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Central injection of nitric oxide synthase inhibitors increases peripheral interleukin-6 and serum amyloid A: involvement of adrenaline from adrenal medulla

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1 Accumulating evidence suggests that plasma levels of interleukin-6 (IL-6), a major cytokine stimulating the synthesis of acute phase proteins, are intimately regulated by the central nervous system (CNS).

2 In the present study, effects of intracerebroventricular (i.c.v) injection of N^G-nitro-L-arginine methyl ester (L-NAME) or 7-nitroindazole, nitric oxide synthase (NOS) inhibitors, on plasma IL-6 levels and peripheral IL-6 mRNA expression were examined in mice.

3 L-NAME (0.1–2 μg per mouse i.c.v.) and 7-nitroindazole (0.2–2 μg per mouse i.c.v.) induced a dose-dependent increase in plasma IL-6 levels and a subsequent increase in circulating serum amyloid A, a liver acute-phase protein. In contrast, an intraperitoneal (i.p.) injection of L-NAME up to the dose of 25 μg per mouse had no effect.

4 Pretreatment with yohimbine (α_2-adrenergic antagonist; 1 mg kg^{-1} i.p.), or ICI-118,551 (β_2-adrenergic antagonist; 2 mg kg^{-1} i.p.), but not with prazosin (α_1-adrenergic antagonist; 1 mg kg^{-1} i.p.), nor betaxolol (β_1-adrenergic antagonist; 2 mg kg^{-1} i.p.), significantly inhibited the central L-NAME-induced plasma IL-6 levels.

5 I.c.v. (50 μg per mouse) or i.p. (100 mg kg^{-1}) pretreatment with 6-hydroxydopamine had no effect on central L-NAME-induced plasma IL-6 levels. However, intrathecal (i.t.) pretreatment with 6-hydroxydopamine (20 μg per mouse) markedly inhibited central L-NAME-induced plasma IL-6 levels. Both yohimbine (1.5 μg per mouse i.t.) and ICI-118,551 (1.5 μg per mouse i.t.) were effective in inhibition of central L-NAME-induced plasma IL-6 levels.

6 There was an elevation of base-line plasma IL-6 levels in adrenalectomized animals. The adrenalectomy-enhanced levels were not further increased by central L-NAME.

7 L-NAME (2 μg per mouse i.c.v.) induced an increase in IL-6 mRNA expression in liver, spleen, and lymph node.

8 These results suggest that NOS activity in the brain tonically down-regulates peripheral IL-6 by inhibiting adrenaline release from the adrenal medulla.

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Abbreviations: CNS, central nervous system; i.c.v., intracerebroventricular; IL-6, interleukin-6; i.p., intraperitoneal; i.t., intrathecal; L-NAME, N^G-nitro-L-arginine methyl ester; NA, noradrenaline; NOS, nitric oxide synthase; 6-OHDA, 6-hydroxydopamine; SAA, serum amyloid A

Introduction

Atherosclerosis continues to be a major health problem. Complications of atherosclerosis, including ischaemic heart disease, stroke, and gangrene, account for over half of annual deaths in the developed countries (Gotlieb *et al*., 1999). Recently it has been known that chronic inflammation plays an important role in the pathogenesis of atherosclerosis: a slight increase in baseline level of plasma acute phase proteins (e.g. C-reactive protein and serum amyloid A), indicators of systemic inflammation, is a risk factor for future coronary heart disease, stroke and peripheral vascular disease in patients with angina pectoris (Haverkate *et al*., 1997; Liuzzo *et al*., 1994), in haemodialysis patients (Zimmermann *et al*., 1999) and in apparently healthy men (Ridker *et al*., 1997; 1998). Thus, it is important to characterize precisely the factors that increase synthesis of acute phase proteins *in vivo*.

Interleukin (IL)-6 is a major cytokine stimulating production of acute phase proteins in the liver (Heinrich *et al*., 1990; Moshage, 1997). Accumulating evidence demonstrates that plasma concentration of IL-6 is intimately regulated by the central nervous system (CNS) (Terreni & De Simoni, 1998): diverse models of stress (Komaki *et al*., 1994; LeMay *et al*., 1990; Papanicolaou *et al*., 1996; Takaki *et al*., 1994; Zhou *et al*., 1993), and central administration of IL-1β (De Simoni *et al*., 1990; 1993; Kitamura *et al*., 1998; Romero *et al*., 1996), tumour necrosis factor (Benigni *et al*., 1996) and lipopolysaccharide (De Luigi *et al*., 1998; De Simoni *et al*., 1995; Gottschall *et al*., 1992; Song *et al*., 1999a) increase plasma levels of IL-6.

