Production of Mice Deficient in Genes for Interleukin (IL)-1α, IL-1β, IL-1α/β, and IL-1 Receptor Antagonist Shows that IL-1β Is Crucial in Turpentine-induced Fever Development and Glucocorticoid Secretion

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Summary

Interleukin (IL)-1 is a major mediator of inflammation and exerts pleiotropic effects on the neuro-immuno-endocrine system. To elucidate pathophysiological roles of IL-1, we have first produced IL-1α/β doubly deficient (KO) mice together with mice deficient in either the IL-1α, IL-1β, or IL-1 receptor antagonist (IL-1ra) genes. These mice were born healthy, and their growth was normal except for IL-1ra KO mice, which showed growth retardation after weaning. Fever development upon injection with turpentine was suppressed in IL-1β as well as IL-1α/β KO mice, but not in IL-1α KO mice, whereas IL-1ra KO mice showed an elevated response. At this time, expression of IL-1β mRNA in the diencephalon decreased 1.5-fold in IL-1α KO mice, whereas expression of IL-1α mRNA decreased >30-fold in IL-1β KO mice, suggesting mutual induction between IL-1α and IL-1β. This mutual induction was also suggested in peritoneal macrophages stimulated with lipopolysaccharide in vitro. In IL-1β KO mice treated with turpentine, the induction of cyclooxygenase-2 (EC 1.14.99.1) in the diencephalon was suppressed, whereas it was enhanced in IL-1ra KO mice. We also found that glucocorticoid induction 8 h after turpentine treatment was suppressed in IL-1β but not IL-1α KO mice. These observations suggest that IL-1β but not IL-1α is crucial in febrile and neuro-immuno-endocrine responses, and that this is because IL-1α expression in the brain is dependent on IL-1β. The importance of IL-1ra both in normal physiology and under stress is also suggested.

Interleukin (IL)-1 is a proinflammatory cytokine which plays an important role in host responses to inflammation and infection (for a review, see references 1–3). IL-1 has pleiotropic activities, as it had been identified originally as an endogenous pyrogen, lymphocyte-activating factor, hemopoietin-1, and osteoclast-activating factor (4). IL-1 consists of two molecular species, IL-1α and IL-1β, which are derived from two distinct genes 50 kb apart on chromosome 2 of the mouse genome (5, 6). Although the amino acid sequence homology of these molecules is only 25% (4), both molecules exert similar but not completely overlapping biological activities through the IL-1 type I receptor (IL-1RI)[1] (7). Although an IL-1 type II receptor (IL-1RII) is also present, this receptor is not considered to be involved in signal transduction, but rather plays a regulatory role as a decoy (8). In addition, another member of the IL-1 gene family, IL-1 receptor antagonist (IL-1ra), binds IL-1 receptors without exerting agonistic activity (9, 10). Three isoforms of IL-1ra protein are synthesized by alternative splicing from a single gene; one is a secreted form with a signal peptide (sIL-1ra), and the other two exist intracellularly (icIL-1raI and icIL-1raII) (11, 12). These three isoforms can inhibit IL-1 activities.

Pathophysiological roles of IL-1 were suggested in inflammation, acute phase responses, host defense against bacterial and viral infection, activation of the immune sys-

[1]*Abbreviations used in this paper:* ACTH, adrenocorticotropic hormone; COX, cyclooxygenase; CRH, corticotropin-releasing hormone; DT, diphtheria toxin A fragment gene; ES, embryonic stem; HPA, hypothalamic-pituitary-adrenal; IL-1RI and IL-1RII, IL-1 type I and II receptors; IL-1ra, IL-1 receptor antagonist; KO, knockout; PEC, peritoneal exudate cells; PGK, phosphoglycerate kinase.

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tem including thymocyte maturation and T helper 2 cell proliferation, bone metabolism including osteoclast activation and secretion of metalloproteases, fever development, and activation of the hypothalamic-pituitary-adrenal (HPA) axis (1, 2). Roles in the neuro-immuno-endocrine system are especially worth noting, since they are important in maintaining homeostasis of the body and controlling the immune system from the central nervous system.

To date, many investigators have made efforts to elucidate the roles of IL-1 in the brain. In this connection, it was shown that both IL-1α and IL-1β could induce fever development and that administration of neutralizing antibodies against IL-1β inhibited bacterial LPS–induced fever development (13, 14). Recently, mice deficient in the IL-1β gene were produced, and Kozak et al. (15) reported that the febrile response to LPS was reduced in these mice. In contrast, Alheim et al. (16) reported that IL-1β knockout (KO) mice were hyperresponsive to LPS-induced fever development. The reason for this discrepancy is not completely clear at present, although the authors suggested the use of LPS from different bacterial strains in the experiments. Turpentine-induced fever was also examined using IL-1β KO mice (17) and IL-1RI KO mice (18). These studies suggest that endogenous IL-1β plays an important role in controlling the febrile response of an animal after local inflammation. However, why IL-1β deficiency is enough to suppress fever development is not clear, since IL-1α and IL-1β are both produced in the inflammation and both IL-1 species can induce febrile responses.

It has been shown that inflammation, tissue damage, or infection affect serum glucocorticoid levels (19). Inflammatory cytokines such as IL-1, IL-6, and TNF-α are suggested to be involved in this activation through the so-called HPA axis (20, 21); these cytokines stimulate the hypothalamus to release corticotropin-releasing hormone (CRH), which then induces secretion of corticotropin (adrenocorticotropic hormone; ACTH) from the pituitary and corticosterone from the adrenal cortex. Both LPS and turpentine, which cause systemic and local inflammation in experimental animals, respectively, enhance corticosterone secretion through activation of the HPA axis (22, 23). Although it was suspected that IL-1 was the major mediator in controlling the HPA axis under such stress (20, 22, 24), a recent study failed to show effects of IL-1β deficiency on the induction of corticosterone after treatment with turpentine (25). These results suggest two possibilities, either that IL-1 is not involved in the activation of the HPA axis, at least at 2 h after turpentine treatment, or that IL-1α compensates for the IL-1β deficiency.

To clarify the roles for each member of the IL-1 gene family in febrile and neuro-immuno-endocrine responses, we produced KO mice carrying a null mutation either in IL-1α, IL-1β, or IL-1ra genes. We also have been the first to succeed in producing IL-1α/β double-KO mice by successive homologous recombinations in an embryonic stem (ES) cell.[2] Using these KO mice, we studied the effects of complete deficiency of IL-1 as well as deficiency of one of these molecules on normal physiology and stress responses induced by turpentine.

Materials and Methods

Construction of IL-1α, IL-1β, and IL-1ra Targeting Vectors. IL-1α, IL-1β, and IL-1ra genomic clones were isolated from mouse 129 genomic phage libraries either from Dr. Anton Berns (The Netherlands Cancer Institute, Amsterdam, The Netherlands) (IL-1α) or from Stratagene (129SvJ Mouse Genomic Library in the FIX II Vector; La Jolla, CA) (IL-1β and IL-1ra) using mouse cDNA probes or a genomic DNA probe (see below), and were partially sequenced before constructing targeting vectors. The neomycin resistance gene (*neo*) (26) and hygromycin B phosphotransferase gene (*hph*) (27) were used as positive selection markers in ES cells under the control of the phosphoglycerate kinase (PGK) 1 promoter. To monitor IL-1 gene expression, the β-galactosidase gene (*lacZ*) (Pharmacia Biotech, Inc., Piscataway, NJ) was inserted in IL-1α and IL-1β genes to produce IL-1-LacZ fusion proteins.

For the IL-1α targeting vector, a 1.5-kb DNA fragment between the Sau3AI and KpnI sites in exon 5 and intron 5, respectively, including the NH_2-terminal coding region for mature IL-1α, was deleted, and the *lacZ*-pA-PGK-*hph*-pA cassette was inserted in place of the DNA fragment. A diphtheria toxin A fragment gene under the control of the MC1 promoter (DT) (28) was ligated at the 5′ end of the vector for negative selection (Fig. 1 *A*). Homologous regions of 5′ and 3′ ends were 1.5 and 5.5 kb, respectively.

