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Research report

Endotoxin inhibitor blocks heat exposure-induced expression of brain cytokine mRNA in aged rats

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Abstract

To investigate the age-related changes in the expression of interleukin-1β (IL-1β) and its related substances in the brain during heat stress, we measured amounts of mRNAs for IL-1β, cyclooxygenase-2 (COX-2), and an inhibitor of nuclear factor (NF)-κB-β (IκB-β) that is known to reflect an activation of NF-κB, in the cortex, cerebellum, and hippocampus using a quantitative real-time capillary PCR method. The basal levels of IL-1β mRNA in aged rats (108–110 weeks old) was significantly higher than those in young animals (10–11 weeks old) in these brain regions. Heat exposure (33 °C) for 1 h enhanced the expression of IL-1β and COX-2 mRNAs in aged rats but not in young ones. The amount of lipopolysaccharide (LPS) assessed by its bioactivity in the cortex increased by heat exposure only in aged rats. To further examine an involvement of LPS in the increase in mRNAs, an endotoxin inhibitor (EI), a synthetic peptide that detoxifies LPS by binding to the toxic component of LPS, lipid A, was intraperitoneally injected before heat exposure in aged rats. An intraperitoneal injection of EI significantly attenuated the heat exposure-induced increases in mRNAs for IL-1β, COX-2, IκB-β, and the LPS activity. Administration of EI also debilitated the heat exposure-induced hyperthermia and responses of plasma ACTH and catecholamines. These findings, taken together, suggest that the bacterial translocation is involved in the mechanisms of the responses to heat exposure in aged rats including the increased expression of mRNAs for IL-1β and its related substances in the brain.

Theme: I: Neural Basis of Behavior
Topic: Aging

Keywords: Heat exposure; Aging; Interleukin-1β; Endotoxin inhibitor; Real-time PCR; Bacterial translocation

1. Introduction

Brain-derived proinflammatory cytokines have various influences on the autonomic, neuroendocrine, and behavioral systems, e.g., on activity of the sympathetic nervous system and the hypothalamic–pituitary–adrenal (HPA) axis and on feeding, sleep, nociception, and peripheral immunity [12,30]. It is also known that these homeostatic functions are affected by aging, suggesting that the age-related changes in production and/or action of cytokines may be involved at least in part in the central mechanisms of aging. For example, it has been reported that neuronal expression of a proinflammatory cytokine, interleukin-1β (IL-1β), increases with aging so as to contribute as a trigger for the age-induced impairment in long-term potentiation [21], and that the increased neuronal expression of IL-1β is suggested to be a feature of the age-related neurodegeneration [6]. IL-1β is synthesized in the brain during systemic administration of lipopolysaccharide (LPS), which is used most commonly as a model for bacterial infection [2,11,15,22,25], as well as various kinds of brain insults such as meningitis and cerebral ischemia [29,38]. In addition to inflammation and infection, noninflammatory stressors such as immobilization also induce IL-1β mRNA in the brain [19].

One of the characteristic aspects of aging is a progressive impairment in the ability to adapt to environmental challenges. In the present study, we sought to investigate whether heat exposure as an environmental stress could induce mRNAs for IL-1β and its related substances such as cyclooxygenase-2 (COX-2), an enzyme induced by IL-1β to produce prostaglandins, and the inhibitor of nuclear factor (NF)-κB-β (IκB-β) that is known to contribute to persistent NF-κB activation [33] in the brain, and whether there are

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differences in the expression of these mRNAs between young and aged rats. An involvement of enteric flora-derived LPS in the induction of IL-1β mRNA during heating was examined by measuring the bioactivity of LPS in the brain tissues. Furthermore, the effects of pre-treatment with an endotoxin inhibitor (EI), a synthetic peptide proved to inactivate LPS toxicity [31,37], on the heat exposure-induced changes in mRNA contents and the LPS activity were also investigated in aged rats. We measured the amount of brain mRNAs quantitatively using a real-time capillary RT-PCR method. This method allows rapid and accurate quantification of the initial transcript copy number by monitoring continuously the fluorescence of amplifying DNA [20].

2. Materials and methods

2.1. Animals

Male Wistar rats 10–11 weeks old (380–420 g, $n=12$) and 108–110 weeks old (650–750 g, $n=36$) were used as young and aged groups, respectively. They were housed individually in a temperature-controlled environment (23 ± 1 °C) with a 12-h-light/dark cycle (light on at 0800 h) and were given free access to food and water.

2.2. Experimental procedure

2.2.1. Experiment 1

Young and aged rats were divided into two groups (young/room temperature, Y/RT; young/heated, Y/H; aged/room temperature, A/RT; and aged/heated, A/H group; $n=6$ in each group). From at least 3 days before the experimental day, rats were daily transported to the temperature- and humidity-controlled testing cabinet (MLR-350 HT, Sanyo, Japan) with their home cages for 1 h to make them familiar with the cabinet. On the experimental day, after the rectal temperature (T_{rec}) was measured, the animals were put into the cabinet without food and water. In the Y/H and A/H groups, the ambient temperature of the testing cabinet was changed to 33 °C from 23 °C within 15 min and kept for 1 h, while in the Y/RT and A/RT groups the ambient temperature was kept at 23 °C. Relative humidity of the cabinet was maintained at around 50%. After 1-h exposure to heat or room temperature, the T_{rec} was again measured. The rats were then deeply anesthetized with ether and transcardially perfused with ice-cold sterile saline containing 5 mM EDTA to exclude blood and any circulating LPS from the brain. The brain was swiftly removed and put into dish with ice-cold saline. The parietal cortex, cerebellar cortex, and the hippocampus were dissected and immediately frozen.