Nitric oxide (NO) is involved in the regulation of a vast array of biological functions (Moncada *et al*., 1991; Schmidt & Walter, 1994). Inhibition of nitric oxide synthase (NOS) in the brain has been reported to induce various peripheral phenomena, including a rise of blood pressure (Cabrera & Bohr, 1995; Matsumura *et al*., 1998; Togashi *et al*., 1992), hyperglycaemia (Uemura *et al*., 1997), and suppression of colonic motor activity (Ohta *et al*., 1996). However, the

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potential involvement of the central NO in the modulation of plasma IL-6 levels has not been investigated. We hypothesized that inhibition of NOS activity in the brain may induce alteration in plasma levels of cytokines. In the present study, we focused on IL-6 and examined the effects of the intracerebroventricular (i.c.v.) injection of N^G-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole, NOS inhibitors, on plasma IL-6 levels and IL-6 mRNA expression in mice.

Methods

Reagents

L-NAME, 7-nitroindazole, phentolamine mesylate, propranolol HCl, prazosin HCl, and ICI-118,551 HCl were from Research Biomedicals International (Natick, MA, U.S.A.), yohimbine HCl from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and betaxolol HCl from Tocris Cookson Ltd. (Bristol, U.K.). 7-Nitroindazole, prazosin HCl, yohimbine HCl were dissolved in saline containing 3.5, 10 and 20% dimethylsulphoxide (v v^{-1}), respectively. Other drugs were dissolved in sterile saline.

Mice and experimental protocol

Male ICR mice weighing 25–30 g (Myung-Jin, Inc., Seoul, Korea) were used in all the experiments. All animal procedures were carried out as approved by the Animal Care and Use Committee at Hallym University College of Medicine. Intracerebroventricular administration was performed following the procedure established by Laursen & Belknap (1986) which was modified from the method of Haley & McCormick (1957). Briefly, the animal slightly anaesthetized with ether was injected at 2 mm lateral to the bregma with a 50 μl Hamilton syringe fitted with a 26-gauge needle, which was adjusted to be inserted 2.4 mm deep. The restraint stress procedure consisted of restraining each animal for 1.5 h in a 50 ml Corning conical tube, with the nose of the mouse at the tip of the tube. Adequate ventilation was provided by means of a hole at the tip of the tube. For the study on the effect of adrenergic receptor antagonists, the antagonist was injected i.p. 15 min prior to the i.c.v. injection of L-NAME. To deplete central and peripheral noradrenaline (NA), respectively, 6-hydroxydopamine HBr (6-OHDA, Sigma) dissolved in sterile 0.1% ascorbic acid (w v^{-1}), was injected at the dose of 50 μg per mouse i.c.v. (Suaudeau *et al*., 1995) and 100 mg kg^{-1} i.p. (Takaki *et al*., 1994), respectively, 3 days before the L-NAME injection. An i.p. injection of 6-OHDA (100 mg kg^{-1}) induced a selective decrease of NA content in the spleen to 13% of control values 3 days after the injection (Song *et al*., 1999b). On the other hand, an i.c.v. injection of 6-OHDA (50 μg per mouse) caused a selective decrease of NA content in the hypothalamus to 14% of control values 3 days after the injection (Song *et al*., 1999b). Adrenalectomy or sham operation was performed by a lateral subcostal approach using pentobarbitone anaesthesia. Adrenalectomized mice were maintained on isotonic saline as a drinking fluid. Two weeks after adrenalectomy i.c.v. injection of L-NAME was performed.

Cytokine and SAA assay

Blood for the cytokine and SAA assay was collected 1.5 h and 24 h after an i.c.v. injection, respectively, from the retro-orbital venous plexus with a heparinized micro-haematocrit capillary tubes. Plasma was separated by centrifugation of the freshly drawn blood and stored at -80°C until assayed. The ELISA kits used for assaying murine cytokine and SAA were from Genzyme (Cambridge, MA, U.S.A.) and Biosource International (Camarillo, CA, U.S.A.), respectively. Assays were performed exactly as described by the manufacturers.