For the IL-1β targeting vector, a 2.45-kb DNA fragment between the HincII and BstXI sites in exons 3 and 5, respectively, including the NH_2-terminal coding region for mature IL-1β, was deleted, and the *lacZ*-pA-PGK-*neo*-pA cassette was inserted in place of the DNA fragment (Fig. 1 *B*). Homologous regions of the 5′ and 3′ ends were 1.5 and 3.9 kb, respectively.

To disrupt all three isoforms of the IL-1ra gene product, a 5.2-kb DNA fragment between the AvaI and SphI sites which contains all the coding regions for the secreted form of the IL-1ra was deleted, and the PGK-*neo*-pA cassette was inserted in place of the DNA fragment. DT was ligated at the 5′ end of the vector for negative selection (Fig. 1 *C*). Homologous regions of the 5′ and 3′ ends were 1.8 and 4.5 kb, respectively.

Production of IL-1α, IL-1β, and IL-1ra KO Mice. ES cells (R1, kindly obtained from Dr. Andras Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada) were electroporated with 20–25 μg of a linearized IL-1α, IL-1β, or IL-1ra targeting vector per 10^7 cells as described previously (29). ES cells were then plated onto mitomycin C–treated NHL7 cells (27) or G418-resistant primary embryonic fibroblast cells and selected with hygromycin B (140 μg/ml; Calbiochem-Novabiochem Corp., La Jolla, CA) for IL-1α, or G418 (180–300 μg/ml; GIBCO BRL, Gaithersburg, MD) for IL-1β and IL-1ra. Resistant colonies were picked up 6–8 d after selection. Homologous recombination was screened by PCR and confirmed by Southern blot analysis using 5′ and 3′ probes. Chimera mice were produced by an aggregation method modified from the original report (30). 10–15 ES cells were aggregated with two (C57BL/6 × DBA/2)F_1 eight-cell stage embryos in a hole on a plastic dish and cultured overnight, and well-formed blastocysts were transferred into the uterus of pseudopregnant female mice. Male chimera mice were then bred with C57BL/6 female mice, and germline transmission was checked by agouti coat color. Mice heterozygous for IL-1α, IL-1β, or IL-1ra mutation were intercrossed to yield homozygous mice for the mutation.

[2]Asano, M., R. Horai, and Y. Iwakura, manuscript in preparation.

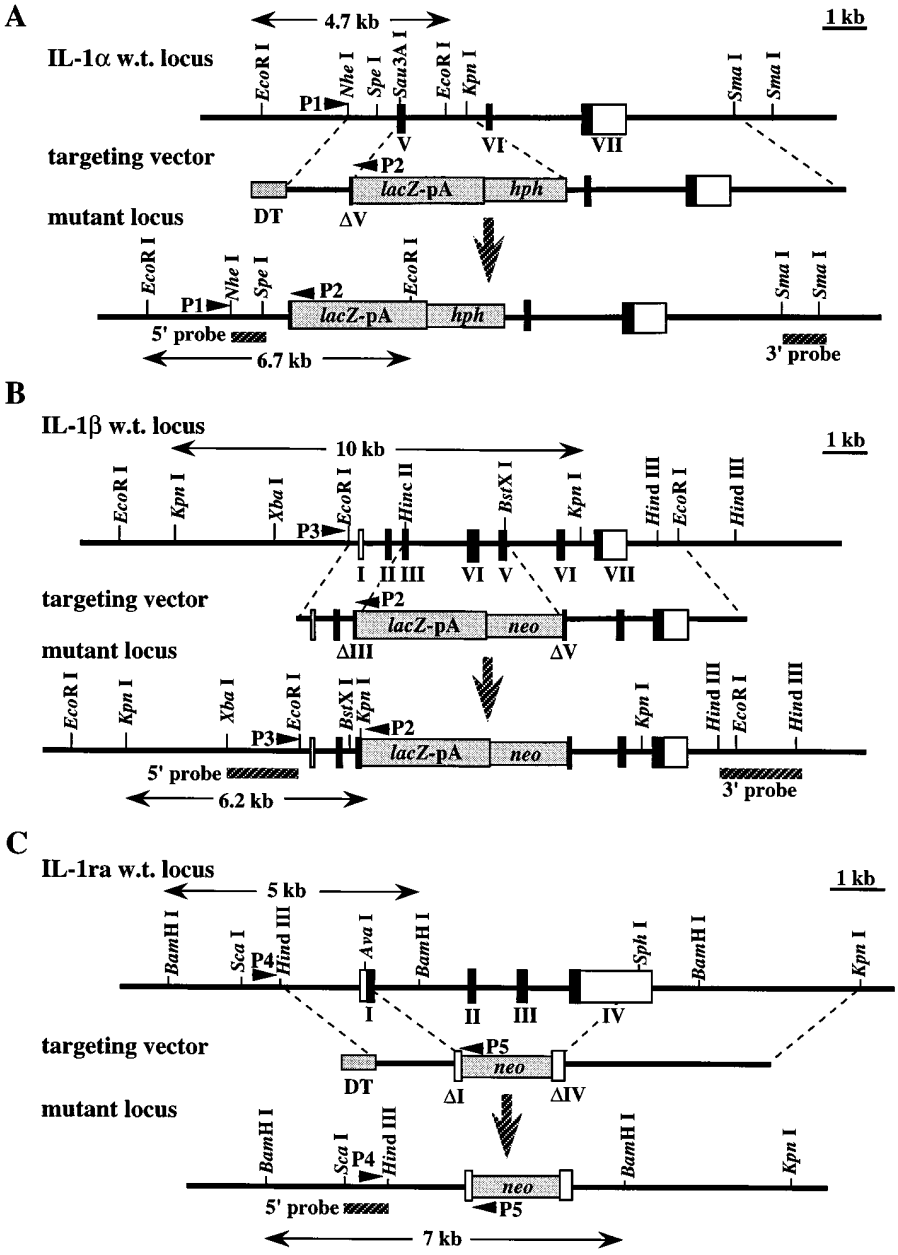

Figure 1. Targeted disruption of the IL-1 gene family by homologous recombination. Genomic loci, targeting vectors, and mutated loci of IL-1α, IL-1β, and IL-1ra genes are indicated. (*A*) Targeting strategy for the IL-1α gene. A DNA fragment including exon 5 was replaced with the *lacZ-PGK-hph-pA* cassette (*LacZ-hph*). MC1-DT was ligated at the 5′ end of the vector for negative selection. (*B*) Targeting strategy for the IL-1β gene. A DNA fragment including exon 3–5 was replaced with the *lacZ-PGK-neo-pA* cassette (*LacZ-neo*). (*C*) Targeting strategy for the IL-1ra gene. A DNA fragment containing all the coding regions for the IL-1ra secretion form was replaced with the *PGK-neo-pA* cassette (*neo*). DT was ligated at the 5′ end of the vector for negative selection. *Arrowheads*, Primers (P1–P5) used for PCR. *Striped lines*, Probes for Southern blot analysis are indicated as 5′ and 3′ probes. *Arrows*, Expected sizes of DNA fragments obtained by restriction enzyme digestions. *Black boxes*, Exons. *White boxes*, Introns. Restriction enzyme sites are also indicated in the maps. *w.t.*, Wild-type.

PCR primers were as follows: P1, 5′-CTT GGC CAT ACT GCA AAG GTC ATG-3′; P2, 5′-GAG GTG CTG TTT CTG GTC TTC ACC-3′; P3, 5′-GCG AAT GTG TCA CTA TCT GCC ACC-3′; P4, 5′-GCT GTG ATA GCA ACA GTT TGT ACC-3′; and P5, 5′-GAC TGC CTT GGG AAA AGC GCC TCC-3′. The PCR reaction buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM for each of the dNTPs, 1 μM primers, and 2.5 U/50 μl *Taq* polymerase (Boehringer Mannheim Corp., Indianapolis, IN). PCR cycles were 94°C for 1 min, 66°C for 2 min, and 72°C for 3 min, over 40 cycles.

