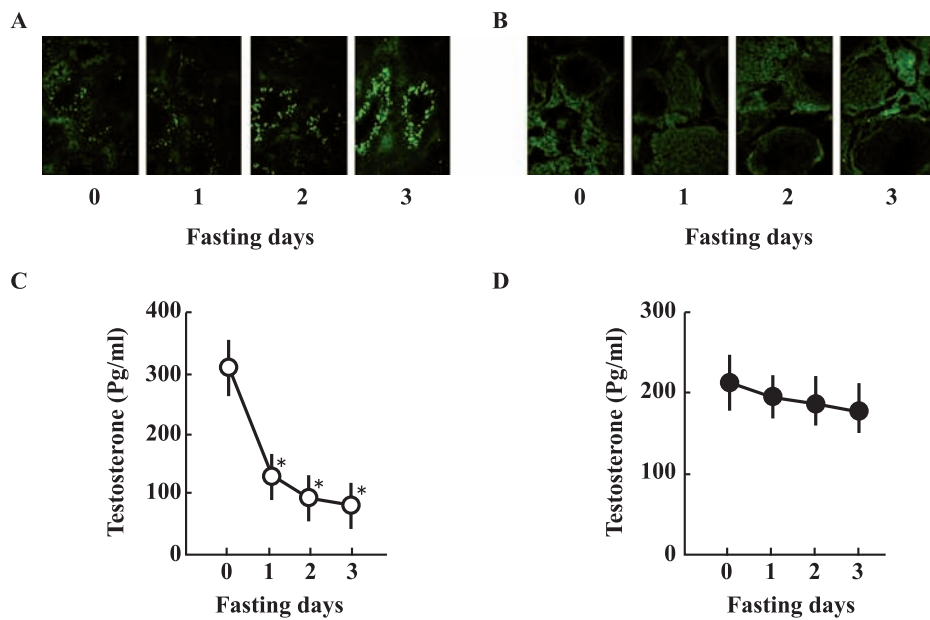


Fig. 6. Effect of fasting on the plasma level of sex hormones and on expression of reproductive organ of tunnel positive cells. At the indicated times of fasting, testis specimens (A) and oviduct specimens (B) were measured using a tunnel kit. The plasma levels of testosterone (C) and estradiol (D) were measured as described in the text. All values are shown as the mean \pm SD. The statistical difference between 0 time and each experimental times is analyzed by Student's *t* test. * p <0.05. Scale bar = 50 μ m.

opiomelanocortin (POMC) peptides. ACTH induces the production and secretion of cortisol, a powerful anti-inflammatory factor. During stress, autonomic nervous system (ANS) also exerts systemic effects on immune system by inducing the secretion of IL-6 in the circulation [25]. Despite its inherent inflammatory activity, IL-6 plays a major role in the overall control of inflammation by stimulating glucocorticoid secretion [26, 27] and by suppressing the secretion of TNF- α and IL-1. Furthermore, catecholamines inhibit IL-12 and stimulate IL-10 secretion [28]. The combined effects of glucocorticoids and catecholamines on the monocyte/macrophage and dendritic cells inhibit innate immunity and Th1-related cytokines, such as IFN- γ and IL-12, and stimulate Th2-related cytokines, such as IL-10 [29].

The present work shows that the plasma level of cortisol and IL-10 increased during the fasting stress. The strong increase in plasma cortisol in male seems to elicit the sex difference in the amount of production of IL-10. Preliminary experiment revealed that lipopolysaccharide (LPS) levels in the blood significantly increased during the fasting, suggesting the translocation of microflora across the intestinal mucosa (data not shown). Consistent with previous observation [30], the increase in plasma IFN- γ was followed by the decrease in IL-10 on day 3 after fasting.

The mucosal IgA, a Th2-related antibody, also increased during the fasting. As a result, B cells in the intestinal mucosa were activated by the increased cortisol. Since the mucosal expression of IgA reflects the increased immunity against the invasion of intestinal pathogens, the increase in the plasma levels of LPS might be the cause of the increased IgA in the intestine [31]. The increased IgA in the mucosal layer might participate in the increased activity to suppress pathogens invading from intestinal lumen under the fasting conditions.

The present works shows that the stress elicited by fasting stimulated the HPA axis and immunological reactions more markedly in male than in females. The reaction in the females was increased by the administration of testosterone. Furthermore, the immunological reaction of the OVX females administered with testosterone was similar to that of the males. The reaction of male animals was reduced by administering estradiol to a level comparable to that of the CAX females administered with estradiol. Furthermore, apoptosis of Leydig cells in the testis was induced by fasting with concomitant decrease in plasma levels of testosterone. In contrast, apoptotic cells were not observed in the ovary of fasted females despite the decrease in plasma estradiol. This observation suggested that sex hormones are responsible for the sex differences in the immunological reactions to fasting stress.

Several studies suggested that estradiol plays a role in the enhancement of stress responses in OVX rats that showed

the enhancement of the HPA response to stress after treatment with estradiol [32–34]. Female rats show enhanced HPA response in the proestrus cycle when plasma levels of estrogen and progesterone are low [34]. Since plasma levels of estradiol and testosterone decrease during the exposure to fasting stress, their decrease might reflect the stimulation of the HPA axis. However, the detailed mechanism request further study.

Previous studies have indicated that the sex difference in the susceptibility to stress might result from sex difference in cytokine production upon stimulation by autoantigens in male and female. Enhanced production of Th2-related cytokines and suppressed expression of Th1 related cytokines have been observed with males as compared with females [35–37]. Since plasma levels of testosterone decreased markedly during the fasting, the activation of Th2 reactions does not last for a long time and, hence, Th1 type reaction increased during the long fasting stress.

These observations suggest that the HPA axis was activated by fasting to cause immunomodulation in which Th2 type reaction predominate during the initial time particularly in male. In contrast, the response to fasting stress occurs mildly in females than in male, thereby immunological reactions occur more mildly in the former than in the latter. The marked sex difference in the immunological responses to fasting might favor the animals to survive under environment where foods were always limited strongly.

Acknowledgments

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