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Immobilization Stress May Increase Plasma Interleukin-6 via Central and Peripheral Catecholamines

Key Words

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6-Hydroxydopamine

Abstract

It has recently been reported that both physical and psychological stress elevate plasma inter-leukin (IL)-6 levels independently of endotoxemia, tissue damage, or inflammation. However, the mechanism of plasma IL-6 elevation in these models is poorly understood. In the present study, plasma IL-6 levels were measured using the IL-6-dependent murine hybridoma subclone B9 cell line, which is commonly used by other investigators. We first demonstrated that an immobilization (IM) stress, a typical psychophysiological stress, increased plasma IL-6 levels. Then the contribution of the hypothalamic-pituitary-adrenal (HPA) axis and the central and peripheral catecholaminergic systems in IM-induced plasma IL-6 elevation were examined because these mechanisms play important roles in host defense against stress. Blood samples were collected through an indwelling jugular venous catheter before, during, and after IM; the number of samples taken serially from each animal was 12–13. Blood cells were resuspended in a saline solution and injected into the animals through the same catheter after each blood collection in order to prevent loss of blood volume. After initiation of restraint, plasma IL-6 levels significantly increased at 60 min and peaked at 90 min in the animals immobilized for either 30 or 120 min. The peak levels of IM-induced plasma IL-6 in the animals immobilized for 120 min ($1,905 \pm 414$ U/ml) were significantly higher than those in the animals subjected to 30 min IM (837 ± 95 U/ml; $p < 0.05$). In the hypophysectomized (Hypox) or adrenalectomized (ADX) rats, the peak levels of IM-induced (60 min) plasma IL-6 were considerably higher than in sham-operated rats (Hypox: $33,281 \pm 4,983$ U/ml at 150 min; ADX: $52,020 \pm 19,231$ U/ml at 120 min). In addition, in order to determine the possible involvement of endogenous catecholamines in IM-induced plasma IL-6 elevation, 6-hydroxydopamine (6-OHDA) was injected into the lateral cerebral ventricle (i.c.v., 100 µg/rat) or jugular vein (i.v., 100 mg/kg) of the rats, and the animals were exposed to IM stress 1 week (i.c.v.) or 3 days (i.v.) after injection. Both i.c.v. and i.v. injections of 6-OHDA markedly attenuated the plasma IL-6 response to IM as compared to the respective vehicle-injected group, whereas the plasma adrenocorticotrophic hormone response to IM stress was reduced only by pretreatment with an i.c.v. injection of 6-OHDA. These data suggest that (1) neither the pituitary nor the adrenal gland is a major source of increased plasma IL-6 induced by IM stress; (2) the HPA axis exhibits a suppressive regulatory role in the IM-induced IL-6 response; (3) both the central and peripheral catecholamines play critical roles in causing IL-6 elevation induced by IM stress, and (4) the involvement of peripheral catecholamines in elevating plasma IL-6 by IM stress is independent of HPA axis activation. Moreover, to determine whether splenocytes are the source of IM-induced plasma IL-6, splenectomized (Splex) rats were examined for their plasma IL-6 response to IM stress. The peak IL-6 levels after IM in Splex animals were significantly higher than those in sham-operated animals ($p < 0.05$). The immune-competent cells in the spleen, as well as the pituitary and adrenal glands do not seem to be a source of the increased levels of plasma IL-6 induced by IM stress.

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Introduction

Although interleukin (IL)-6 was originally identified as a soluble immune-tissue-derived factor which promotes the proliferation and differentiation of lymphocytes [1], many reports have detailed the nonimmune functions of IL-6, such as the production of acute-phase proteins in liver cells [2], the stimulation of the hypothalamic-pituitary-adrenal (HPA) axis [3-5], activity as an endogenous pyrogen [6], its cytoprotective function against lethal irradiation [7], and neurotrophic functions [8,9]. These pleiotropic activities of IL-6 seem to play important roles in the host defense mechanism [10].