Mice were kept under specific pathogen–free conditions in an environmentally controlled clean room at Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo. All equipment and supplies, including cages, water bottles, wooden chips, and food pellets, were sterilized. The experiments were carried out according to institutional ethical guidelines for animal experiments and safety guidelines for gene manipulation experiments. Sex- and age-matched adult mice were used for each experiment.

Southern Blot Hybridization Analysis. Genomic DNA was isolated from ES cell clones and mouse tails. DNA (10 μg) digested with an appropriate restriction enzyme was electrophoresed on a 0.7% agarose gel and transferred to a nylon membrane (Gene Screen Plus; NEN Research Products, Boston, MA). Hybridization was performed at 42°C with ^{32}P-labeled probes in a buffer containing 5× SSCP (0.75 M NaCl, 75 mM sodium citrate, 50 mM NaH_2PO_4, 5 mM EDTA), 50% formamide, 2× Denhardt's solution, 1% SDS, and 100 μg/ml salmon testis DNA. Membranes were washed in 2× SSC containing 1% SDS at room temperature, and 0.1× SSC containing 1% SDS at 65°C. Probes were labeled with [^{32}P]dCTP using a random priming method

(Amersham Corp., Arlington Heights, IL). The NheI-SpeI fragment was used as the 5′ probe, and the SmaI-SmaI fragment was used as the 3′ probe to analyze the targeted IL-1α gene. In the case of IL-1β, the XbaI-EcoRI fragment 5′ upstream of the targeting vector and the HindIII-HindIII fragment 3′ downstream of the vector were used as probes. The ScaI-HindIII fragment 5′ upstream of the targeting vector was used for IL-1ra.

Northern Blot Hybridization Analysis. Total RNA was isolated from peritoneal exudate cells (PEC) by an acid guanidinium thiocyanate-phenol-chloroform extraction method (31), and poly A^+ RNA was purified using the QuickPrep Micro mRNA purification kit (Pharmacia Biotech, Inc.). Total RNA or poly A^+ RNA was electrophoresed on a 1.2% denatured agarose gel and transferred to a nylon membrane. Hybridization was performed at 42°C with ^{32}P-labeled DNA probes in a buffer containing 50% formamide, 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 5× Denhardt's solution, 10% dextran sulfate, 0.1% sodium diphosphate, 1% SDS, and 200 μg/ml salmon testis DNA, and membranes were washed in 2× SSC containing 1% SDS at room temperature, and in 0.1× SSC containing 1% SDS at 65°C. Radioactivities were measured using the BAS-2000 system (Fuji Photo Film Co., Tokyo, Japan).

Probes. Mouse IL-1α and IL-1β cDNAs (32, 33) were kindly given by Dr. Tetsuo Sudo (Toray Industry, Kanagawa, Japan), mouse cyclooxygenase (COX) 1 and -2 cDNAs (34) were from Dr. Shozo Yamamoto (Tokushima University School of Medicine, Tokushima, Japan), mouse IL-6 and TNF-α cDNAs (35, 36) were from Dr. Takashi Yokota (Institute of Medical Science, University of Tokyo, Tokyo, Japan), and mouse β-actin cDNA (37) was from Dr. Tetsu Akiyama (Institute for Microbial Disease, Osaka University, Osaka, Japan). Mouse IL-1ra cDNA (38) and mouse IL-1ra 523-bp genomic DNA (39) were amplified from spleen and ES cells, respectively. The PCR primers used to amplify mouse IL-1ra cDNA were 5′-CCT CGG GAT GGA AAT CTG CTG-3′ and 5′-AGG CCT CGG CAG TAC TAT TGG-3′, and to amplify mouse IL-1ra genomic DNA were 5′-GAC TCG GAG TAC CTG TCA TGC-3′ and 5′-GCT CTG GAC ATA TGG CAT GTG-3′. PCR cycles were 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min, over 40 cycles.

Isolation and Culture of Peritoneal Macrophages. PEC were recovered from mice 4 d after intraperitoneal injection of 2 ml of 2% thioglycollate broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 10% FCS. After 10^6 PEC were allowed to adhere to 60-mm dishes for several hours or overnight, they were incubated in either the presence or absence of 10 μg/ml LPS.

Fever Induction and Body Temperature Measurement. Intraperitoneal body temperature of male mice was measured using an electric thermometer and tips (ELAMS® system; BioMedic Data System, Inc., Maywood, NJ) with an accuracy of ±0.1°C. Mice were anesthetized with nembutal, and the tip was implanted into their peritoneal cavity. Each animal was housed in a separate cage and allowed to recover for 1 wk. Regular day–night body temperature rhythm was monitored for another week before the experiment. The room temperature was kept at 24 ± 1°C. Mice at ~3 mo of age were injected subcutaneously with 150 μl of turpentine oil (Nakarai Tesque, Inc., Kyoto, Japan) into both hindlimbs. Body temperature was measured at noon every day until the mice returned to normal body temperature.

Corticosterone Measurement. For turpentine-induced HPA axis activation, groups of five mice were injected with either saline or 100 μl of turpentine and decapitated at 2 and 8 h after administration. 20 μl of the serum was diluted to 1 ml with distilled water and extracted with 4 ml diethyl ether. The extracts were radioimmunoassayed using a specific antibody to corticosterone as described previously (40). The relative cross-reactivities of antibodies to testosterone, progesterone, and deoxycorticosterone were <0.1, 4, and 10%, respectively. The lower limit of detection in the serum was 10 ng/ml.

Statistical Analysis. Student's *t* test was used for statistical evaluation of the results.

Results

Production of IL-1α, IL-1β, IL-1α/β, and IL-1ra KO Mice. Genomic DNAs for mouse IL-1α, IL-1β, and IL-1ra genes were isolated from 129 genomic phage libraries. Since the mouse IL-1α genomic clones have not been reported, we cloned them using mouse IL-1α cDNA as probe. The resulting IL-1α gene clones covered 15 kb of the gene, which contained homologous sequences corresponding to exons 5–7 of the human IL-1α gene (41). We also cloned 17 kb of the IL-1β gene (42) containing all the exons (1–7), and 28 kb of the IL-1ra gene (39) containing all the exons (1–4) of the secreted form of IL-1ra. Targeting vectors for the IL-1α, IL-1β, and IL-1ra genes were constructed as described in Materials and Methods (Fig. 1). ES cells were electroporated with each targeting vector, and either hygromycin B (IL-1α) or G418 (IL-1β and IL-1ra)–resistant colonies were selected and screened by PCR for homologous recombination. ES clones targeted for both IL-1α and IL-1β genes were obtained using IL-1β–targeted ES cells by a successive introduction of the IL-1α targeting vector.[2] Homologous recombination was con-

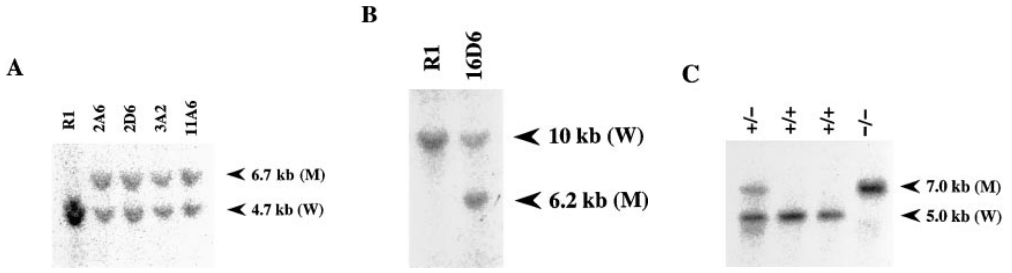

Figure 2. Southern blot analysis of DNA from cloned ES cells and from transgenic mouse tails bred by heterozygous intercross. Hybridization was carried out using 5′ probes of each gene as shown in Fig. 1. All the bands detected correspond to either the wild-type (*W*) or mutant allele (*M*), as expected from the genome structure shown in Fig. 1 (*arrows*). (*A*) DNA from IL-1α KO ES cells digested with EcoRI. The number shows the ES clone number, and *R1* shows the parental ES cells. (*B*) DNA from IL-1β KO ES cells digested with KpnI. (*C*) DNA from IL-1ra KO offspring digested with BamHI. +/+, +/−, and −/−, Expected genotypes.