Reverse transcription-PCR

Pituitary, adrenals, liver, spleen, iliac lymph nodes, and thymus were collected at 1 h after L-NAME administration. The number of animals used for each experiment was seven for pituitary, five for adrenals, and three for the rest of the tissues. Total cellular RNAs in the aqueous phase were precipitated with cold isopropyl alcohol. Isolated RNA samples were subjected to spectrophotometric analysis at 260 and 280 nm, and samples were stored at -70°C until used. The RNA was denatured by incubating it at 70°C for 5 min and then chilled quickly to 4°C. cDNA synthesis was conducted on 1–3 μg total RNA. The reaction mixture for the synthesis of cDNA by RT reaction included the following: 15 mM $MgCl_2$; 5× reaction buffer containing 375 mM KCl and 250 mM Tris-HCl (pH 8.3); 100 mM each dATP, dCTP, dGTP, and dTTP (Pharmacia, Uppsala, Sweden); ribonuclease inhibitor (RNasin, 40 u μl^{-1}, Promega, Madison, WI, U.S.A.); and Moloney murine leukaemia virus reverse transcriptase (200 u μl^{-1}, Gibco BRL, Gaithersburg, MD, U.S.A.). One to 3 μg of sample RNA was added to 20 μM oligo$(dT)_{16}$ primer, RT master mix, which contained 10 mM each dNTP, and 1 U of RNasin. The RT reaction mixture was incubated in a Techine-PHCZ thermal cycler at 25°C for 10 min, 37°C for 60 min, 99°C for 5 min, and 4°C for 5 min, and stored at -20°C. The PCR mixture contained the following: 15 mM $MgCl_2$; 10× reaction buffer containing 500 mM KCl, 100 mM Tris-HCl (pH 8.3), and 0.01% (w v^{-1}) gelatin; and Taq DNA polymerase (5 u μl^{-1}, Perkin-Elmer, Wellesley, MA, U.S.A.). The primers for IL-6 and β-actin were synthesized at Bohan Biomedical Inc. (Seoul, S. Korea). The sequences of these primers were as previously described (Faulkner *et al*., 1995); β-actin, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ (348 bp); IL-6, 5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′, 5′-TCTGACCACAGTGAGGAATGTCCAC-3′ (155 bp). For each reaction, 30 μl of master mix containing 10× reaction buffer, 0.5 U of Taq DNA polymerase, and 20 μM each primer was added to a tube containing 3 μl of the cDNA synthesized in the RT reaction. The tubes were incubated in a thermal cycler at 95°C for 2 min (once), 94°C for 45 s, 67°C for 2 min, and 72°C for 3 min (28–30 cycles), and 72°C for 10 min, and then held at 4°C. PCR products were visualized by ethidium bromide staining after agarose (1.2%) gel electrophoresis.

Statistical analysis

Statistical analysis was carried out by one-way (Figures 1C,D and 2) or two-way (Figures 1A,B and 3–6) ANOVA. Bonferroni test was used for *post hoc* comparisons. P values of <0.05 were considered to indicate statistical significance.

Results

Effect of i.c.v. L-NAME on plasma IL-6 levels

To examine whether inhibition of central NOS affects plasma IL-6 levels, we injected i.c.v. either saline or various doses of L-NAME (0.1–2 μg per mouse), and plasma IL-6 levels were

measured 1.5 h after the injection. As shown in Figure 1A, an i.c.v. administration of L-NAME elicited a dose-dependent increase in the plasma IL-6 levels. The increase was statistically significant from the dose of 0.1 μg/mouse ($P<0.05$). When i.c.v. L-NAME administration was combined with restraint stress, a known stimulus for high plasma IL-6 (Song *et al*., 1996; Takaki *et al*., 1994; Zhou *et al*., 1993), there was no further increase in plasma IL-6 levels (Figure 1A). A time-course study revealed a peak at 2 h after L-NAME (2 μg per mouse i.c.v.) injection (Figure 1B). In contrast, intraperitoneal (i.p.) administration of L-NAME up to the dose of 25 μg per mouse did not affect the plasma IL-6 levels (data not shown), which excludes the possibility that the i.c.v. administered L-NAME might leak into general circulation and act in the peripheral tissues to increase plasma IL-6 levels. As L-NAME is a non-selective inhibitor of NOS, the effect of 7-nitroindazole, a selective inhibitor of neuronal NOS (Moore *et al*., 1993), was examined. An i.c.v. injection of 7-nitroindazole (0.2–2 μg per mouse) dose-dependently increased the plasma IL-6 levels (Figure 1C), which suggests that the source of NO is from neuronal NOS. L-NAME (2 μg per mouse i.c.v.) and 7-nitroindazole (2 μg per mouse i.c.v.) also significantly increased plasma levels of IL-1β (Figure 1D). However, L-NAME (2 μg per mouse i.c.v.) did not affect plasma levels of TNF-α (Figure 1D). Because IL-6 is a key cytokine for acute-phase protein synthesis (Gauldie *et al*., 1987), plasma levels of serum amyloid A (SAA), a liver acute-phase protein (Jensen & Whitehead, 1998), were measured 24 h after the injection; Figure 2 shows an increased SAA level in L-NAME or 7-nitroindazole-treated animals.

Effects of adrenoceptor antagonists on central L-NAME-induced plasma IL-6 levels

The catecholamine systems are involved in the increase of plasma IL-6 level induced by several CNS stimuli, such as stress (Soszynski *et al*., 1996; Takaki *et al*., 1994), and central injection of lipopolysaccharide (Finck *et al*., 1997; Song *et al*., 1999a) and nicotine (Song *et al*., 1999b). To examine the involvement of adrenoceptors in the central L-NAME-induced plasma IL-6 levels, we injected i.p. either phentolamine (an α-adrenoceptor antagonist) or propranolol (a β-adrenoceptor

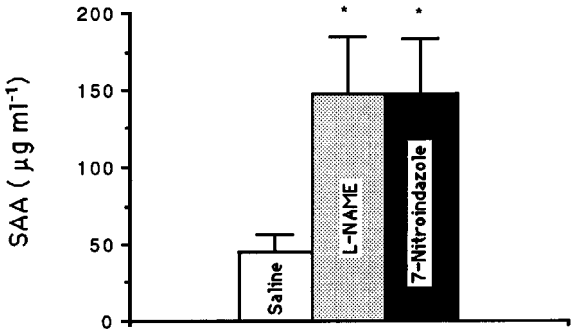

Figure 2 Effects of an i.c.v. injection of L-NAME and 7-nitroindazole on the plasma SAA levels. Either saline (5 μl per mouse), L-NAME (2 μg per mouse), or 7-nitroindazole (2 μg per mouse) was administered i.c.v. and blood was collected 24 h after the injection. The data are means±s.e.mean ($n=8-16$). *$P<0.05$, significantly different from saline-treated control.

