Effects of Odorants on the Hypothalamic–Pituitary–Adrenal Axis and Interleukin-6 (IL-6) and IL-6 Receptor mRNA Expression in Rat Hypothalamus after Restraint Stress

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Abstract

We recently reported that stress alters interleukin (IL)-6 and IL-6 receptor (IL-6R) mRNA levels in the hypothalamus. Odorants are reported to exert anti-stress effects. The aim of our study was to determine the effects of odorants on IL-6 and IL-6R mRNA expression in the hypothalamus, using reverse transcriptase-polymerase chain reaction and on serum levels of adrenocortico-tropic hormone (ACTH) and corticosterone in rats exposed to stress. Control rats were not exposed to stress; test control rats were exposed to 4 h stress then immediately killed. In other groups, rats were exposed to the same stress followed by 30 min exposure air, dimethoxymethylbenzene (DMMB), or citralva. In the air group, IL-6 and IL-6R mRNA levels were significantly reduced and serum levels of ACTH and corticosterone significantly increased relative to the control. Exposure to DMMB signifi-cantly augmented IL-6 mRNA expression but restored that of IL-6R mRNA, did not change serum corticosterone level relative to that of the air group and significantly reduced ACTH. In comparison, citralva restored the expression of IL-6 and IL-6R mRNAs and significantly increased serum ACTH and corticosterone levels. Our results indicate that citralva enhances stress-induced acti-vation of the hypothalamic–pituitary–adrenal axis by corticotropin-releasing hormone (CRH)-mediated stimulation of IL-6, while DMMB enhances the beneficial action of IL-6 without affecting CRH.

Key words: ACTH, brain cytokines, citralva, corticosterone, dimethoxymethylbenzene, reverse transcriptase-polymerase chain reaction

Introduction

Several odorants have been reported to have anti-stress effects. Tuberose, labdanum, oakmoss and lemon (Shibata *et al.*, 1990, 1991; Fujiwara *et al.*, 1998) rescue stress-induced immunosuppression as evaluated using plaque-forming cell assays in mice. Denda *et al.* (2000) reported that dimethoxy-methylbenzene (DMMB), but not citralva, exhibited an anti-stress effect, using the cutaneous barrier recovery rate in mice and human skin. Our research interest is in the effects of these odorants on brain function and we designed the present study to investigate the effects of odorants on mRNA expression of interleukin-6 (IL-6) and its receptor (IL-6R) in the hypothalamus as well as on the hypo-thalamic–pituitary–adrenal (HPA) axis in rats exposed to stress.

IL-6 was identified in 1985 as a cytokine produced by blood monocytes (Hirano *et al.*, 1985). Its diverse biological actions in the immune and hematopoietic systems are well known. In addition to such the peripheral actions, recent studies indicate that IL-6 is an important regulator of cellular function in the central nervous system. Neurons and glia not only respond to various cytokines via their recep-tors, but also produce them (Sei *et al.*, 1995; Groul and Nelson, 1997). Although the physiological roles of brain cytokines remain unclear, it has been reported that IL-6 may act as a neurotrophic factor. IL-6, like nerve growth factor, can induce neuronal differentiation and support the survival of neurons (Hama *et al.*, 1989, 1991; Yamada and Hatanaka, 1994; Umegaki *et al.*, 1996; Yamada *et al.*, 1997; Nakajima *et al.*, 2002). Several studies have also suggested that IL-6 might activate the HPA axis at the level of the hypothalamus (Naitoh *et al.*, 1988; Fukata *et al.*, 1989; Navarra *et al.*, 1991; Tominaga *et al.*, 1991). IL-6 stimulates the release of corticotropin-releasing hormone (CRH) (Navarra *et al.*, 1991; Lyson and McCann, 1992) and the induction of IL-6R in the paraventricular nucleus supports the hypothesis for CRH-mediated stimulation of the HPA axis by IL-6 (Vallières and Rivest, 1999). Although recent studies reported that CRH-independent actions of IL-6 on

the pituitary and adrenal may also occur to augment the HPA axis (Ray and Melmed, 1997; Arzt *et al.*, 1999; Bethin *et al.*, 2000), CRH is, however, required to support the normal amplitude of HPA axis responsiveness (Jacobson *et al.*, 2000).