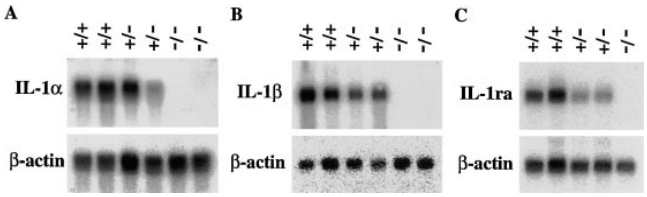

Figure 3. Northern blot analysis of RNA from macrophages from IL-1α (*A*), IL-1β (*B*), and IL-1ra (*C*) KO mice. Thioglycollate-induced peritoneal macrophages were prepared from wild-type (+/+), heterozygous (+/−), or homozygous (−/−) mice. RNA was isolated from the macrophages after incubation with 10 μg/ml LPS for 12 h (*A*) or 6 h (*B* and *C*). Total RNA (5–10 μg) was loaded on a denatured agarose gel and hybridized with either an IL-1α, IL-1β, IL-1ra, or β-actin probe.

firmed by Southern blot analysis. Genomic DNAs from cloned ES cells (Fig. 2, *A* and *B*), or from transgenic offspring (Fig. 2 *C*) were isolated and digested with either EcoRI (Fig. 2 *A*), KpnI (Fig. 2 *B*), or BamHI (Fig. 2 *C*) and analyzed by Southern blot hybridization using 5′, 3′, and *neo* or *hph* probes (Fig. 1). Hybridization with a 5′ probe detected an expected band length of 4.7, 10, or 5.0 kb, corresponding to the wild-type alleles of the IL-1α, IL-1β, or IL-1ra genes, respectively, as well as a 6.7-, 6.2-, or 7.0-kb DNA band corresponding to the mutant alleles, respectively (Fig. 2). All the bands hybridized with the 3′ probe as well as with the *neo* or *hph* probe were also of expected length (data not shown), indicating that one of the wild-type alleles in the targeted ES clones was replaced correctly by the mutant gene. The targeting efficiency of the IL-1α, IL-1β, and IL-1ra genes was 4/282 (1.4%), 1/501 (0.2%), and 3/462 (0.6%), respectively. Chimera mice were produced from these targeted ES cell clones, and germline transmission of the targeted gene was observed in three of four clones (IL-1α), one of one clone (IL-1β), and two of three clones (IL-1ra). IL-1α/β double-KO mice were also obtained similarly.[2]

To confirm deficiency of these genes, peritoneal macrophages were prepared from these KO mice, and expression of the IL-1 and IL-1ra genes after LPS stimulation was examined by Northern blot hybridization analysis. No IL-1α, IL-1β, or IL-1ra mRNA was detected in any of the IL-1α, IL-1β, or IL-1ra KO mice, respectively, indicating complete disruption of the IL-1 and IL-1ra genes (Fig. 3). Deficiency was also confirmed in IL-1α/β double-KO mice.[2]

We found that homozygous pups not only with the IL-1α, IL-1β, or IL-1ra mutation but also those with the IL-1α/β mutation were born in a Mendelian ratio, indicating that IL-1 and IL-1ra are dispensable during mouse embryogenesis. Mutant mice homozygous for IL-1α and IL-1β, as well as for IL-1α/β, were healthy and fertile in specific pathogen–free conditions. Mutant mice homozygous for IL-1ra also developed normally before weaning at 4 wk old and were fertile. However, their growth became retarded after 6–8 wk of age (Table 1). This abnormality was more significant in older mice. On the other hand, neither growth retardation nor acceleration was observed in IL-1α/β KO mice (Table 2). These observations suggest a beneficial role for IL-1ra in growth and homeostasis.

Mutual Induction of IL-1α and IL-1β In Vitro. Macrophages are a major producer of inflammatory cytokines, including IL-1, IL-6, and TNF-α. To examine the effects of IL-1 deficiency on the induction of other cytokines, thioglycollate-activated macrophages were obtained from the peritoneal cavity, and expression levels of various inflammatory cytokine genes were measured by Northern blot hybridization analysis at 0, 3, 6, and 12 h after stimulation with LPS in vitro. Macrophages from IL-1α KO mice expressed only half of the IL-1β mRNA compared with those from wild-type mice (Fig. 4 *A*). Macrophages from IL-1β KO mice also expressed a lower level of IL-1α mRNA (Fig. 4 *B*). These results suggest that IL-1α can induce expression of IL-1β, and vice versa. Expression levels of IL-1ra, IL-6, and TNF-α were not affected significantly in these mice (Fig. 4, *C–E*).

Failure of Febrile Response against Turpentine-induced Local Inflammation in IL-1β but not IL-1α KO Mice. Fever development is a systemic response that can be elicited by a multitude of exogenous stimuli. IL-1α, IL-1β, TNF-α, and

Table 1. *Body Weight of Heterozygous (+/−) and Homozygous (−/−) Littermates of IL-1ra KO Mice*

	Males		Females	
Age	+/−	−/−	+/−	−/−
wk				
4	14.3 ± 2.1 (18)	14.4 ± 2.4 (13)	13.3 ± 1.7 (14)	12.4 ± 1.8 (17)
6	23.0 ± 2.8 (10)	20.2 ± 2.3 (8)*	17.6 ± 1.7 (10)	17.1 ± 1.1 (13)
8	26.0 ± 3.0 (10)	22.4 ± 1.8 (5)*	19.6 ± 1.6 (10)	18.3 ± 1.3 (15)*
10	29.1 ± 3.4 (14)	24.3 ± 2.1 (7)‡	21.2 ± 0.8 (11)	19.4 ± 1.7 (18)‡
12	31.6 ± 1.6 (6)	25.4 ± 1.3 (10)‡	22.0 ± 0.9 (7)	19.1 ± 2.4 (14)‡

The numbers in parentheses show the number of mice used in this experiment. *$P < 0.05$, ‡$P < 0.01$.

Table 2. *Body Weight of Control (+/+, +/−) and Homozygous (−/−) Littermates of IL-1α/β KO Mice*

	Males		Females	
Age	+/+,+/−	−/−	+/+,+/−	−/−
wk				
4	15.8 ± 2.3 (24)	15.3 ± 1.5 (17)	15.1 ± 1.9 (9)	13.5 ± 2.4 (19)
8	25.3 ± 2.8 (15)	24.7 ± 2.2 (9)	ND	ND
10	28.3 ± 2.9 (12)	27.1 ± 2.2 (28)	ND	ND
12	28.4 ± 3.3 (34)	27.6 ± 2.9 (27)	22.0 ± 2.1 (17)	21.0 ± 3.2 (21)

The numbers in parentheses show the number of mice used in this experiment.

IL-6 have been suggested as putative endogenous pyrogens (13). Subcutaneous injection with turpentine in the hindlimbs of mice causes local inflammation and induces fever. We examined the role of IL-1 in the turpentine-induced fever model using IL-1α/β KO mice. As shown in Fig. 5 *A*, febrile response in IL-1α/β KO mice was reduced significantly compared with wild-type mice, suggesting that either IL-1α or IL-1β, or both, is important for fever induction. Then, to discriminate the roles for IL-1α and IL-1β, IL-1α and IL-1β KO mice were examined. In IL-1β KO mice, body temperature did not rise significantly after turpentine treatment, in contrast to the turpentine-injected control mice (Fig. 5 *B*). However, in IL-1α KO mice, body temperature rose as in control mice from day 1 to day 5 after the injection, and returned to normal by day 6 (Fig. 5 *B*). These results were confirmed in another experiment. These observations indicate a critical role for IL-1β but not IL-1α in the febrile response to local inflammation.