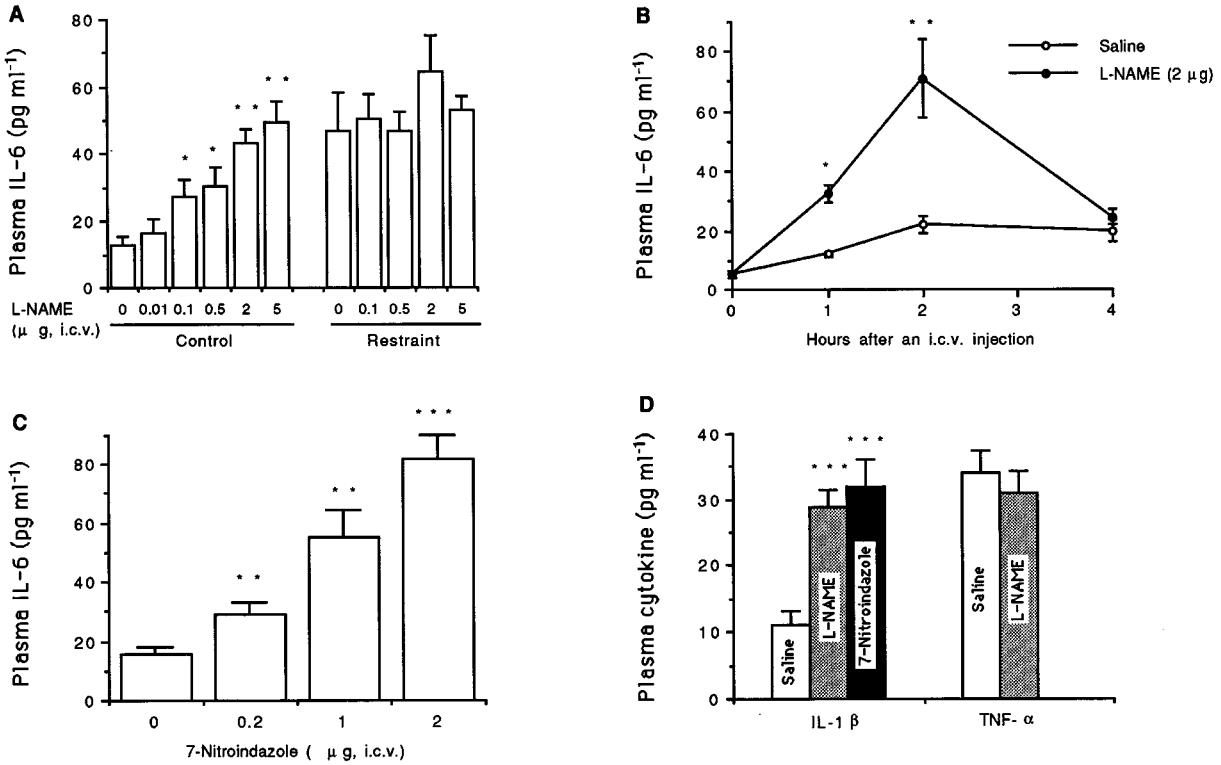

Figure 1 (A) Effects of L-NAME injected i.c.v. on the plasma IL-6 levels. Either saline (5 μl per mouse i.c.v.) or various doses of L-NAME (0.1–2 μg per mouse) were administered i.c.v. and blood was collected 1.5 h after the injection. For restraint group, the stress was applied for 1.5 h immediately after the L-NAME injection. (B) Time course of the effect of L-NAME injected i.c.v. on plasma IL-6 levels. Blood samples were obtained from one group of animals immediately after L-NAME (2 μg per mouse i.c.v.) or saline injection (value at time point 0), whereas other groups of animals were allowed to rest for the indicated intervals before blood samples were obtained. (C) Dose-dependent increase in plasma IL-6 levels by an i.c.v. injection of 7-nitroindazole, a selective inhibitor of neuronal NOS. (D) Effects of L-NAME and 7-nitroindazole injected i.c.v. on plasma IL-1β and TNF-α levels. The data are means±s.e.mean ($n=8-16$). *$P<0.05$; **$P<0.01$; ***$P<0.001$, significantly different from saline-treated control.