2.2.2. Experiment 2

Additional aged rats were divided into four groups ($n=6$ in each group). Endotoxin inhibitor (EI, Bachem, Switzerland) was dissolved with saline at 0.5 mg/ml just before use. After acclimation to the testing cabinet, saline (S) or EI (0.5 mg/kg) was intraperitoneally (ip) injected just before exposure to room temperature or hot environment (S/RT, S/H, EI/RT, and EI/H groups, respectively). After deep anesthesia, blood was taken by cardiac puncture for the measurement of the serum concentration of ACTH, epinephrine (E), norepinephrine (NE), and dopamine (DA). Animals were then transcardially perfused, and the brain samples were taken in the same manner as Experiment 1.

2.3. Quantitative measurement of mRNA in the brain

Total RNA was isolated from the samples using magnetic beads (MagExtractor system, Toyobo, Japan) after homogenizing the tissues. Primers were designed with GENETYX-MAC (Software Development, Japan) based on the sequences obtained from the GenBank Sequence Database and synhesized by Greiner Japan. Primer pairs were chosen to flank at least one intron. To standardize the content of mRNAs, mRNA for one of the house keeping genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also quantitated. The sequences of primers are as follows: IL-1β, sense = 5′-ACGACCTGCTAGTGTGTGAT-3′, antisense = 5′-CCGACCATTGCTGTTTCCTA-3′; COX-2, sense = TCCAACCTCTCCTACTACAC-3′, antisense = 5′-GTTGCACGTAGTCTTCGATC-3′; IκB-β, sense = 5′-GAGTGTTGGTGACTGAGAGA-3′, antisense = 5′-ggatgacagctacatggagt-3′; and GAPDH, sense = AAAAGGGTCATCATCTCCGC-3′, antisense = 5′-CAGCATCAAAGGTGGAGGAA-3′. The predicted sizes of PCR products are 440 bp for IL-1β, 625 bp for COX-2, 294 bp for IκB-β, and 544 bp for GAPDH, respectively.

To make copy number standards for each PCR product, reverse transcription (RT)-PCR was performed using RTG RT-PCR Beads (Amersham Pharmacia Biotech, USA) with 37 °C, 60 min followed by 94 °C, 45 s denaturation, 60 °C, 45 s annealing, and 72 °C, 90 s extension in a conventional thermal cycler. Part of the PCR product was examined for purity by gel electrophoresis, and the rest was purified using spin column purification kit (SUPREC-02, Takara, Japan). The copy number (copies/microliter) of each product standards was calculated from its OD_{260}. A standard solution of each product was then diluted serially to 10^2–10^7 copies/μl solutions.

First-strand cDNA of the experimental samples was generated using RTG First-Strand Beads (Amersham Pharmacia Biotech) with random hexamers. cDNA samples and serially diluted copy number standards were then simultaneously amplified in a real-time fluorescence thermal cycler (LightCycler LC32, Idaho Technology, USA) using capillary tubes. A reaction mixture containing 3 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.3), 250 ng/μl bovine serum albumin, 200 μM each dNTP, 0.5 μM each primer, 0.04 U/μl *Taq* DNA polymerase (Platinum *Taq* DNA Polymerase High Fidelity, Life Technologies, Gibco, USA), and template (1

μl of cDNA solution/10 μl mixture) was used. In addition, double-stranded DNA (dsDNA)-sensitive fluorescent dye, SYBR Green I (1:30,000, Molecular Probes, USA), was added to the reaction mixture for detecting PCR products at each cycle. Amplification reactions (9 μl PCR mixture/one capillary tube) were conducted for 45 cycles of heating at 20 °C/s to 94 °C (denaturation, 0 s), cooling at 20 °C/s to 55 °C (annealing, 3 s), and heating at 5 °C/s to 72 °C (extension, 15 s). Fluorescence was acquired during the extension phase of each cycle. After amplification, a melting curve was acquired by heating at 20 °C/s to 95 °C, cooling at 20 °C/s to 60 °C and slowly heating at 0.2 °C/s to 94 °C with fluorescence data collection at 0.2 °C intervals. The PCR products were then run on an ethidium bromide-stained 2% agarose gel and photographed under UV illumination. Finally, the PCR fragments were sequenced directly after purification with primers used in the original amplification reaction by an automated DNA sequencer (Perkin-Elmer Applied Biosystems model 377 Prism DNA Sequencer).

2.4. Measurement of bioactive LPS

The tissue samples of the parietal cortex were homogenized using FastPREP (Savant Instrument, USA) and tubes with zirconia/silica beads (Green, BIO 101, USA) in 400 μl of LPS free physiological solution at 4 °C. After centrifugation at 20,000 × *g* for 10 min, the supernatant was taken for LPS assay. LPS activity was measured by a highly sensitive chromogenic LPS determination system (Endospecy, Seikagaku, Japan) [35]. The supernatant (5 μl) were duplicately put into wells in a 96-well plate (Toxipet Plate 96F, Seikagaku). After adding 20 μl of LPS-free distilled water, the plate was incubated at 37 °C for 10 min. A 100-μl sample of main reagent of Endospecy, a mixture of limulus lysate and chromogenic substance, was then added and further incubated at 37 °C for 45 min in an incubator installed in a microplate reader (Microplate Reader MR5000, Dynatech Labs, USA). Subsequently, 50 μl of 0.04% $NaNO_2$, 50 μl of 0.3% ammonium sulfate, and 50 μl of 0.07% *N*-(1-naphthylo)-ethylenediamine (DIA-MP set, Seikagaku) were added into all wells. The absorbance was measured at 550 nm against a reference of 630 nm. The absorbance curve was obtained by the serial dilution of the standard LPS solution (Et-2, *Escherichia coli* O111: B4, 0.424 EU [176 pg]/ml, Seikagaku). The sensitivity of this system is less than 1 pg/ml in standard LPS solution.