It has recently been demonstrated that noninflammatory stress increased the plasma IL-6 level independently of endotoxemia, tissue damage, or inflammation [11-13]. Zhou et al. [11] showed that electrical footshock, as well as restraint and conditioned aversive stress increased plasma IL-6. They suggested that footshock-induced plasma IL-6 came from the adrenal gland [14] because adrenalectomy (ADX) attenuated the elevation of IL-6 levels. Contrary to this report, we [13] demonstrated that hemorrhage/reinfusion-induced plasma IL-6 elevation was enhanced by ADX. This enhancement was abolished by implanting a corticosterone pellet in the subcutaneous tissue to maintain high corticosterone levels in the plasma. These reports suggest two different contributions of the adrenal gland to stress-induced changes in plasma IL-6 levels. Firstly, it may be a source of IL-6 and, secondly, it may suppress IL-6 production in peripheral tissues through increased corticosterone release into the plasma [15-17]. In this study, we intended to clarify whether or not the HPA axis and the adrenal gland are involved in immobilization (IM)-induced IL-6 increase using hypophysectomized (Hypox) and ADX animals.

IM stress stimulates the release of catecholamines from the adrenal medulla and sympathetic nerve terminals [18]. Exogenous catecholamine, when subcutaneously injected, stimulates the IL-6 increase in plasma [19, 20], while a mitogen-induced lymphocyte proliferation [21] and a natural killer cytotoxicity [22] were suppressed by endogenous catecholamines. Furthermore, the central catecholaminergic mechanism, especially the α_1 -adreno receptor, mediates the release of corticotropin-releasing factor (CRF) into the portal vein in the median eminence of the hypothalamus resulting in the activation of the HPA axis [23,24]. In turn, CRF, which is induced by psychosocial stress in the brain, enhances the activity of the central catecholaminergic neurons [25] and activates the peripheral sympathetic nervous system [26, 27].

These evidences suggest that both the peripheral and central catecholaminergic mechanisms are involved in the plasma IL-6 response to IM stress. In order to verify this possibility, 6-hydroxydopamine (6-OHDA) was injected into the lateral cerebral ventricle (i.c.v.) or jugular vein (i.v.) to deplete the catecholamines in the central or peripheral tissues, respectively [28], and the plasma IL-6 response to IM stress was examined.

IL-6 is produced by many different cell types, including splenic monocytes and lymphocytes [10]. The spleen is also known as a key organ through which the brain modulates immune functions via the sympathetic nervous system [29-32]. Therefore, to determine whether or not the splenocytes are a possible source of plasma IL-6 which is increased by IM stress, the plasma IL-6 response to IM stress was examined in splenectomized (Sp⁻) animals.

Materials and Methods

General Procedure

Adult male CD rats weighing 300-400 g from Charles River Breeding Laboratories (Wilmington, Mass., USA) were used throughout the study. Animals were housed in a light- (12 h light-dark cycle, light on at 06.00 h), temperature- (23°C), and humidity-controlled room with food and water available ad libitum. All procedures were approved by the Advisory Committee for Animal Resources of Tulane University. All animals were implanted with an indwelling jugular venous catheter filled with heparinized saline solution (50 U/ml) 1 or 3 days before the experiment, under ether anesthesia. Blood samples (0.7 ml) were taken through the jugular vein catheter 12-13 times (-30 to 300 or 360 min) before and after the initiation of restraint. Each sample was collected in an ice-chilled tube containing heparin (10 U, Sigma), Trasylol (aprotinin; 500 kIU, Mobay), and EDTA (2 mg, Mallinckrodt). After centrifugation of the blood samples, the plasma (0.3 ml) was removed and frozen at -80°C. Blood cells were resuspended in physiological saline (0.3 ml) and injected through the same catheter after each blood collection to prevent loss of blood volume.