antagonist) 15 min prior to the L-NAME injection. As shown in Figure 3A, pretreatment with either phentolamine mesylate (2 mg kg^{-1}) or propranolol HCl (10 mg kg^{-1}) inhibited the L-NAME (2 μg per mouse i.c.v.)-induced plasma IL-6 levels. Next, we determined the involvement of the subtypes of α- and β-adrenoceptors by injecting i.p. either prazosin HCl (an α_1-adrenoceptor antagonist; 0.5 mg kg^{-1}), yohimbine HCl (an α_2-adrenoceptor antagonist; 1 mg kg^{-1}), betaxolol HCl (a β_1-adrenoceptor antagonist; 2 mg kg^{-1}), or ICI-118,551 HCl (a β_2-adrenoceptor antagonist; 2 mg kg^{-1}) 15 min prior to the central L-NAME injection. As shown in Figure 3B,C, pretreatment with yohimbine or ICI-118,551, but not prazosin nor betaxolol, significantly inhibited the L-NAME (2 μg per mouse i.c.v.)-induced plasma IL-6 levels, which suggests the involvement of α_2- and β_2-adrenoceptors.

Intracerebroventricular (50 μg per mouse) and intraperitoneal (100 mg kg^{-1}) administration of 6-OHDA, which depletes central and peripheral stores of NA, respectively, did not affect the central L-NAME-induced plasma IL-6 response (Figure 4A,B). However, intrathecal pretreatment with 6-OHDA (20 μg per mouse) which depletes spinal stores of NA, markedly inhibited the central L-NAME-induced plasma IL-6 response (Figure 5A). Intrathecal pretreatment with yohimbine HCl (1.5 μg per mouse) or ICI-118,551 HCl (1.5 μg per mouse) 15 min prior to the central L-NAME injection effectively inhibited the central L-NAME-induced plasma IL-6 response (Figure 5B).

Because catecholamine content in adrenal medulla is not reduced by peripheral injection of 6-OHDA (Takahashi *et al*., 1993), the effect of adrenalectomy on central L-NAME-

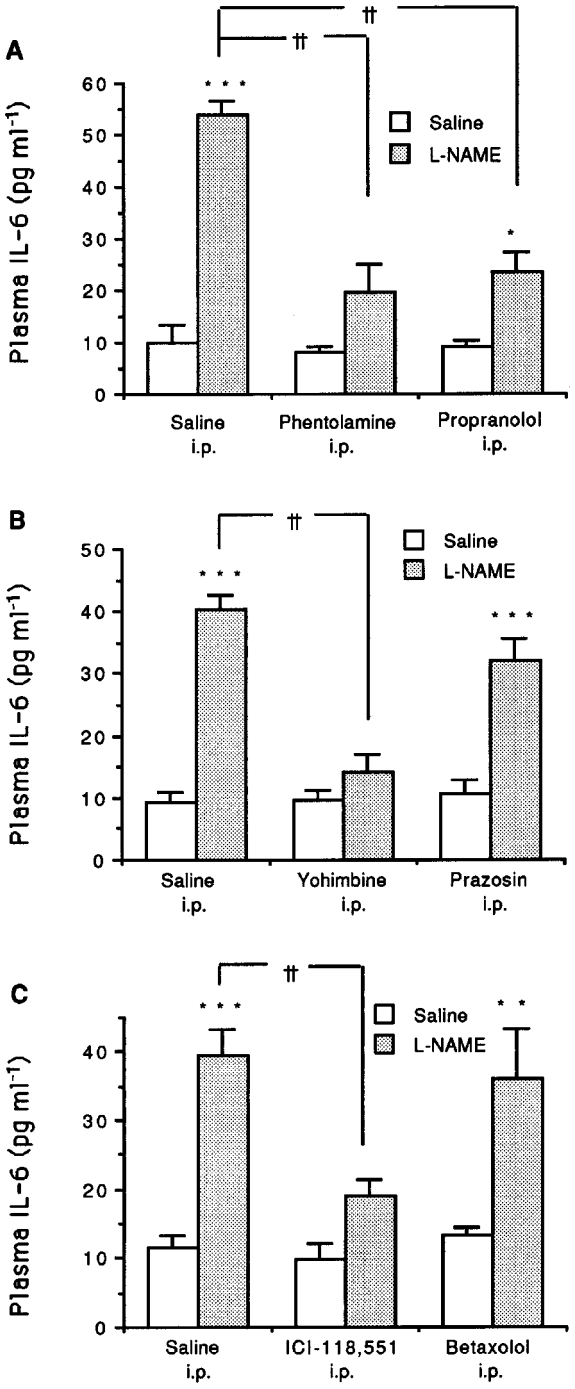

Figure 3 Effects of adrenoceptor antagonists, on the i.c.v. L-NAME-induced plasma IL-6 levels. Animals were intraperitoneally pretreated with phentolamine mesylate (2 mg kg^{-1}), propranolol HCl (10 mg kg^{-1}), prazosin HCl (0.5 mg kg^{-1}), yohimbine HCl (1 mg kg^{-1}), betaxolol HCl (2 mg kg^{-1}), or ICI-118,551 HCl (2 mg kg^{-1}) 15 min prior to the central L-NAME (2 μg per mouse i.c.v.) injection, and blood was collected 2 h after the L-NAME injection. The data are means$\pm$s.e.mean of 8–14 animals. *$P<0.05$, **$P<0.01$, ***$P<0.001$, significantly different from the respective saline-treated controls. ††$P<0.01$.