2.5. Statistics

All data were expressed as mean ± S.E.M. The statistical analysis for the differences in mRNA, LPS contents, plasma levels of ACTH and catecholamines, and rectal temperature was performed by one-way analysis of variance (ANOVA), followed by Newman–Keuls' post hoc test. $P < 0.05$ was considered to be statistically significant.

3. Results

Aged rats showed no abnormal appearance such as tumor, bleeding, and infection of the skin except for the fat bodies. Aged groups were significantly heavier in body weight than were young groups (Table 1), but there were no differences among aged groups (Tables 1 and 2).

3.1. Experiment 1

3.1.1. Changes in body temperature after heat exposure

The T_{rec} measured before heat exposure was not different among the four groups of rats (Table 1). The T_{rec} in Y/H and A/H rats significantly increased after heat exposure (33 °C, 1 h), and there was no significant difference in the increase in T_{rec} between the Y/H (2.7 ± 0.3 °C) and A/H (2.2 ± 0.1 °C) groups. Both groups showed no visible salivation during heating. The T_{rec} did not change in Y/RT and A/RT rats.

3.1.2. Effects of heat exposure on IL-1β and COX-2 mRNAs

Using the LightCycler software, the fluorescence of each sample was normalized after the background was subtracted. The amplification curves of cDNA standards demonstrated that the cycle numbers at which the fluorescence started to be detected were dependent on the initial cDNA concentration (Fig. 1A, thin curves), i.e., the fewer the starting copies, the more PCR cycles were required for detecting PCR products within a range from 10^2 to 10^7 copies/μl. The fraction cycle numbers were determined at the crossing points of each amplification curve with the threshold fluorescence (Noise Band in Fig. 1A that was

Table 1
Body weight and effects of heat exposure on body temperature in young and aged rats

Group	BW (g)	T_{rec} (pre) (°C)	T_{rec} (post) (°C)	ΔT_{rec} (°C)
Y/RT ($n=6$)	412.5 ± 5.1	36.9 ± 0.3	37.0 ± 0.2	0.1 ± 0.3
Y/H ($n=6$)	413.7 ± 8.8	36.9 ± 0.2	39.2 ± 0.2[a]	2.7 ± 0.3[a]
A/RT ($n=6$)	695.8 ± 31.8[b]	36.8 ± 0.3	37.5 ± 0.2	0.7 ± 0.2
A/H ($n=6$)	704.8 ± 32.1[b]	36.6 ± 0.1	38.8 ± 0.1[a]	2.2 ± 0.1[a]

Data are expressed as means ± S.E.M. Young and aged rats are 10–11 and 108–110 weeks old, respectively. Y/RT, young/room temperature (23 °C for 1 h); Y/H, young/heated (33 °C for 1 h); A/RT, aged/room temperature; and A/H, aged/heated. BW, body weight; T_{rec} (pre), rectal temperature before heat exposure; T_{rec} (post), after exposure; and ΔT_{rec}, differences between T_{rec} (pre) and T_{rec} (post).

There was an overall group effect in BW, T_{rec} (post) and ΔT_{rec} [one-way ANOVA, $F(3,20)=45.9$, $P<0.01$, $F(3,20)=8.9$, $P<0.01$, and $F(3,20)=18,5$, $P<0.01$, respectively], and no significance in T_{rec} (pre) [$F(3,20)=0.43$, $P>0.05$].

[a] $P<0.01$ compared with RT groups (Newman–Keuls' post hoc test).

[b] $P<0.01$ compared with Y groups.

Table 2
Body weight and effects of endotoxin inhibitor on heat exposure-induced hyperthermia in aged rats

Group	BW (g)	T_{rec} (pre) (°C)	T_{rec} (post) (°C)	ΔT_{rec} (°C)
S/RT ($n=6$)	656.5 ± 40.2	37.5 ± 0.2	37.6 ± 0.2	0.1 ± 0.1
EI/RT ($n=6$)	681.6 ± 42.4	37.6 ± 0.2	37.6 ± 0.2	0.1 ± 0.2
S/H ($n=6$)	655.7 ± 18.6	37.7 ± 0.2	39.7 ± 0.3[a]	2.1 ± 0.1[a,b]
EI/H ($n=6$)	708.1 ± 28.1	37.8 ± 0.2	39.0 ± 0.2[a]	1.2 ± 0.2[a]