On the other hand, body temperature changes in IL-1ra KO mice were significantly greater than those in turpentine-injected control mice on day 1 ($P < 0.01$) (Fig. 5 *C*). Body temperature in IL-1ra KO mice did not return to normal circadian temperature even 1 wk after turpentine injection (Fig. 5 *C*). These results suggest a regulatory function for IL-1ra in the febrile response.

Suppression of IL-1α Expression in the Brain of IL-1β KO Mice, but Less Marked Suppression of IL-1β Expression in IL-1α KO Mice, after Treatment with Turpentine. We examined the effects of IL-1 and IL-1ra deficiency on the expression of related genes in the brain after turpentine injection, in order to ascertain why IL-1β but not IL-1α deficiency causes suppression of fever development by this reagent. In wild-type mice, induction of both IL-1α and IL-1β mRNA was observed as early as 6 h after turpentine treatment and reached a peak at 12 h, coincident with the development of fever (Fig. 6 *A*). Both mRNA levels were comparable, ranging 10–20-fold higher than in untreated control mice. Since both IL-1α and IL-1β can induce febrile response, these results indicate that the reason why IL-1α deficiency does not affect fever induction is not due to the inability of

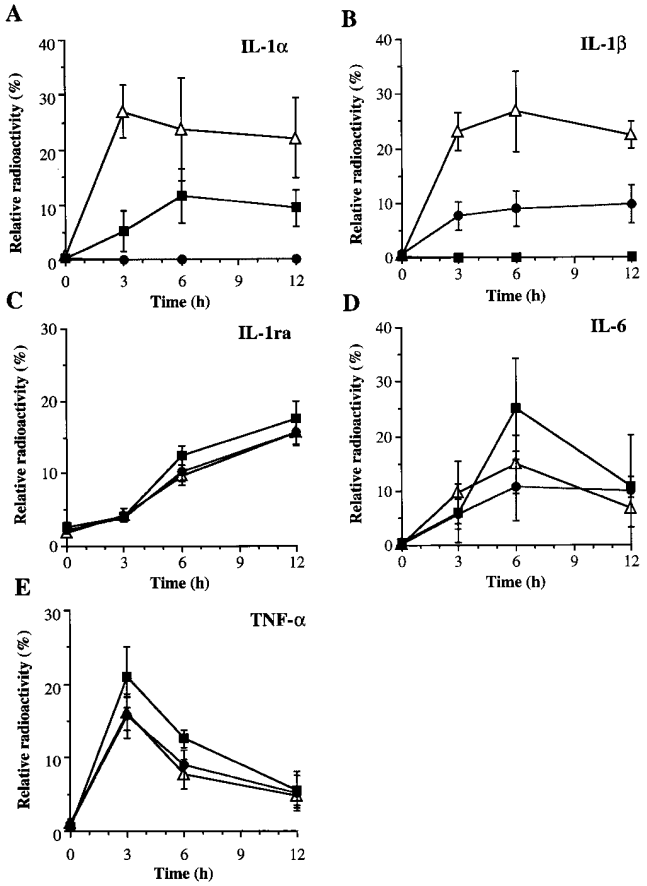

Figure 4. Expression of inflammatory cytokine genes in peritoneal macrophages from IL-1α and IL-1β KO mice. Thioglycollate-induced peritoneal macrophages were prepared from wild-type (*open triangles*) and homozygous IL-1α (*filled circles*) and IL-1β (*filled squares*) KO mice. Macrophages were treated with 10 μg/ml LPS for 0, 3, 6, and 12 h, and the total RNA was isolated. Northern blot analysis was carried out using each cytokine probe and a β-actin probe. Relative radioactivity of each cytokine mRNA was calculated as a percentage of β-actin mRNA. (*A*) IL-1α; (*B*) IL-1β; (*C*) IL-1ra; (*D*) IL-6; and (*E*) TNF-α mRNA. The values represent the mean ± SD of three independent experiments.

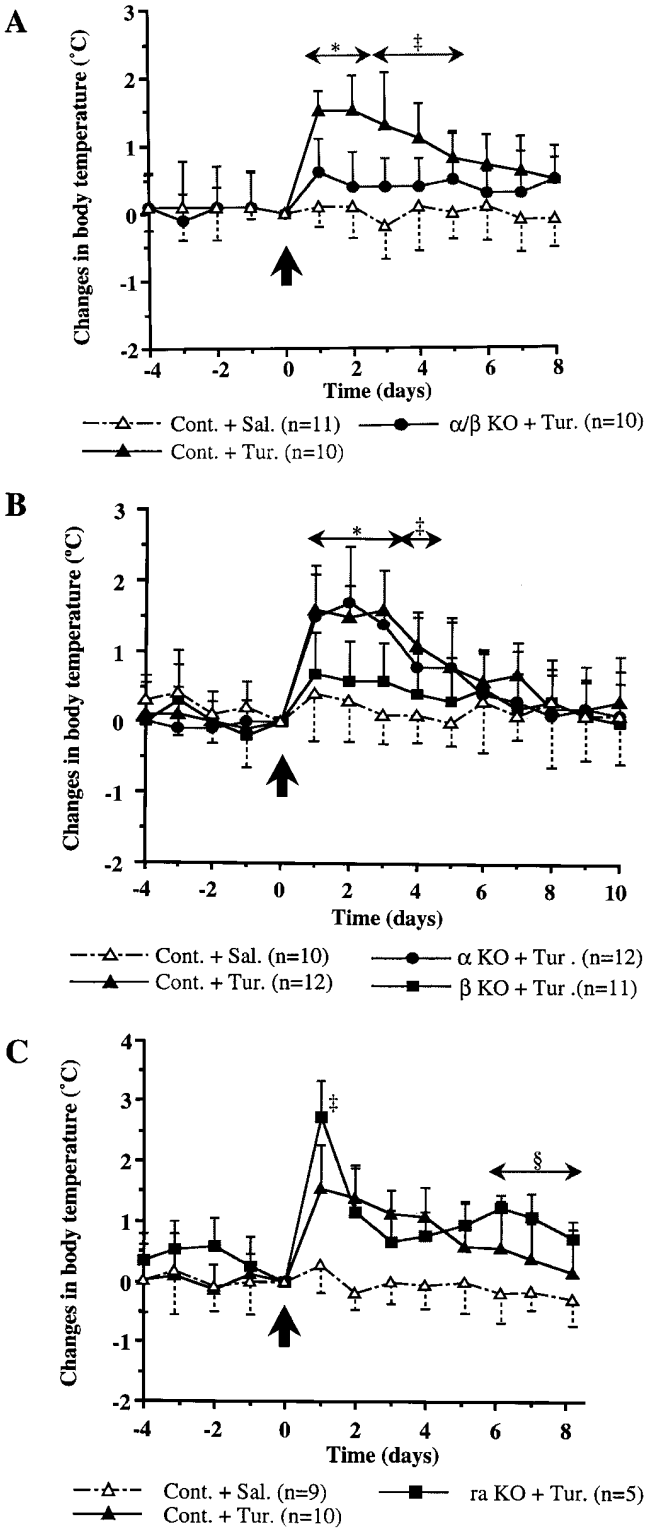

Figure 5. Fever induction after turpentine injection. Wild-type mice (*Cont.*) and homozygous IL-1 KO mice (*KO*) (male) were implanted with transmitters to monitor body temperature. Mice were injected subcutaneously with either saline (*Sal.*) or turpentine oil (*Tur.*) in both hindlimbs. Fever development was monitored in IL-1α/β (*A*), IL-1α and IL-1β (*B*), and IL-1ra (*C*) KO mice. *Arrowheads*, Time point of turpentine injection. The number of mice used for each experiment is shown in parentheses. Fever development was compared between these KO mice and wild-type mice injected with turpentine. **P* <0.001, ‡*P* <0.01, §*P* <0.05. These experiments were repeated twice with similar results.