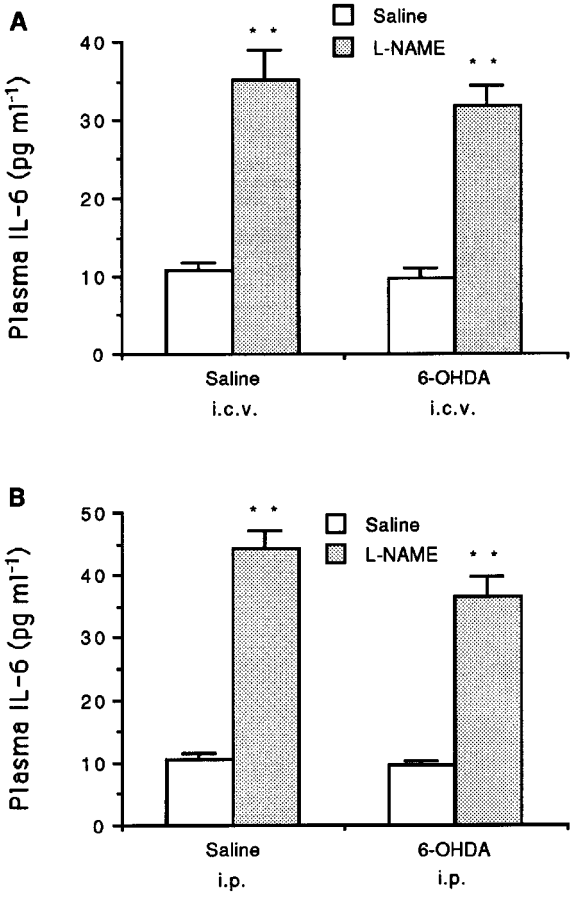

Figure 4 Effects of pretreatment of mice with (A) i.c.v. or (B) i.p. 6-hydroxydopamine (6-OHDA) on the L-NAME (2 μg per mouse)-induced increase in the plasma IL-6 levels. Animals were pretreated with 6-OHDA (A) i.c.v. (50 μg per mouse), (B) i.p. (100 mg kg^{-1}) 3 days prior to the central L-NAME (2 μg per mouse i.c.v.) injection, and blood was collected 2 h after the L-NAME injection. The data are means$\pm$s.e.mean of 8–14 animals. **$P<0.01$, significantly different from the respective saline-treated controls.

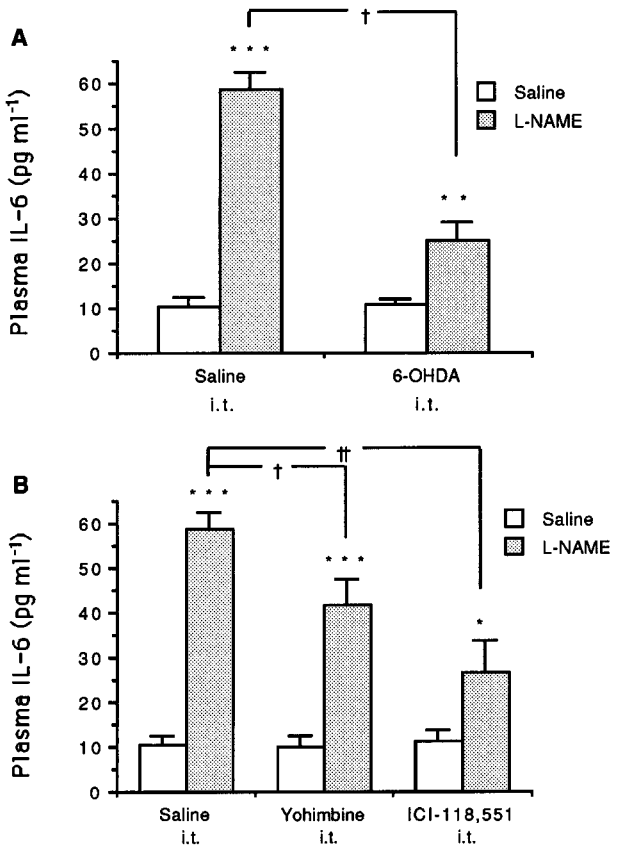

Figure 5 Effects of intrathecal (i.t.) pretreatment with (A) 6-hydroxydopamine (6-OHDA), (B) yohimbine or ICI-118,551 on the i.c.v. L-NAME-induced plasma IL-6 levels. Animals were pretreated with i.t. (A) 6-OHDA (20 μg per mouse) and (B) yohimbine HCl (1.5 μg per mouse) or ICI-118,551 HCl (1.5 μg per mouse), 3 days and 15 min, respectively, prior to the central L-NAME (2 μg per mouse i.c.v.) injection, and blood was collected 2 h after the L-NAME injection. The data are means$\pm$s.e.mean of 8–14 animals. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, significantly different from the respective saline-treated controls. $\dagger P<0.05$, $\dagger\dagger P<0.01$.