Data are expressed as means ± S.E.M. Rats are 108–110 weeks old. Saline (S) or endotoxin inhibitor (EI) was injected intraperitoneally just before exposure. S/RT, saline/room temperature (23 °C for 1 h); EI/RT, endotoxin inhibitor/room temperature; S/H, saline/heated (33 °C for 1 h); and EI/H, endotoxin inhibitor/heated. BW, body weight; T_{rec} (pre), rectal temperature before heat exposure; T_{rec} (post), after exposure; and ΔT_{rec}, differences in T_{rec} between T_{rec} (pre) and T_{rec} (post).
There was an overall group effect in T_{rec} (post) and ΔT_{rec} [one-way ANOVA, $F(3,20)=28.9$, $P<0.01$, and $F(3,20)=12.9$, $P<0.01$, respectively], and no significance in BW and T_{rec} (pre) [$F(3,20)=2.73$, $P>0.05$, and $F(3,20)=0.98$, $P>0.05$, respectively].
[a] $P<0.01$ compared with RT groups.
[b] $P<0.01$ compared with EI groups (Newman–Keuls' post hoc test).

usually about 30% of the full scale). The number of copies initially present in the unknown sample was calculated by the least-squares best fit of the standards that was made based on the inverse correlation between the fraction cycle numbers and the log of the original concentration of the standard templates. The melting curve was produced by measuring fluorescence during slow denaturation of the double-stranded DNA products after the completion of the amplification. The fluorescence of all PCR products gradually decreased with temperature and then suddenly dropped to the almost zero level at the melting temperature, indicating that each capillary contained the single PCR product. The melting temperature of each PCR product was 88.9 °C for GAPDH and 87.0 °C for IL-1β. The single product of each capillary was confirmed by gel electrophoresis (Fig. 1B). Furthermore, the sequences showed an identity of 95–99% with data from Genbank.

To correct a variation of mRNA content in each sample, the concentration of mRNAs was normalized to the corresponding GAPDH mRNA concentration obtained in the same method. As shown in Fig. 2A, there were no differences in IL-1β mRNA levels between Y/H and Y/RT rats in the cortex, cerebellum, and hippocampus. However, expression of IL-1β mRNA in aged rats significantly increased by heating in the cortex and cerebellum (A/H vs. A/RT, $P<0.01$, Newman–Keuls' test). In addition, the basal levels of IL-1β mRNA of aged rats (A/RT) were significantly higher than those of young rats (Y/RT) in all brain regions.

As shown in Fig. 2B, the COX-2 mRNA levels did not change after heat exposure in young animals. The basal levels of COX-2 mRNA of aged (A/RT) rats were not different from those of young (Y/RT) rats. However, the COX-2 mRNA levels in aged rats significantly increased by heat exposure in the cortex and cerebellum but not in the

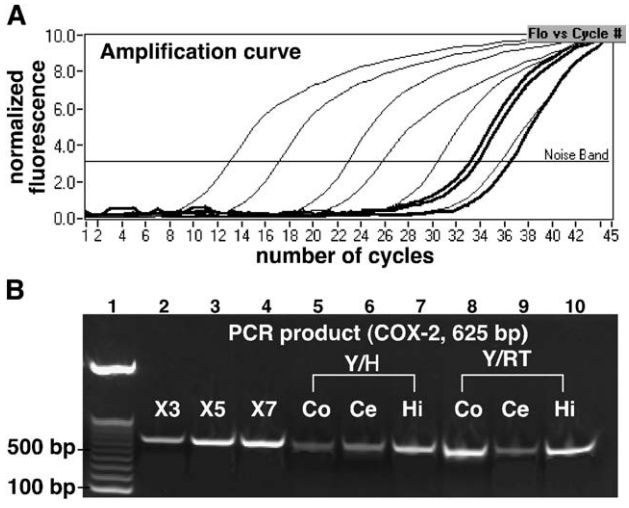

Fig. 1. Real-time capillary PCR method. (A) Normalized amplification curves of the serially diluted standards (thin curves, 10^2–10^7 copies/μl from right to left) and samples from cortex, cerebellum, and hippocampus of the Y/H group (thick curves, from left to right). Reconstructed from data for quantification of COX-2 mRNA. Vertical axis, normalized fluorescence, and horizontal axis, cycle numbers. Noise band, threshold fluorescence (see text for details). (B) PCR products for COX-2 in the gel of cDNA standards (lane 2–4, 10^3, 10^5, and 10^7 copies/μl), cortex (Co, lane 5), cerebellum (Ce, lane 6), hippocampus (Hi, lane 7) of young/heat exposed (Y/H) and young/room temperature exposed (Y/RT) groups (lane 8–10) and markers (lane 1). The length of PCR products was 625 bp.