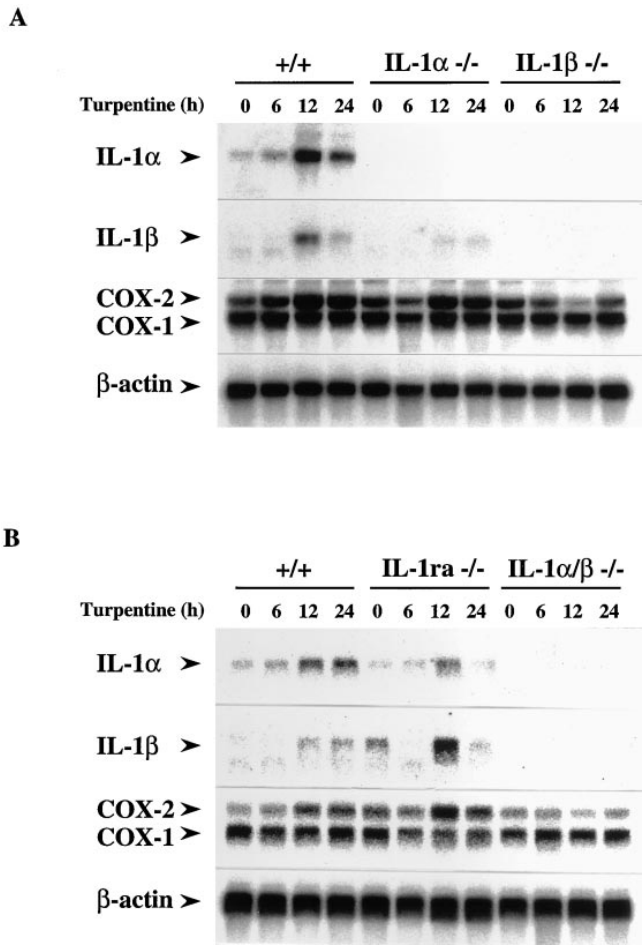

Figure 6. Time course of mRNA expression specific for the IL-1α, IL-1β, COX-1, and COX-2 genes in whole brain after turpentine injection. Wild-type (+/+) or homozygous (−/−) mice were injected with 100 μl turpentine subcutaneously in the left hindlimb. Mice were killed at 0, 6, 12, and 24 h after turpentine injection, and poly A^+ RNA was isolated from whole brain. Poly A^+ RNA (5–10 μg) was electrophoresed on a denatured agarose gel and hybridized with specific probes. β-actin was used as a control. (*A*) IL-1α and IL-1β KO mice; (*B*) IL-1ra and IL-1α/β KO mice.

turpentine to induce IL-1α in the brain. Interestingly, we found that IL-1α mRNA expression is hardly detected in IL-1β KO mice, suppressed >30-fold compared with wild-type mice (Fig. 6 *A*). However, IL-1β mRNA could still be detected in IL-1α KO mice, although the expression was 10-fold lower than in control mice. These observations were confirmed in two other experiments. Thus, it was shown that IL-1β deficiency abrogated not only IL-1β but also IL-1α expression in the brain, and that IL-1α deficiency did not suppress expression of IL-1β completely.

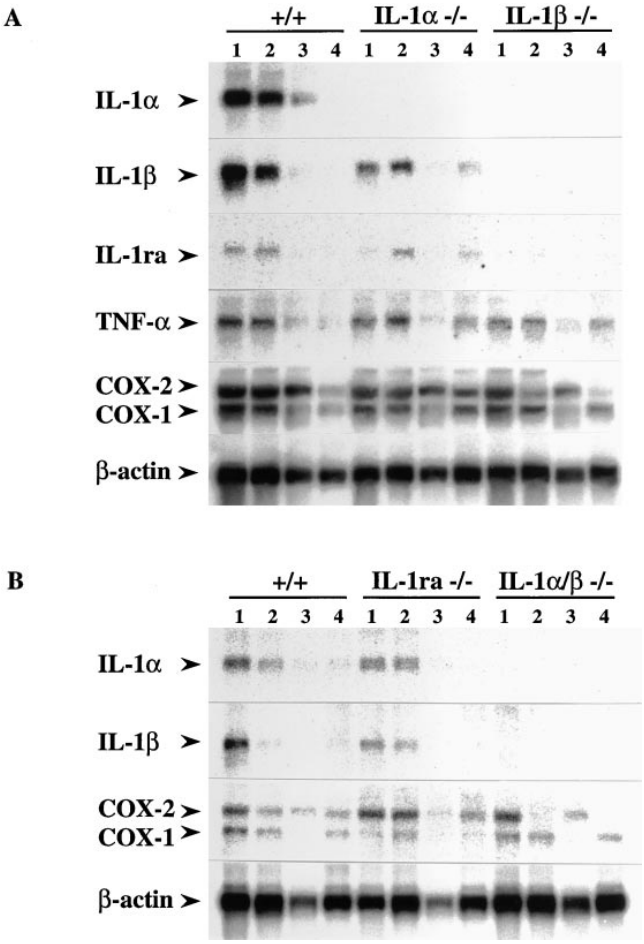

Figure 7. Region specificity of mRNA expression in the brain at 12 h after turpentine injection. Samples were pooled from four mice of each genotype. Lane *1*, Cerebral cortex; lane *2*, diencephalon; lane *3*, hippocampus; lane *4*, cerebellum. Poly A^+ RNA (5–10 μg) was purified from each part of the brain and hybridized with probes as indicated. (*A*) IL-1α and IL-1β KO mice; (*B*) IL-1ra and IL-1α/β KO mice.

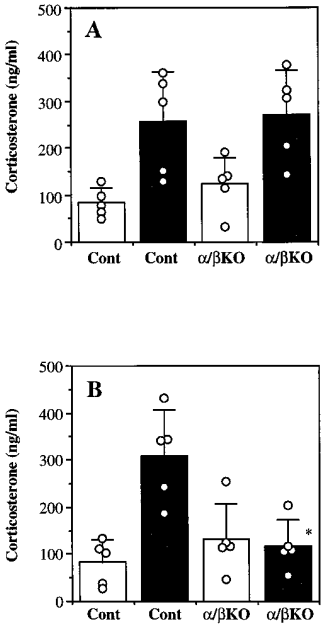

Figure 8. Turpentine-induced serum corticosterone levels in IL-1α/β KO mice. Control (*Cont*) and IL-1α/β KO mice were injected subcutaneously with saline (*white bars*) or turpentine (*black bars*), and serum samples ($n = 5$) were obtained at 2 h (*A*) and 8 h later (*B*). Corticosterone levels were then measured by radioimmunoassay. Although corticosterone levels at 2 h were similar, the levels at 8 h were significantly higher in wild-type than in IL-1α/β KO mice. *$P < 0.01$.

IL-1β mRNA expression in IL-1ra KO mice was augmented 2.5-fold compared with wild-type mice 12 h after injection with turpentine, whereas IL-1α mRNA expression was not affected (Fig. 6 *B*). These results were consistent with enhanced febrile response in IL-1ra KO mice (Fig. 5 *C*). As expected, neither the IL-1α nor IL-1β mRNA was detected in IL-1α/β double-KO mice (Fig. 6 *B*).

To correlate IL-1 expression with fever development, the expression levels of IL-1α and IL-1β mRNA in specific regions in the brain after turpentine injection were examined by Northern blot hybridization analysis. Intense expression of both IL-1α and IL-1β mRNA was observed in the cerebral cortex and diencephalon in wild-type mice, whereas expression levels were low in the hippocampus and cerebellum (Fig. 7 *A*). The expression level of IL-1β in the diencephalon, where body temperature is thought to be controlled, was 1.5-fold lower in IL-1α KO mice, whereas IL-1α as well as IL-1β mRNA could not be detected in IL-1β KO mice, indicating that IL-1α expression was >30-fold lower than in wild-type mice (Fig. 7 *A*). These data are consistent with the data shown in Fig. 6 *A*, in which whole brain was used for the analysis, although the effect of IL-1α deficiency on IL-1β expression is less marked in the diencephalon than in the whole brain. We confirmed these results in two independent experiments. These results indicate that the expression of the IL-1α gene in the diencephalon is dependent mostly on the expression of IL-1β, and that IL-1β expression is relatively independent of IL-1α expression.