We have recently demonstrated that restraint stress affected mRNA levels of IL-6 and IL-6R in rat brain regions, including hypothalamus, and that those changes might be related to activation of the HPA axis as well as neurotrophic effects (Shizuya *et al.*, 1997, 1998; Miyahara *et al.*, 2000). In this study, we investigated the central effects of two odorants, DMMB and citralva, which were reported by Denda *et al.* (2000) to have anti-stress effects, on IL-6 and IL-6R mRNA expression levels in the rat hypothalamus.

Methods

Animals

Male Wistar rats, 12 weeks old, were purchased from SLC (Shizuoka, Japan) and used throughout the experiments. All rats were housed in cages in a quiet room and provided with pelleted diet and water *ad libitum*. Room temperature was controlled ($20 \pm 2°C$) and lights were on between 6.00 a.m. and 6.00 p.m.. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Mie University School of Medicine.

Stress and exposure to odorant

Rats were individually exposed to restraint stress by wrapping wire gauze around them in the prone position for 4 h. At 1.00 p.m., immediately after release from restraint stress, one group of rats (0 min group, $n = 6$) was decapitated immediately. The other rats were exposed to air (air group, $n = 6$), DMMB (DMMB group, $n = 6$), or citralva (citralva group, $n = 6$) for 30 min. The apparatus for odorant application consisted of an air pump, flow meter, glass tube covered with a mantle heater (maintained at ~100°C), a chamber (height 14 cm, diameter 25 cm), flow meter and an air pump. All these components were connected by tube in the above order. One rat was placed in the chamber at any one time. Triethyl citrate was used as solvent for dilution and 10% solution DMMB and citralva were used in the experiment. For odorant application, the volatile odorant liquid was injected into the glass tube at 0.1 ml/h using an infusion pump. The chamber was provided with two holes. One hole was connected to the glass tube and the odorized air was delivered into the chamber by driving air through the glass tube. The flow rate was maintained at 2.0 l/min using a flow meter. Through the other hole, the equal volume of air was exhausted by driving air. The flow rate was also maintained at 2.0 l/min using a flow meter. Rats of the control group ($n = 6$) were not exposed to stress or odorants, while those of the air group were not exposed to odorants.

After decapitation, brains were immediately removed and the hypothalamus was rapidly dissected out by the method of Glowinski and Iversen (1966) and stored in a freezer at –80°C until analysis. The hypothalamus included the ventromedial nucleus, paraventricular nucleus, arcuate nucleus, anterior hypothalamic area and lateral hypothalamic area.

Isolation of total RNA

Total RNA was extracted from the hypothalamus by the method of Chomczynski and Sacchi (1987). Tissue samples were lysed in the presence of guanidinium thiocyanate and phenol. The RNA was extracted with chloroform and isopropanol, precipitated twice in ethanol, redissolved in diethylpyrocarbonate-treated water (H_2O/DEPC) and quantitated spectrophotometrically. The total RNA sample from the hypothalamus was used for the experiment.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

PCR is an *in vitro* method for amplifying specific DNA sequences and enzymatically generates millions or billions of exact copies. RNA molecules can be analysed with PCR by first converting them to cDNA by reverse transcription using the enzyme reverse transcriptase. The resultant DNA transcript (first-strand cDNA) can then be amplifid by the normal PCR process (Sullivan, 1995).

Levels of mRNAs for IL-6, IL-6R and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were determined by a semiquantitative RT-PCR method. RT was performed with a First-Strand cDNA Synthesis Kit using the protocol recommended by the supplier (Pharmacia Biotech, Tokyo, Japan). The RT reaction was allowed to proceed for 60 min at 37°C. Primers for IL-6R were synthesized by Takara Shuzo (Shiga, Japan). The following specific primers were prepared: sense primer, 5′-GACTTCTCCCCCACCG-TATC-3′; antisense primer, 5′-TCCTCACAGCCCCT-TCTTTC-3′. Primers for IL-6 (sense and antisense) have been described previously (Minami *et al.*, 1991). Primers for G3PDH (sense primer, 5′-ACCACAGTCCAT-GCCATCAC-3′; antisense primer, 5′-TCCACCACCCT-GTTGCTGTA-3′) were obtained from Clontech (Palo Alto, CA). The lengths of the amplified products were 509 bp for IL-6 mRNA, 365 bp for IL-6R mRNA and 452 bp for G3PDH mRNA. To normalize signals from different samples of RNA, G3PDH mRNA was co-amplified as an internal standard. Amplification reactions with oligonucleotide primers were stopped before the end of the exponential phase (IL-6, 33 cycles; IL-6R, 29 cycles; G3PDH, 23 cycles). In the next step, 20 μl of the reaction mixture was heated at 95°C for 5 min to inactivate the reverse transcriptase and subjected to PCR with specific primers for IL-6, IL-6R or G3PDH in separate tubes.