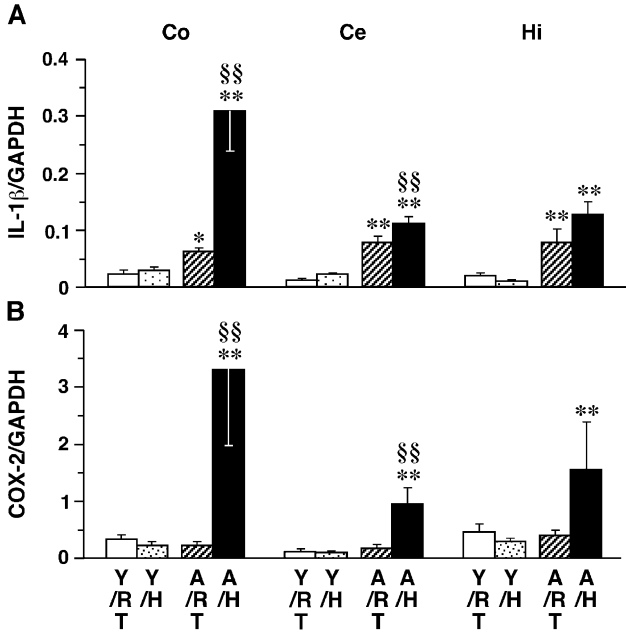

Fig. 2. Effects of heat exposure on IL-1β (A) and COX-2 (B) mRNAs in young and aged rats. Co, cortex; Ce, cerebellum; and Hi, hippocampus. Y/RT, young/room temperature exposed; Y/H, young/heat exposed; A/RT, aged/room temperature exposed; and A/H, aged/heat exposed group. ANOVA showed an overall difference in each brain region of both IL-1β and COX-2 mRNAs. IL-1β, Co, $F(3,20)=34.9$, $P<0.01$; Ce, $F(3,20)=35.1$, $P<0.01$; Hi, $F(3,20)=10.5$, $P<0.01$, and COX-2, Co, $F(3,20)=10.7$, $P<0.01$; Ce, $F(3,20)=8.9$, $P<0.01$; Hi, $F(3,20)=11.8$, $P<0.01$. $^{**}P<0.01$ and $^{*}P<0.05$, A/RT vs. Y/RT and A/H vs. Y/H. $^{§§}P<0.01$, A/H vs. A/RT (Newman–Keuls' post hoc test).

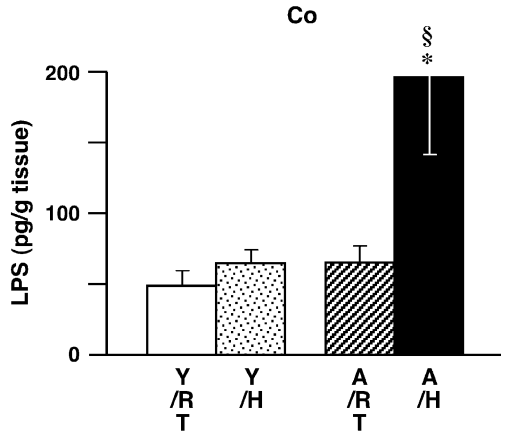

Fig. 3. Effects of heat exposure on LPS contents in young and aged rats. Co, cortex; Y/RT, young/room temperature exposed; Y/H, young/heat exposed; A/RT, aged/room temperature exposed; and A/H, aged/heat exposed group. There was an overall significance [ANOVA, $F(3,20)=18.9$, $P<0.01$]. *$P<0.05$, A/H vs. Y/H. $^{\S}P<0.05$, A/H vs. A/RT (Newman–Keuls' post hoc test).

hippocampus (A/RT vs. A/H), although the A/H group showed a higher level of COX-2 mRNA than the Y/H group in all regions.

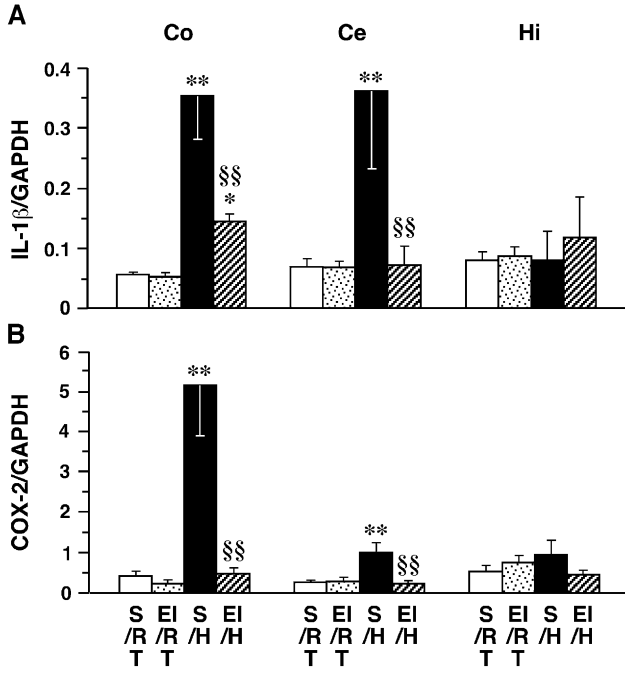

Fig. 4. Effects of endotoxin inhibitor on the heat exposure-induced increase in IL-1β (A) and COX-2 (B) mRNAs in aged rats. Co, cortex; Ce, cerebellum; and Hi, hippocampus. S/RT, saline injected/room temperature exposed; EI/RT, endotoxin inhibitor injected/room temperature exposed; S/H, saline injected/heat exposed; and EI/H, endotoxin inhibitor injected/heat exposed group. ANOVA showed an overall difference in Co and Ce, but not in Hi of both IL-1β and COX-2 mRNAs. IL-1β, Co, $F(3,20)=18.5$, $P<0.01$; Ce, $F(3,20)=5.6$, $P<0.01$; Hi, $F(3,20)=0.80$, $P>0.05$, and COX-2, Co, $F(3,20)=14.7$, $P<0.01$; Ce, $F(3,20)=7.4$, $P<0.01$; Hi, $F(3,20)=1.1$, $P>0.05$. **$P<0.01$ and *$P<0.05$, S/H vs. S/RT and EI/H vs. I/RT. $^{\S\S}P<0.01$, EI/H vs. S/H (Newman–Keuls' post hoc test).