The expression level of IL-1ra mRNA in the diencephalon of IL-1α KO mice was similar to that in wild-type mice (Fig. 7 *A*). In contrast, the IL-1ra expression level was much reduced (<1/10 of the wild-type level) in IL-1β KO mice (Fig. 7 *A*). IL-1α and IL-1β mRNA expression levels in the diencephalon of IL-1ra KO mice were 1.5- and 2-fold higher than those of wild-type mice (Fig. 7 *B*). These results suggest the possibility that IL-1ra functions as a suppresser of IL-1 expression under stress.

Induction of COX-2 but not COX-1 by IL-1 in the Brain. IL-1 is known to induce transcription of the COX-2 gene (43), an enzyme that catalyzes synthesis of prostaglandins, including prostaglandin E_2 (PGE_2). Since PGE_2 is a potent mediator of the febrile reaction in the body (13), COX-2 expression during turpentine-induced fever was examined. As shown in Fig. 6 *A*, expression of the COX-2 but not the COX-1 gene was induced threefold in the brain after treatment with turpentine, coincident with peak expression of IL-1 at 12 h. This induction was also observed in IL-1α KO mice, although the level was slightly lower than in wild-type mice. In contrast, induction of COX-2 expression in the brain after treatment with turpentine was not observed in IL-1β KO mice, indicating that IL-1β is the major mediator of COX-2 induction (Fig. 6 *A*).

COX-2 expression in IL-1ra KO mice was 1.5-fold higher at 12 h compared with wild-type mice, correlating with the augmented expression of IL-1β in these mice, whereas COX-1 expression level was not affected (Fig. 6 *B*). These results suggest that COX-2 expression level is controlled by a balance between IL-1 and IL-1ra.

COX-2 expression in the diencephalon was not induced in IL-1β KO mice after treatment with turpentine, whereas its expression was induced similarly in IL-1α KO as in wild-type mice (Fig. 7 *A*). COX-2 expression was 2.5-fold higher in IL-1ra KO compared with wild-type mice (Fig. 7 *B*). In these KO mice, the mRNA expression level of TNF-α, a possible mediator of fever development in the brain, was not affected (Fig. 7 *A*). These data show that COX-2 is induced mainly by IL-1β, and that this induction is antagonized by IL-1ra.

Suppression of HPA Axis Activation in IL-1 KO Mice. Acute inflammatory stimuli are known to elevate circulating corticosterone levels through activation of the HPA axis. To further elucidate the roles of IL-1 in the neuro-immuno-endocrine system, we examined the effects of IL-1 as well as IL-1ra deficiency on serum corticosterone levels after treatment with turpentine.

When mice were treated with turpentine, serum corticosterone levels increased two- to fourfold at 2 h after injection. At this point, the induction level did not differ between IL-1α/β KO and wild-type mice (Fig. 8 *A*). However, at 8 h after turpentine injection, the level was significantly lower in IL-1α/β KO compared with wild-type mice, in which the level was elevated three- to fivefold (Fig. 8 *B*; $P < 0.01$).

Then, to discriminate molecular species of IL-1 involved, IL-1α and IL-1β KO mice were injected with turpentine, and the serum corticosterone level at 8 h was measured. As shown in Fig. 9, *A* and *B*, the serum corticosterone level in IL-1β KO mice was not elevated ($P < 0.001$), whereas corticosterone levels in IL-1α KO mice were elevated similarly to that in control mice. These data clearly show that IL-1 is not responsible for turpentine-induced activation of the HPA axis at the initial phase but is responsible for the secondary rise of corticosterone, and that IL-1β but not IL-1α is a major mediator of this activation.

The effects of IL-1ra deficiency were also examined. As shown in Fig. 9 *C*, corticosterone levels in IL-1ra KO mice at 8 h after turpentine injection were elevated similarly to heterozygous control mice. Thus, IL-1ra did not seem necessary for the regulation of serum corticosterone level after treatment with turpentine.

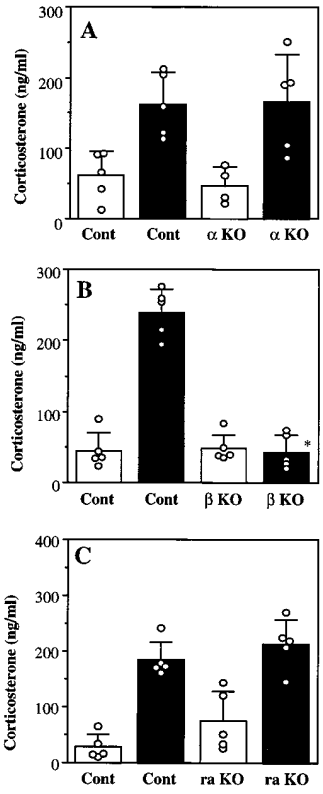

Figure 9. Turpentine-induced serum corticosterone levels in IL-1α (*A*), IL-1β (*B*), and IL-1ra (*C*) KO mice. IL-1α KO (*α KO*), IL-1β KO (*β KO*), and IL-1ra KO (*ra KO*) mice were injected subcutaneously with saline (*white bars*) or turpentine (*black bars*), and serum samples ($n = 5$, except for saline-injected IL-1α KO mice) were obtained 8 h later. The corticosterone level in IL-1α or IL-1ra KO mice is similar to that in wild-type mice, whereas that in IL-1β KO mice is significantly lower. *$P < 0.001$.

Discussion

Production of IL-1α, IL-1β, IL-1α/β, and IL-1ra KO Mice. Although mice deficient in either IL-1β, IL-1ra, or IL-1RI have been produced previously (17, 44–46), to our knowledge, IL-1α and IL-1α/β double-KO mice have not yet been available. Since IL-1α and IL-1β have similar biological activities, and since both molecules can bind with both IL-1RI and IL-1RII, the effects of IL-1 deficiency could only be observed in IL-1α/β double-KO mice. In this report, to ascertain the pathophysiological roles of IL-1 in normal physiology and under stress, we first produced both IL-1α KO and IL-1α/β double-KO mice, together with IL-1β and IL-1ra KO mice, and then compared the phenotypes of these KO mice with each other.

All of these KO mice were fertile, and the pups were born healthy, indicating that IL-1 is not necessary for embryogenesis and reproduction, although previous studies suggested the importance of IL-1 in gonadotropin release, oogenesis, testosterone synthesis, and maintenance of pregnancy (47, 48). After birth, IL-1α/β as well as IL-1α and IL-1β KO mice developed normally. In contrast, the growth after weaning was retarded in IL-1ra KO mice, consistent with a previous report (45). We found that the growth retardation was first observed between 6 and 8 wk of age, later becoming increasingly obvious. Since some cytokines, including IL-1 and TNF-α, induce anorexia (49, 50), the defect observed in IL-1ra KO mice could be due to appetite suppression caused by IL-1 overproduction in the satiety center in the brain. However, it should be noted that the growth of IL-1α/β KO mice was not accelerated compared with their wild-type or heterozygous littermates. These observations suggest that the low level of IL-1 detected in untreated wild-type mice is not inhibitory for body growth, provided that IL-1ra is present, and only higher levels of IL-1, or lack of IL-1ra, or both, causes suppression. However, it is also possible that IL-1ra may antagonize the binding of molecules other than IL-1 that exert

suppressive effects on body growth, or it may directly promote body growth.