The total reaction mixture for PCR contained 10 mM Tris–HCl (pH 8.3), 20 mM KCl, 2.5 mM $MgCl_2$, 0.02 mg/ml BSA, 0.5 mM each dNTP, 20 pmol sense and antisense primers, 2.5 U Taq DNA polymerase and 5 μCi [α-^{32}P] dCTP. PCR involves a repetitive series of temparature cycles

with each cycle comprising three stages: denaturation of the template DNA at >91°C to separate the strands of the target molecule, then cooling to ~50 °C to allow annealing to the template of single-stranded oligonucleotide primers which are specifically designed to flank the region of DNA of interest, and finally, extension of the primers by DNA polymerase at ~72°C. One cycle of PCR doubles the number of target DNA molecules, since the newly synthesized strands can themselves act as templates in the next round of amplification. Thus, the logarithmic growth in product occurs (Sullivan, 1995). The conditions for PCR amplification of IL-6R and G3PDH cDNAs were 94°C for 1 min, 55°C for 40 s and 72°C for 1 min and for IL-6 cDNA 94°C for 30 s, 70°C for 40 s and 72°C for 1 min 90 s. The PCR products were separated on a $1 \times$ Tris-borate EDTA-3.5% polyacrylamide gel and detected by electric autoradiography (InstantImager; Packard, CT). The radioactivity of each specific cDNA was compared with that of cDNA for G3PDH and the result was expressed relative to the control value.

Serum ACTH and corticosterone concentrations

Serum concentrations of ACTH and corticosterone were measured by radioimmunoassay (Szafarczyk *et al.*, 1979; Armario and Castellanos, 1984).

Statistical analysis

IL-6 and IL-6R mRNA levels were normalized by reference to G3PDH mRNA level, quantitated from the same blot, for confirmation of equal loading and presented as the mean ± SEM of six experiments. Each experiment was repeated three times. Differences between groups were examined for statistical significance using one-factor analysis of variance (ANOVA) and Fisher's test. A P value <0.05 denoted the presence of a statistically significant difference.

Results

Table 1 shows the effects of restraint and odorant inhalation on IL-6 mRNA, IL-6R mRNA levels in the hypothalamus, and serum ACTH and corticosterone concentrations. One-way ANOVA showed a significant difference for IL-6 mRNA [$F_{(4,25)} = 143.128$, $P < 0.0001$], IL-6R mRNA in the hypothalamus [$F_{(4,25)} = 31.320$, $P < 0.0001$], serum ACTH [$F_{(4,25)} = 77.128$, $P < 0.0001$] and corticosterone [$F_{(4,25)} = 186.604$, $P < 0.0001$] between the groups. Relative to the control rats (not exposed to stress), restraint stress for 4 h (0 min group) caused a significant reduction of IL-6R mRNA expression level in the hypothalamus and significant increases in serum ACTH and corticosterone. However, it did not result in any change in IL-6 mRNA level in the hypothalamus.

Within 30 min of release from restraint stress, the expression of IL-6 and IL-6R mRNAs in the hypothalamus in the air group became significantly lower than the control, although the expression level of IL-6R mRNA was not different from that of the 0 min group. Serum ACTH returned toward the control level, but remained high compared with the control, while serum corticosterone continued to be significantly elevated.

Exposure to DMMB for 30 min after release from restraint stress significantly increased the expression of IL-6 mRNA, and restored that of IL-6R mRNA, which did not differ from the control. Serum ACTH level did not differ from control, but was significantly low compared with the other groups. Serum corticosterone remained significantly high compared with the control, but did not differ from the 0 min or air groups.

Inhalation of citralva for 30 min after release from restraint stress significantly restored the expression levels of IL-6 and IL-6R mRNAs in the hypothalamus and the levels were the same as control. Inhalation of citralva significantly increased serum levels of ACTH and corticosterone. Serum ACTH was significantly high compared with the control, air and DMMB groups, while it was significantly low compared with the 0 min group. Serum corticosterone was significantly high compared with all other groups.