IM Stress

Rats were adapted to experimental conditions by daily handling to avoid manipulative stress. On the experimental day, cages were placed in a quiet room for at least 1 h to enable the animals to adapt to the experimental conditions. Rats were restrained for 30, 60, or 120 min in the supine position by taping their limbs to holders. In general, IM stress at room temperature over 24 h or more produces a minimal degree of gastric lesioning [33]. However, in the animals exposed to restraint for 120 min, no apparent histological changes in the stomach membrane which could have affected IL-6 production in the damaged tissue were seen.

Hypox, ADX, and Splex

Hypophysectomized (Hypox) rats of the CD strain were purchased from Charles River. They were allowed to drink tap water.

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containing 0.9% NaCl and given sugar cubes. Plasma adrenocorticotrophic hormone (ACTH) was not detectable in any of the Hypox rats. IM stress was imposed 2 weeks after the operation. In the other animals, bilateral ADX or Splex was aseptically performed under ketamine (100 mg/kg body weight) + xylazine (8 mg/kg body weight, i.m.) anesthesia, 1 week before the IM stress. ADX rats were also given physiological saline ad libitum.

Lev. and i.v. Injection of 6-OHDA

One week before the experiment, a stainless steel guide cannula (22G) was implanted stereotaxically into the right lateral ventricle (AP -0.8, L 1.5, H 4.0 mm) according to a brain atlas [34] under pentobarbital anesthesia (50 mg/kg body weight, i.p.). Microinjection was carried out through an inner cannula (26G) which was connected to a fixed but flexible connector (C313CS, Plastic One) that allowed the rats to move freely. To deplete central catecholamines, we injected 6-OHDA (100 µg/rat, Sigma) into the cerebral ventricle, 1 week before the IM stress. 6-OHDA was dissolved in 10 µl of artificial cerebrospinal fluid (128 mM NaCl, 2.6 mM KCl, 1.3 mM CaCl₂, 20 mM NaHCO₃, 1.3 mM Na₂HPO₄, pH 7.35) containing 0.1% bovine serum albumin and 0.1% ascorbic acid, and injected over 20 min using a microinjection pump. The content of norepinephrine in the hypothalamus was expected to decrease to one third that of the vehicle-injected rats 1 week after the injection [35]. The i.v. injection of 6-OHDA (100 mg/kg body weight dissolved in saline solution containing 0.4% ascorbic acid) was made through an indwelling jugular venous catheter 3 days before the IM stress to deplete the endogenous catecholamines in peripheral tissues [28].

Fig. 1. Effect of IMj plasma 10^{-6} (a) and ACTH (b) levels (in the same animals). Arrows indicate the initiation of restraint. Each point represents the mean $\pm$ SE (n = 7). * p $\leq$ 0.05, ** p $\leq$ 0.01 vs. values of the no IM group; † p $\leq$ 0.05, †† p $\leq$ 0.01 vs. values of the 30-min-IM group.

Plasma ACTH Concentration

Plasma ACTH levels were determined by RIA using an anti-human ACTH antibody which was gifted from the National Hormone and Pituitary Program of the National Institute of Diabetes. Human ACTH (1-39) is used as reference standard for plasma ACTH.

Statistical Analysis

All data were expressed as the mean $\pm$ SE. We first analyzed all graphs by two-way (group and time factor) analysis of variance (ANOVA). When the p value was $\leq 5\%$ in both factors, we checked the significance of the difference between each value at each sampling time using the Tukey test or Student t test.

Results

Plasma IL-6 and ACTH Responses to IM

During and after IM stress, plasma IL-6 increased depending on the time of restraint (group factor: d.f. = 2, $F = 60.9$, $p \leq 0.001$; time factor: d.f. = 12, $F = 9.5$, $p \leq 0.001$; fig. 1a). Plasma IL-6 levels started to rise within

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Fig. 2. Effect of Hypox (a) and ADX (b) on the IM-induced (1 h) plasma IL-6 increase. Each point represents the mean $\pm$ SE ($n = 6$).