Mutual Induction between IL-1α and IL-1β. We showed that IL-1β mRNA levels in IL-1α KO macrophages after treatment with LPS were two- to threefold lower than in wild-type macrophages, and that IL-1β deficiency also caused a similar reduction in IL-1α expression. In agreement with our results, Fantuzzi et al. also reported that IL-1α protein production in IL-1β–deficient macrophages was approximately half that of wild-type macrophages after stimulation with LPS in vitro (51). The finding that IL-1α production in macrophages from IL-1β converting enzyme KO mice, in which mature IL-1β could not be produced, was about fivefold lower than in wild-type macrophages is also consistent with our results (52). These results suggest that IL-1 is involved in the induction of IL-1. In agreement with this notion, it was reported previously that both IL-1α and IL-1β were induced by recombinant IL-1α in vivo and in vitro (53). In contrast to these observations, Shornick et al. reported that IL-1α mRNA level was not affected by IL-1β deficiency in LPS-treated macrophages (44). Although the reason for this discrepancy is not clear at present, it is possible that the difference in LPS concentrations for macrophage stimulation may affect the results. Another possibility is that a mutant product which still has the ability to induce IL-1α might be produced in their studies, since their targeting construct contained only an insertional mutation in exon 4, in contrast to Zheng et al. (17) and our constructs, in which functional exons were deleted. The expression level of IL-1ra and TNF-α was not affected significantly in IL-1α and IL-1β KO mice, suggesting that these cytokines are regulated independently of IL-1. Although the expression level of IL-6 in IL-1β KO mice seemed greater than in IL-1α KO mice at 6 h after LPS stimulation, the difference was not statistically significant.

In contrast to the effects of IL-1α or IL-1β deficiency on the production of IL-1 in vitro, the effects were much more marked in the brain; IL-1α mRNA was reduced >30-fold in IL-1β KO mouse brain after induction with turpentine compared with wild-type mouse brain, and IL-1β mRNA levels were reduced 5- to 10-fold in IL-1α KO mice. It was remarkable that IL-1α induction in the brain was much more dependent on the presence of IL-1β than IL-1β induction was dependent on IL-1α. The mechanism that activates both IL-1α and IL-1β genes through a single IL-1 receptor is considered to be an efficient way of amplifying the IL-1 signal. We reported previously that mutual induction was also observed between IFN-α and IFN-β, both of which bind to the same receptor (54). Thus, the presence of two homologous genes with similar biological activity and the same receptor specificity seems to be one of the widely used mechanisms for cells to amplify the response against weak stimuli. The data presented in this paper show that this mechanism plays an important role in the induction of IL-1 in the brain.

The expression level of IL-1ra was reduced markedly in IL-1β KO mouse brain. This observation is consistent with reports that IL-1β is a potential inducer of IL-1ra (55, 56), and suggests a feedback mechanism of IL-1 activity.

Recently, it was reported that insertion of the *neo* gene into the host chromosome might disturb the expression of neighboring genes (57). Therefore, it is possible that the observed suppressive effect of IL-1α and IL-1β deficiency on the expression of the other molecule may be the result of *neo* or *hph* gene insertion into these loci, which are only 50 kb apart on chromosome 2. However, it seems unlikely that independent insertional events of different genes into different loci, that is, insertion of the *lacZ-hph* gene into the IL-1α gene or of the *lacZ-neo* gene into the IL-1β gene, can cause similar suppression on both genes. Furthermore, since suppression of IL-1α production has also been reported in IL-1β–converting enzyme KO mice, in which insertion of the *neo* gene occurred in a totally different locus (chromosome 9), we believe that mutual induction of IL-1α and IL-1β is not an artificial result caused by transgene insertion but rather a characteristic of these genes.

Role of IL-1 in Turpentine-induced Fever. Subcutaneous injection of turpentine causes local inflammation and induces fever. It is known that the hypothalamus is involved in temperature control (58). This study showed that fever development was not induced in IL-1α/β KO mice, indicating that IL-1 is involved in fever induction. We also found that this fever induction was abolished in IL-1β but not IL-1α KO mice. Involvement of IL-1β in turpentine-induced fever has also been reported by Zheng et al. using IL-1β KO mice (17). These observations do not necessarily imply that the fever induction is caused mainly by IL-1β, since IL-1α as well can cause a febrile reaction in mice (16), and both IL-1α and IL-1β are induced in turpentine-treated mice (Fig. 6). These results indicate that IL-1α deficiency in the febrile reaction can be compensated for with IL-1β, but IL-1β deficiency cannot be compensated for. To explain this, we found that significant levels of IL-1β expression were still observed in the brains of IL-1α KO mice after treatment with turpentine, although the level was 1/5–1/10 that in wild-type mice. Further, IL-1α expression in IL-1β KO mice was <1/30 that in wild-type mice and thus hardly detectable. These data indicate that the IL-1β gene is crucial for febrile response, and that the absence of both IL-1α and IL-1β expression in IL-1β KO mice is the reason why febrile response is abolished in IL-1β KO mice. Consistent with these observations, we found that febrile response was enhanced in IL-1ra KO mice associated with enhanced expression of IL-1β. However, it is possible that IL-1 signaling is enhanced due to the absence of the competitor molecule in IL-1ra KO mice. Although we failed to detect IL-6 mRNA in the brain after turpentine injection by Northern blot analysis (data not shown), it is most likely that IL-6 is a major mediator downstream of IL-1β in this fever development, since central injection of LPS or IL-1β could not elicit a fever response in IL-6 KO mice (18, 59).

It is known that IL-1 induces COX-2 in the brain (60). We found that induction of COX-2 was greatly suppressed in the brain of IL-1β as well as IL-1α/β KO mice, but

COX-1 expression was not affected in these KO mice. It is noteworthy that the suppression of COX-2 induction was especially prominent in the diencephalon, whereas expression in the cerebral cortex and hippocampus was minimally affected. Since expression of IL-1 was observed both in the cortex and diencephalon, these observations indicate region-specific control of COX-2 by IL-1 in the brain: the expression in the diencephalon is dependent on IL-1, whereas the expression is independent of IL-1 in other brain regions. Although the mechanism for the region-specific induction of COX-2 by IL-1 remains to be elucidated, these results suggest a complex regulatory mechanism for COX-2 expression. The regulatory role of IL-1 in COX-2 expression in the diencephalon was also suggested by the fact that COX-2 expression in the diencephalon was enhanced in IL-1ra KO mice. These observations are consistent with the notion that the absence of COX-2 induction in the diencephalon is responsible for the defect in febrile response in IL-1 KO mice.

Role of IL-1 in the HPA Axis-mediated Stress Response. It is well known that corticosterone secretion from the adrenal gland is controlled by ACTH released from the pituitary, and ACTH by CRH from the hypothalamus. Since previous studies suggested involvement of IL-1 in the regulation of CRH secretion from the hypothalamus (61, 62), we examined glucocorticoid levels after induction with turpentine in IL-1 KO mice. We found that the corticosterone level in the serum of IL-1α/β KO mice was significantly low at 8 h after turpentine injection, although corticosterone was induced similarly in KO mice as in wild-type mice at 2 h after the injection (Fig. 8). These results suggest two different mechanisms in the activation of the HPA axis after treatment with turpentine. The initial rise in glucocorticoid secretion seems to be caused by direct activation of the hypothalamus either through neurological pathways or through other cytokines than IL-1 induced by turpentine. On the other hand, the second rise is thought to be mediated by the increase of IL-1 in the brain. This second rise in corticosterone level was also suppressed in IL-1β KO mice, but not in IL-1α KO mice. This indicates that deficiency of IL-1α in activating the HPA axis can also be compensated for with IL-1β, as in the febrile response. However, IL-1β deficiency cannot be compensated for, probably because of the strong suppression of IL-1α expression in IL-1β KO mice. Although Fantuzzi and Dinarello reported that corticosterone levels were not affected by IL-1β deficiency, this is not inconsistent with our results, since they only examined levels 2 h after treatment with turpentine (25).

In summary, the results presented in this report indicate importance of IL-1 in maintaining homeostasis of the body by controlling fever development and glucocorticoid synthesis. We suggest that a network formed by IL-1α, IL-1β, and IL-1ra regulates the expression of these molecules with each other, and this may contribute an efficient response to inflammation or infection. These KO mice should be useful to investigate further the roles of IL-1 in normal physiology and under stress.

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