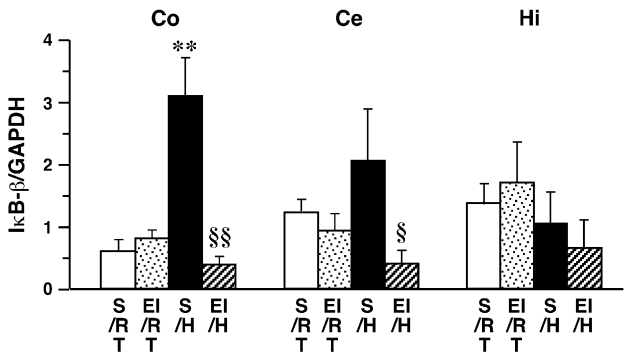

Fig. 5. Effects of endotoxin inhibitor on the heat exposure-induced increase in IκB-β mRNA in aged rats. Co, cortex; Ce, cerebellum; and Hi, hippocampus. S/RT, saline injected/room temperature exposed; EI/RT, endotoxin inhibitor injected/room temperature exposed; S/H, saline injected/heat exposed; and EI/H, endotoxin inhibitor injected/heat exposed group. ANOVA showed an overall difference in Co and Ce, but not in Hi. Co, $F(3,20)=14.3$, $P<0.01$; Ce, $F(3,20)=3.4$, $P<0.05$; Hi, $F(3,20)=0.84$, $P>0.05$. **$P<0.01$, S/H vs. S/RT. $^{\S\S}P<0.01$ and $^{\S}P<0.05$, EI/H vs. S/H (Newman–Keuls' post hoc test).

3.1.3. Effects of heat exposure on LPS activity of the cortex

The LPS activity of the cortex in young rats did not change after heat exposure (Y/RT vs. Y/H). Although the basal level of LPS activity in aged rats (A/RT) was not significantly different from that of young rats (Y/RT), the LPS activity significantly increased after heat exposure (A/RT vs. A/H, $P<0.05$, Fig. 3).

3.2. Experiment 2

3.2.1. Effects of EI on heat exposure-induced hyperthermia

As shown in Table 2, ΔT_{rec} in the EI/H group (1.2 ± 0.2 °C) was significantly smaller than that in the S/H group

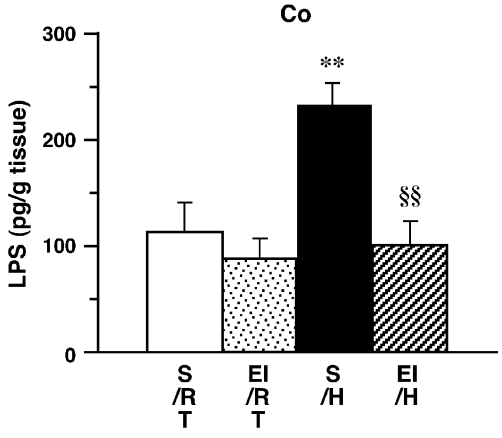

Fig. 6. Effects of endotoxin inhibitor on the heat exposure-induced increase in LPS content in aged rats. Co, cortex. S/RT, saline injected/room temperature exposed; EI/RT, endotoxin inhibitor injected/room temperature exposed; S/H, saline injected/heat exposed; and EI/H, endotoxin inhibitor injected/heat exposed group. There was an overall significance [ANOVA, $F(3,20)=5.7$, $P<0.01$]. **$P<0.01$, S/H vs. S/RT. $^{\S\S}P<0.01$, EI/H vs. S/H (Newman–Keuls' post hoc test).

(2.1 ± 0.1 °C), indicating that the heat exposure-induced increase in T_{rec} was attenuated by pretreatment with EI. Administration of EI did not affect the T_{rec} at room temperature.

3.2.2. Effects of EI on heat exposure-induced increase in brain IL-1β, COX-2, and IκB-β mRNAs

As shown in Fig. 4A, the amount of IL-β mRNA in aged rats increased by heat exposure in the cortex and cerebellum, but not in the hippocampus (S/H group) as observed in Experiment 1 (Fig. 2A). However, the enhanced expression was attenuated in the cortex and completely suppressed in the cerebellum, respectively, in rats pretreated with EI before heat exposure (EI/H group). EI did not alter the basal level of IL-1β mRNA content at room temperature (S/RT vs. EI/RT). Similarly, the increased expression of COX-2 mRNA was completely suppressed by EI treatment in the cortex and cerebellum (Fig. 4B).

As shown in Fig. 5, amount of IκB-β mRNA in the cortex also increased in the S/H group, and pretreatment with EI suppressed the expression of the mRNA. IκB-β mRNA content in the cerebellum did not significantly increase in S/H group, but it was significantly lower in the EI/H than that in the S/H group.