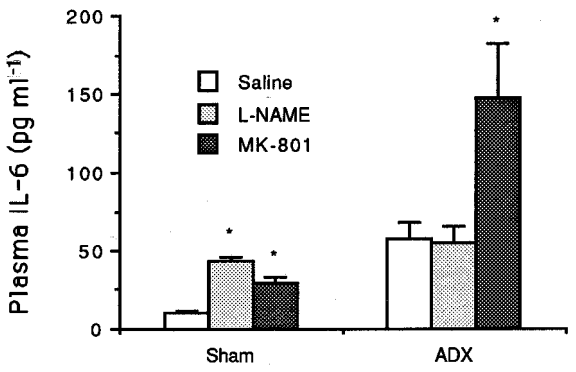

Figure 6 Effects of L-NAME and MK-801 on plasma IL-6 levels in sham operated or adrenalectomized (ADX) mice. Sham operation or adrenalectomy was performed 2 weeks prior to the central injection of L-NAME (2 μg per mouse) or MK-801 (1 μg per mouse), and blood was collected 2 h after the injection. The data are means$\pm$s.e.mean of 8–10 animals. $^*P<0.05$, significantly different from the respective saline-treated controls.

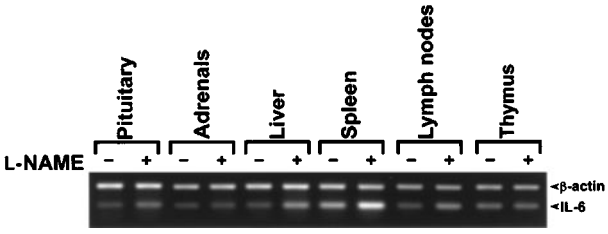

Figure 7 Effect of L-NAME on IL-6 mRNA expression in various peripheral organs. Either saline or L-NAME (2 μg per mouse) was administered i.c.v. and IL-6 mRNA expression was evaluated at 1 h after the injection. The gel shown is a representative one from three repeated experiments. The number of animals used for each experiment was seven for the pituitary, five for the adrenals, and three for the rest of the organs.

induced plasma IL-6 levels was examined. Glucocorticoid negatively regulates IL-6 synthesis (Ray *et al*., 1990), which is shown by the increased basal plasma IL-6 levels in adrenalectomized mice (Figure 6). As shown in Figure 6, the adrenalectomy-enhanced levels were not further increased by central L-NAME. In contrast, i.c.v. injection of MK-801, a non-competitive *N*-methyl-D-NAME aspartate (NMDA) receptor blocker, which also increases plasma IL-6 levels (Song *et al*., 1996) induced an accentuated increase in plasma IL-6 in the adrenalectomized animals.

Effect of i.c.v. L-NAME on IL-6 mRNA expression

To identify the source of the central L-NAME-induced plasma IL-6 levels, IL-6 mRNA expression was examined with RT–PCR in pituitary, adrenals, liver, spleen, lymph nodes, and thymus 1 h after L-NAME administration. I.c.v. injection of L-NAME (2 μg per mouse) induced an increase in IL-6 mRNA expression in liver, spleen, and lymph node (Figure 7).

Discussion

Accumulating evidence indicates that systemic immune function is intimately modulated by the CNS (Madden & Felten, 1995; Straub *et al*., 1998). We found in the present study that inhibition of NOS activity in the brain induced an increase in plasma IL-6 levels and IL-6 mRNA expression in several peripheral organs, and a subsequent increase in circulating SAA. These results suggest that these peripheral IL-6 responses are tonically inhibited by central NO. The finding that 7-nitroindazole, a selective neuronal NOS inhibitor, increases plasma IL-6 suggests that the source of NO is from neuronal NOS. To our knowledge, this is the first report of central NO modulation of peripheral cytokine expression and plasma cytokine levels. This tonic inhibition of plasma cytokine levels extended to plasma IL-1β but not TNF-α.