Discussion

Our results showed that restraint stress for 4 h reduced the expression level of IL-6R mRNA, but not IL-6 mRNA, in the hypothalamus immediately after stress, and that levels of IL-6 and IL-6R mRNAs were decreased 30 min after the

Table 1 Effects of 4 h restraint and odorants on IL-6 and IL-6R mRNAs in the hypothalamus and HPA axis

	IL-6 mRNA (%control)	IL-6R mRNA (%control)	Serum ACTH (pg/ml)	Serum corticosterone (ng/ml)
Control	100.0 ± 4.0	100.0 ± 4.0	74.3 ± 5.9	127.3 ± 7.2
0 min	103.0 ± 4.7	55.5 ± 3.7[a]	249.7 ± 15.0[a]	340.8 ± 16.2[a]
Air	34.8 ± 3.4[a]	49.0 ± 3.7[a]	103.7 ± 7.8[a]	308.2 ± 11.9[a]
DMMB	245.7 ± 11.6[a]	103.3 ± 7.7	49.7 ± 7.2[b]	317.8 ± 11.5[a]
Citralva	105.8 ± 4.9	110.7 ± 5.6	200.2 ± 10.2[a]	617.0 ± 15.5[a]

Data are mean ± SEM. For each group, $n = 6$ rats.
[a]$P < 0.05$, compared with other equivalent values (one-way ANOVA followed by Fisher's test).
[b]$P < 0.05$, compared with the others except the control (one-way ANOVA followed by Fisher's test).

stress. Such alterations were consistent with our previous studies (Shizuya *et al.*, 1997, 1998; Miyahara *et al.*, 2000). Short-term (30–60 min) restraint stress reduced levels of IL-6 mRNA and IL-6R mRNA in the hypothalamus. However, the expression of mRNAs for IL-6 and IL-6R in this region tended to return toward the control level during long-term (2–4 h) restraint stress. During this period, serum ACTH and corticosterone continued to be elevated. The stress-induced elevation of plasma levels of ACTH and corticosterone might result in decreased expression of IL-6 and IL-6R mRNAs in the hypothalamus via suppression of secretion of CRH, whereas IL-6 might be needed during long-term stress and act as a neurotrophic factor. Although the relationship between IL-6 and the HPA axis, and the role of IL-6 as a neurotrophic factor are not yet well clarified, under stress there may be two potential actions of IL-6; one involving suppression potential by negative feedback of the HPA axis and the other an enhancing potential via its requirement as a neurotrophic factor.

Our results showed that 4 h restraint stress resulted in increased serum levels of ACTH and corticosterone. Furthermore, 4 h restraint stress followed by exposure to air for 30 min reduced serum ACTH, although it was still significantly higher than the control. Exposure to DMMB did not affect the serum corticosterone level, but significantly decreased serum ACTH. These results suggest that DMMB enhanced a feedback inhibition of the HPA axis. On the other hand, exposure to citralva significantly increased serum levels of ACTH and corticosterone. Serum corticosterone was highest in the citralva group, suggesting that citralva enhanced the stress response.

DMMB significantly increased the expression of IL-6 mRNA and reduced the stress-enhanced expression of IL-6R mRNA toward the control level. The associated decrease in serum ACTH indicates that enhanced activity of IL-6 is not a stimulant for the HPA axis, but rather that the HPA axis is suppressed. Since IL-6 can act as a neurotrophic factor (Hama *et al.*, 1989, 1991; Yamada and Hatanaka, 1994; Umegaki *et al.*, 1996; Yamada *et al.*, 1997; Nakajima *et al.*, 2002), we postulate that enhanced activity of IL-6 induced by DMMB might play an important role in the survival of neurons during stress-associated damage. On the other hand, citralva restored the expression of IL-6 and IL-6R mRNAs. In this case, enhanced IL-6 activity may stimulate the HPA axis at the level of the hypothalamus (Naitoh *et al.*, 1988; Fukata *et al.*, 1989; Navarra *et al.*, 1991; Tominaga *et al.*, 1991) via a CRH-mediated mechanism (Navarra *et al.*, 1991; Lyson and McCann, 1992), i.e. inhalation of citralva may enhance the stress response or may itself act as a stress.

Our findings here indicated that one odorant (citralva) might activate the HPA axis via the IL-6 system, whereas the other (DMMB) might exert anti-stress effects. The latter might be related to a neurotrophic effect. IL-6 might there-fore be a molecule that has both beneficial and harmful potential (Gadient and Otten, 1997) and the relationships between IL-6 and odorants remain of interest in this respect, although further studies are needed.

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