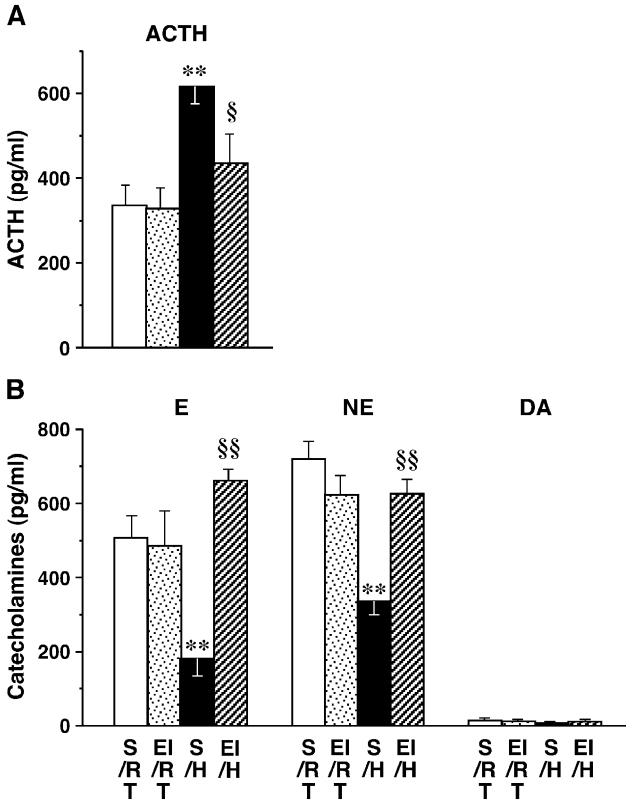

Fig. 7. Effects of endotoxin inhibitor on the heat exposure-induced changes in plasma ACTH (A) and catecholamines (B) in aged rats. E, epinephrine, NE, norepinephrine, and DA, dopamine. S/RT, saline injected/room temperature exposed; EI/RT, endotoxin inhibitor injected/room temperature exposed; S/H, saline injected/heat exposed; and EI/H, endotoxin inhibitor injected/heat exposed group. ANOVA showed an overall difference in ACTH, E, and NE, but not in DA. ACTH, $F(3,20)=6.7$, $P<0.01$; E, $F(3,20)=10.7$, $P<0.01$; NE, $F(3,20)=13.5$, $P<0.01$; DA, $F(3,20)=0.68$, $P>0.05$. $^{**}P<0.01$, S/H vs. S/RT. $^{§§}P<0.01$ and $^{§}P<0.05$, EI/H vs. S/H (Newman–Keuls' post hoc test).

3.2.3. Effects of EI on heat exposure-induced increase in brain LPS activity

As shown in Fig. 6, the LPS activity of the cortex increased by heat exposure as observed in Experiment 1 (Fig. 3). The enhanced activity was completely suppressed by pretreatment with EI (EI/H group). Administration of EI did not affect the LPS activity at room temperature.

3.2.4. Effects of EI on heat exposure-induced changes in plasma ACTH and catecholamines

Plasma concentration of ACTH was elevated in the S/H group compared with the RT groups, and the increased ACTH level was significantly attenuated by EI treatment (EI/H group) (Fig. 7A). On the other hand, plasma epinephrine (E) and norepinephrine (NE) decreased in the S/H group, but pretreatment with EI also blocked the decrease in E and NE (EI/H group). Plasma dopamine (DA) level was not affected by heating or administration of EI (Fig. 7B).

4. Discussion

In the present study, the quantitative analysis of cytokine mRNA contents was performed by a real-time capillary RT-PCR method. This method is based on the log-linear correlation between the initial concentration of cDNA and the PCR curve that is made by continuously detecting dsDNA-specific fluorescent dye [20]. Since all of the dsDNAs, including nonspecific products, could be detected by this dye, the sensitivity might be less than that of the sequence-specific probes. However, by eliminating the fluorescence of the dsDNAs whose melting temperature is below the desired product and by using anti-*Taq* antibody, the contamination of the nonspecific dsDNAs is thought to be reduced to the minimum. The production of only the desired template was confirmed by single peak of the melting curve and sequencing PCR products. We have observed that the log-linearity of the initial cDNA concentration vs. the amplifying curve was within a range from 10^2 to 10^7 copies/μl.

It has been reported that IL-1β and its mRNA in various brain regions increases in rats, mice, and rabbits after inflammatory stress such as occurs with systemic administration of LPS [2,11,15,22,25] or subcutaneous injection of formalin [40]. In addition, noninflammatory stress such as occurs with immobilization has been also reported to induce IL-1β mRNA in the rat hypothalamus [19]. All of these experiments were performed in the young adult animals but not in aged ones. The present study demonstrated that an expression of brain IL-1β mRNA during heat stress was different in young and aged rats. Although heat exposure

(33 °C, 1 h) did not affect IL-1β mRNA levels in the cortex, cerebellum, and hippocampus of young rats, aged animals showed a marked increase in IL-1β mRNA in these brain regions (Fig. 2A), suggesting an elevated sensitivity of cytokine production. It has been shown that the LPS-stimulated production of IL-1β in gingival fibroblast cells is enhanced in aged rats compared with that in the young animals [23]. This finding is compatible with the present result showing an elevated sensitivity of IL-1β mRNA expression in aged rats. Furthermore, the basal level of IL-1β mRNA was significantly higher in aged rats than in young ones. This may be consistent with the previous reports demonstrating an increase in neuronal expression of IL-1β in aged rats [21] and the increased intracellular levels of IL-1β in monocytes of older subjects [24].

Since we measured mRNA contents in the brain tissues using RT-PCR method, the characteristics of the IL-1β-producing cells were not known in the present study. The previous reports have shown that IL-1 in the central nervous system is synthesized by glial cells in the parenchyma, including perivascular regions following peripheral administration of LPS [5,26]. In addition, it has been also shown that systemic injection of LPS induces COX-2 mRNA in cerebral endothelial cells [17,27] as well as perivascular microglia [8]. Since the animals were transcardially perfused with sterile saline before collecting brain samples, involvement of cytokine and COX-2-producing cells in the circulation, such as macrophages, should have been excluded.