The central and/or peripheral NA system has been implicated in various central stimuli-induced peripheral IL-6 responses; pretreatment of animals with either i.c.v. or i.p. 6-OHDA has been shown to effectively inhibit the immobilization stress (Takaki *et al*., 1994) – and central lipopolysaccharide (Song *et al*., 1999a) – induced peripheral IL-6 responses. In contrast, pretreatment of animals with i.p. but not i.c.v. 6-OHDA has been shown to effectively inhibit peripheral IL-6 responses induced by central injection of nicotine (Song *et al*., 1999b) and SR-95,531, a $GABA_A$ receptor antagonist (Song *et al*., 1998). The central L-NAME-induced plasma IL-6 response is different from the above mentioned stimuli, in that neither i.c.v. nor i.p. pretreatment with 6-OHDA affected the central L-NAME-induced IL-6 response (Figure 4A,B). However, intrathecal (i.t.) pretreatment with either 6-hydroxydopamine (50 μg/mouse) or adrenoceptor antagonists (yohimbine and ICI-118,551) effectively inhibited central L-NAME-induced

plasma IL-6 levels. This result suggests that NA or adrenaline in the spinal cord mediates the central NOS inhibition-induced IL-6 responses. Furthermore, intraperitoneal administration of yohimbine and ICI-118,551 effectively inhibited the central L-NAME-induced plasma IL-6 levels (Figure 3A–C), and central L-NAME did not further increase the adrenalectomy-enhanced plasma IL-6 levels. This result suggests that adrenaline released from adrenal medulla mediates the central NOS inhibition-induced IL-6 responses. In support of this contention, systemic injection of adrenaline induces an increase in plasma IL-6 levels (DeRijk *et al*., 1994; Van Gool *et al*., 1990), and adrenaline infusion in isolated perfused rat liver was shown to increase IL-6 production (Liao *et al*., 1995). In addition, an i.c.v. administration of L-NAME has been shown to induce a marked increase in plasma levels of adrenaline (Uemura *et al*., 1997). To our knowledge, this is the first case for the CNS stimulus-induced peripheral IL-6 responses which is predominantly dependent on adrenal glands, but not on the peripheral sympathetic nervous system.

Because α_2- and β_2-adrenoceptors are opposite in the modulation of intracellular cyclic AMP levels, it is difficult to speculate that these receptors are located on the same cells modulating IL-6 responses in the same way. As we administered α_2- and β_2-adrenoceptor antagonists either systemically or intrathecally, the exact localization of these receptors involved in the modulation of IL-6 responses is unclear from the present study. Further studies are needed to delineate the exact localization (including pre- or post-synaptic, and on the IL-6 producing cells or other regulating sites) of these receptors involved in the modulation of central NOS inhibitor-induced plasma IL-6 levels. It was previously reported that the central nicotine-induced plasma IL-6 levels were also similarly inhibited by systemically administered α_2- and β_2-adrenoceptor antagonists (Song *et al*., 1999b).

We previously showed that i.c.v. administration of MK-801, a non-competitive NMDA receptor blocker, increases plasma IL-6 levels in mice (Song *et al*., 1996), suggesting that plasma IL-6 levels are tonically inhibited *via* NMDA receptors in the brain. NMDA receptor stimulation is one of the well-established stimuli for the increase of NOS activity in the brain (Garthwaite, 1991). Therefore, tonic activation of NOS activity *via* NMDA receptor may underlie the NMDA receptor-mediated tonic inhibition of plasma IL-6 levels (Song *et al*., 1996). However, the results of the present study do not support this possibility, because adrenalectomy blocked the plasma IL-6 increase induced by L-NAME but not by MK-801 (Figure 6). Furthermore, adrenoceptor antagonists inhibited the plasma IL-6 increase induced by L-NAME (Figure 3A–C) but not by MK-801 (unpublished observation). Thus it is suggested that NOS activity that is responsible for the tonic inhibition of plasma IL-6 levels is not related to the activation of NMDA receptors.

When immobilization stress is combined with an i.c.v. administration of agents that induce an increase in plasma IL-6 levels, i.e. MK-801, SR-95,531 (a γ-aminobutyric acid $(GABA)_A$ receptor antagonist), and 2-hydroxysaclofen (a $GABA_B$ receptor antagonist), the plasma IL-6 levels are additively increased (Song *et al*., 1996; 1998). However, in the present study, there was no additional increase in plasma IL-6 levels when immobilization stress was combined with an i.c.v. administration of L-NAME (Figure 1A). This result suggests that there is an interaction between immobilization stress and inhibition of NOS activity in the brain, which remains to be defined.

Among the various organs examined, spleen, lymph nodes and liver displayed a marked increase in IL-6 mRNA expression in response to i.c.v. L-NAME. This result suggests that central NOS inhibition-induced IL-6 may particularly influence immune and acute phase responses. In addition to these effects, the increased circulating IL-6 may potentially exert its very diverse biological functions (Akira *et al*., 1993; Hirano, 1998).

It has been reported that NO directly down-regulates IL-6 production stimulated by lipopolysaccharide or IL-1β in various cells, including macrophages, chondrocytes and enterocytes (Deakin *et al*., 1995; Henrotin *et al*., 1998; Meyer *et al*., 1995; Persoons *et al*., 1996). We present a novel physiological function of NO, i.e. NO in the brain tonically inhibits peripheral IL-6 and subsequent acute-phase protein synthesis. Inhibition of peripheral cytokine system by tonic activity of NOS in the brain may be one of the important mechanisms for systemic immunomodulation by the CNS.

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