30 min after the initiation of restraint and reached peak levels at 90 min in both the 30-min- and 120-min-IM animals. The mean peak value of plasma IL-6 during 120 min IM was $1,905 \pm 414$ U/ml which was significantly higher than that during 30 min IM (837 ± 95 U/ml; $p \leq 0.05$). In 120-min-IM rats, the plasma IL-6 remained elevated until 300 min after initiation of restraint. The peak times of plasma ACTH concentration in 30-min- and 120-min-IM animals were within 15 min after initiation of the restraint as shown in figure 1b (group factor: d.f. = 2, $F = 58.8$, $p \leq 0.001$; time factor: d.f. = 12, $F = 46.2$, $p \leq 0.001$). No significant difference in plasma ACTH levels between these two groups was shown except at 90 and 120 min ($p \leq 0.05$).

Plasma IL-6 Response to IM in Hypox Rats and Plasma IL-6 and ACTH Response to IM in ADX Rats Both the Hypox and ADX animals showed much greater and prolonged elevation of plasma IL6 in response to IM

stress (1 h) compared to the sham-operated animals (fig. 2a, b). The mean peak value of the IM-induced IL-6 increase in Hypox animals ($33,281 \pm 4,983$ U/ml at 150 min) was higher than that in ADX animals ($52,020 \pm 19,231$ U/ml at 120 min; $p < 0.01$). As shown in figure 3, the basal value of plasma ACTH (275 ± 44 pg/ml) in ADX rats was significantly higher than that of the sham-operated rats (104 ± 25 pg/ml; $p < 0.01$). ADX rats also showed higher concentrations of plasma ACTH (945 ± 74 pg/ml) 15 min after initiation of restraint when compared to the values found in sham-operated rats (676 ± 58 pg/ml; $p < 0.05$).

Fig. 3. Effect of ADX on IM-induced (1 h) ACTH release. 0 min indicates the basal level of plasma ACTH before restraint. 15 min indicates a sampling time after initiation of restraint. Each column represents the mean $\pm$ SE ($n = 6$). ** $p < 0.01$ compared to the basal level in each group; * $p < 0.05$, *** $p < 0.01$ comparing the experimental groups at each time point.

Plasma IL-6 and ACTH Responses to IM in 6-OHDA-Injected Rats

The i.c.v. injection of 6-OHDA (100 μ g/rat) 1 week before the experiment significantly attenuated the IM-induced plasma IL-6 response (group factor: d.f. = 1, $F = 61.9$, $p < 0.001$; time factor: d.f. = 11, $F = 10.9$, $p < 0.001$; fig. 4a). The peak value of IM-induced IL6 response (252 ± 61 U/ml) in animals given 6-OHDA i.c.v. was significantly lower than that in vehicle-injected animals (891 ± 88 U/ml; $p < 0.01$). The i.v. injection of 6-OHDA (100 mg/kg) 3 days before IM stress also attenuated the resulting IL-6 increase as shown in figure 4b (group factor: d.f. = 1, $F = 62.4$, $p < 0.001$; time factor: d.f. = 11, $F = 17.0$, $p < 0.001$). Moreover, i.c.v. administered 6-OHDA significantly depressed the ACTH response to IM stress (i.c.v. of 6-OHDA: 574 ± 9.9 pg/ml, i.c.v. of vehicle: 820 ± 68 pg/ml; $p < 0.01$), whereas i.v. injected 6-OHDA did not (fig. 5a, b).

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Fig. 4. Effect of i.c.v. (a) and i.v. (b) injection of 6-OHDA on the IM-induced (1 h) plasma IL-6 increase. Each point represents the mean $\pm$ SE (n = 5). * p < 0.05, ** p < 0.01 compared to the vehicle-injected animals.

Fig. 5. Effect of i.c.v. (a) and i.v. (b) injection of 6-OHDA on IM-induced (1 h) ACTH release. Each column represents the mean $\pm$ SE (n = 5). ** p < 0.01 compared to the basal level in each group; * p < 0.01 comparing the experimental groups.

Fig. 6. Effect of Splex on IM-induced (1 h) plasma IL-6 increase. Each point represents the mean $\pm$ SE (n = 5). * p < 0.05 compared to the sham-operated animals.