The levels of COX-2 mRNA in the cortex and cerebellum also markedly increased after heat exposure in aged rats but not in young ones (Fig. 2B). The present study demonstrated that heat exposure induced an increase in expression of IκB-β mRNA in the cortex of aged animals (Fig. 5, S/H group). It has been suggested that IκB-β is a stress protein inducible by hyperthermia and that an increased IκB-β expression in a non-phosphorylated form contributes to persistent activation of NFκB [33]. Since transcription of COX-2 gene is enhanced by activation of NF-κB, the increase in COX-2 mRNA after heat exposure in aged rats might result from an elevation of IL-1β in an IL-1β-dependent manner [7,32]. However, it is also possible that COX-2 is induced by activation of NF-κB independently of IL-1β.

We found that LPS in the cortex significantly increased after heat exposure in aged rats but not in the young, while the basal level of LPS was not different between aged and young rats (Fig. 3). The method for LPS measurement we used was sensitive to lipid A that is the active component of LPS endotoxins responsible for the stimulation of immune cells. Therefore, the increased LPS in the cortex should have pathophysiological significance. Wyckoff et al. [39] have suggested that LPS activates immune cells either after binding to the LPS binding protein, then to CD14 molecule and one of the toll-like receptor superfamily, TLR-4 [36], or direct binding to LPS receptors such as CD11c/CD18 [13] and L-selectin [16], thereby inducing an activation of NF-κB, which leads to the enhanced expression of NF-κB-inducible cytokine genes including IL-1β [18].

We investigated whether the increased LPS activity in the cortex of aged rats was responsible for the expression of IL-1β, COX-2, and IκB-β mRNAs during heat exposure. For this purpose, we used an EI, a synthetic peptide that binds to lipid A with high affinity, detoxifies LPS, and prevents the LPS-induced cytokine release in vivo [31]. This peptide has been also shown to suppress the febrile response to LPS, raise the survival rate after LPS injection, and have very low toxicity and lethality compared with a cyclic peptide antibiotic, polymyxin B. As shown in Figs. 4 and 5, pretreatment with EI significantly attenuated the heat exposure-induced increase in IL-1β, COX-2, and IκB-β mRNAs in the cortex and cerebellum. Furthermore, LPS activity in the cortex was also suppressed by EI treatment (Fig. 6). These findings, taken together, suggest that the enhancement of mRNA expression is attributable to an increase in LPS activity in the brain.

Pretreatment with EI attenuated the hyperthermia (Table 2) and ACTH response (Fig. 7A) induced by heat exposure. In addition, the decrease in E and NE after heat exposure (Fig. 7B), which can be suggested to occur because of diminished secretion of catecholamines in the hot environment [28], was blocked by EI administration. These findings indicate that the responses of body temperature, the HPA axis, and the adrenal sympathetic nervous system to heat exposure are also, at least in part, mediated by the increased LPS activity in the brain.

It is quite possible that the increased LPS in the brain may be derived from the indigenous bacteria in the gastrointestinal tract (bacterial translocation). Generally, three primary mechanisms that promote the bacterial translocation have been proposed: (1) intestinal bacterial overgrowth due to excessive oral antibiotics, bowel obstruction, etc., (2) deficiencies in host immune defenses due to immunosuppressive agents, lymphoma, etc., and (3) increased permeability or damage to the intestinal mucosal barrier due to thermal injury, hemorrhagic shock, etc. [3]. It has been demonstrated that noninflammatory stress such as occurs with cold exposure [1], hemorrhage [14], and immobilization [34] induces leakage of the gastrointestinal bacteria-derived LPS into the circulation. In these reports, gut ischemia has been suggested to be one of the main mechanisms of the bacterial translocation.

Endotoxemia has been reported in adult patients during heatstroke [4]. During heat exposure or heatstroke, the activation of heat loss mechanisms such as an increase in skin blood flow and sweating (salivation in rats) evokes an increase in the systemic blood volume and a decrease in the central blood volume. This redistribution of the blood results in a decrease in the splanchnic blood flow, i.e., gut ischemia, leading to the increase in plasma endotoxin [9]. Hales and Sakurada [10] have demonstrated that the decrease in blood flow in the gastrointestinal tract is much

larger in aged rabbits than in young ones, while the increase in the cardiac output is smaller in aged animals. They suggested that the greater redistribution of blood flow away from the gut of the aged rabbit is a compensatory response for the relatively poor capacity to increase cardiac output to increase or maintain blood flow in heat loss tissues such as skin, tongue, and nasal mucosa. This hypothesis may well account for the heat exposure-induced increase in LPS activity in the brain in aged rats but not in the young in the present study. Finally, it is possible that the permeability of the blood–brain barrier increases in aged rats during heat stress, thereby resulting in the increased LPS in the brain.

In conclusion, the present study demonstrated that expression of mRNAs for a proinflammatory cytokine, IL-1β, and its related substances such as COX-2 and IκB-β increased in the brain during heat stress probably due to the bacterial translocation in aged rats but not in the young. In addition, the bacterial translocation might be involved in the mechanisms of the heat exposure-induced hyperthermia and responses of the HPA axis and the sympathetic nervous system in aged animals. These findings may explain, at least in part, the age-related impairment in the adaptive responses to environmental stress.

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