Plasma IL-6 and Responses to IM in Splex Rats Splex enhanced the IM-induced plasma IL-6 increase as shown in figure 6 (group factor: d.f. = 1, F = 20.1, p < 0.001; time factor: d.f. = 1, F = 43.7, p < 0.001). The peak levels of plasma IL-6 after IM stress in Splex and sham-operated rats were $1,172 \pm 130$ U/ml and 803 ± 46 U/ml, respectively. The difference in the values between the two groups was significant at 60 and 90 min after initiation of restraint (p < 0.05).

Discussion

The present results showed that plasma IL-6 significantly increased in response to IM stress depending on the duration of restraint. The peak response time of IL-6 elevation was 90 min after initiation of restraint in both 30-min- and 120-min-IM animals, while that of plasma ACTH occurred within 15 min. In addition, the plasma corticosterone levels in the same animals exposed to IM stress peaked at 30 min (data not shown). When the plasma corticosterone was depleted by ADX, increases in the plasma IL-6 concentration in response to IM stress were considerably enhanced (41-fold greater than those in the sham-operated animals). In addition, the IM-induced IL-6 elevations were prolonged for more than 5 h after the initiation of restraint. Even

in the case of plasma ACTH depletion by Hypox, both the peak levels and time course of the IM-induced *IL-6* response showed the same tendency with the ADX animals (fig. 2a, b), while the peak value in the Hypox group was lower than that in the ADX group. Moreover, plasma ACTH in ADX rats seems to be

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up-regulated, since both resting plasma ACTH and the plasma ACTH value 15 min after initiation of restraint showed significantly higher levels compared to that in sham-operated animals. These data suggest that not only plasma ACTH, but plasma *IL-6* in response to IM stress is also affected by plasma corticosterone levels. Our previous study using the hemorrhage/reinfusion model demonstrated the negative correlation between plasma *IL-6* and corticosterone levels [13]. Many other reports obtained by in vivo [15, 16] and in vitro experiments [16] also suggest that plasma corticosterone suppresses *IL-6* production in peripheral tissues.

Plasma *IL-6* levels were positively correlated with the mortality of patients who suffered septic shock [37]. Many kinds of endotoxins and endogenous pyrogens induce *IL-6* expression in cells such as fibroblasts, endothelial cells, and monocytes [10]. However, there is little experimental evidence which helps to identify the source of stress-induced plasma *IL-6* without endotoxemia or tissue damage. Since i.v. injection of lipopolysaccharide, the most popular endotoxin, induces more than 100-fold higher levels of *IL-6* in plasma as compared to a hemorrhage/reinfusion procedure [13], a different mechanism might be involved in plasma *IL-6* elevation in response to psychophysiological stress. Although the adrenal gland is considered to be a source for *IL-6* elevation induced by electrical footshock [11], the present data, obtained in an IM stress model, do not support this possibility. Neither the pituitary [38, 39] nor the adrenal gland [14] seem to be a major source of IM-induced plasma *IL-6*. Moreover, since Splex rats showed an enhanced rather than a reduced response of plasma *IL-6* to IM stress (fig. 6), the spleen, which contains many immune-competent cells, is also an unlikely source of IM-induced plasma *IL-6*. The source of plasma *IL-6* induced by noninflammatory stress has yet to be precisely determined.

In the present study, we demonstrated that depletion of endogenous catecholamines in the central or peripheral tissues significantly attenuated IM-induced *IL-6* elevation. IM-induced ACTH release, which is mediated by α_1 -adrenoreceptors [23], was not affected by the i.v. injection of 6-OHDA. However, the i.c.v. injection of 6-OHDA attenuated both IM-induced *IL-6* and ACTH elevations in plasma as shown in figures 4 and 5. Because the blood-brain barrier prohibits systemically injected 6-OHDA from entering into brain tissue [28], the stimulation by peripheral catecholamine of the plasma *IL-6* response to IM stress seems to be independent of central mechanisms such as the activation of the HPA axis. In addition, Gool et al. [20] have shown that s.c. injection of epinephrine

(0.2 mg/rat) increases plasma *IL-6* levels, and that pretreatment with β -blocker attenuates this response. We also found that intraperitoneal injection of epinephrine (0.03 and 0.15 mg/rat) increased plasma *IL-6* levels in a dose-dependent manner [unpubl. obs.]. These data, which indicate that exogenous epinephrine stimulates *IL-6* production in plasma, support our present interpretation. The peripheral epinephrine or norepinephrine is mainly released from the adrenal medulla or sympathetic nerve terminals, respectively, in response to IM stress [18]. However, circulating epinephrine derived from the adrenal medulla was not involved in IM-induced *IL-6* production because plasma *IL-6* response to IM stress was enhanced in ADX animals (fig. 2b). Norepinephrine release from the sympathetic nerve terminals may be critical for IM-induced plasma *IL-6* production. However, the splenocytes, whose functions are modulated by norepinephrine released from the sympathetic nerve terminals [22], do not seem to contribute to the plasma *IL-6* elevation in response to IM stress. On the contrary, *IL-6* production by splenocytes was suppressed by footshock stress [11]. Our finding also demonstrated the enhanced *IL-6* response to IM stress in Splex animals (fig. 6). Immune-competent cells are unlikely to be a source of plasma *IL-6* increased

by IM stress. The target sites of released catecholamines, which directly or indirectly produce $11^{\wedge}6$, have yet to be determined.

When we discuss the involvement of central catechol-aminergic systems in response to stress, the mediation of central CRF must be considered. The release of CRF into the hypophyseal portal veins, resulting in the secretion of ACTH by the pituitary gland, is regulated by central catecholamines [23,24], and, in turn, central CRF induced by stress activates the catecholaminergic neurons in the brain [25]. Furthermore, central CRF which is independent of the HPA axis modulates immune function via the sympathetic nervous system [40]. Actually, it has been reported that i.c.v. injection of CRF increases plasma IL-6 [41]. However, we recently demonstrated that continuous i.c.v. injection of ct-helical CRF (50 μ g/rat), a CRF antagonist, before and during IM stress, did not attenuate IM-induced IL-6 elevation while ACTH release was reduced [42]. Therefore, central CRF activated by catecholamines is unlikely to be a critical factor in IM-induced plasma IL-6 elevation. Simoni et al. [43] have indicated that opiates, as well as CRF, mediate the plasma $11^{\wedge}6$ elevation which is induced by central injection of ELI-p. Since IL-1p mRNA in the hypothalamus was increased 30 min after initiation of IM stress and reached a maximum at 60 min [44], a central opiate mechanism might be involved in the plasma EL-6 response to IM stress.

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Furthermore, another possibility for the target site of centrally injected 6-OHDA are the axon terminals of noradrenergic neurons in the intermediolateral cell column of the spinal cord which contains sympathetic preganglionic neurons [45]. Depletion of catecholamines in the spinal cord [28] may reduce the activity of sympathetic preganglionic neurons, and consequently attenuate the release of catecholamines from the nerve terminals in the peripheral tissue [46]. The specific mechanism of action of the central catecholamines concerned with IM-induced plasma $11^{\wedge}6$ elevation also remains to be determined.

In the present study, we have used the B9 cell line to measure the $11^{\wedge}6$ concentration in plasma. Although the B9 cell assay is commonly used for measurement of plasma IL-6 levels by many investigators [11-13,20, 36,41],

we cannot exclude the possibility that B9 cell growth is stimulated by other factors than TL-6 which might also increase in response to IM. It has recently been reported that EL-11, in addition to $11^{\wedge}6$, a growth factor with multiple effects on both hematopoietic and nonhematopoietic cell populations [47], stimulates proliferation of B9 cells in a dose-dependent manner [48]. All data obtained in the B9 cell assay have to be confirmed by a specific assay for $11^{\wedge}6$ in the future.

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