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IL-1 signal is transduced through type I receptor (IL-1RI). We have recently reported that LPS augments IL-1RI mRNA expression in the hepatocytes of mice in vivo, and the augmentation is mediated by the interaction of IL-1, IL-6, and glucocorticoid (GC). In this study, we examined whether IL-1RI mRNA expression level in the hepatocytes reflects those of cell surface molecule and IL-1 signaling. When primary cultured murine hepatocytes were treated with dexamethasone (Dex) or IL-6, these two reagents synergistically up-regulated IL-1RI mRNA expression in the cells. Radioiodinated IL-1 binding experiment showed that the level of binding was also up-regulated by the treatment with Dex and IL-6. Scatchard analysis revealed that the number of IL-1R increased. The increased binding of IL-1 was completely inhibited by an Ab against murine IL-1RI, indicating that Dex and IL-6 augmented the expression of cell surface IL-1RI molecule. When hepatocytes were pretreated with Dex and IL-6, the activation of IL-1R-associated kinase was augmented in response to IL-1, indicating that IL-1 signaling was also augmented. In addition, IL-1 treatment following administration of the combination of Dex and IL-6 into mice markedly increased the serum level of serum amyloid A. These results indicate that GC and IL-6 augment the expression of cell surface IL-1RI in hepatocytes, as well as IL-1 signaling and IL-1R-associated kinase activation, through up-regulation of IL-1RI mRNA level, which represents a novel regulatory network between IL-1, GC, and IL-6. The Journal of Immunology, 1999, 162: 4260 – 4265.

I nterleukin-1, mainly produced by macrophages, plays an important role in inflammation and immunologic reactions through activation of a variety of genes and through affecting the function and growth of various cells and organs (1). Two distinct types of IL-1 (α and β) bind common receptors on cell surface and exert almost the same activities (2–5). There are two types of IL-1 receptors. Type I IL-1RI (IL-1RI) is mainly expressed in T cells and fibroblasts, and Type II IL-1RI (IL-1RII) is in B cells, macrophages, and neutrophils (6–8). IL-1RII is liberated and works as a decoy target for its ligand, and only IL-1RI participates in IL-1 signaling (9). IL-1RI is also expressed in B cells, macrophages, and neutrophils and transduces IL-1 signal into the cells (10, 11). Systemic or constitutive local production of IL-1 is implicated in a variety of disease, including septic shock, rheumatoid arthritis, osteoporosis, type I diabetes, crystal arthritis, idiopathic dilated cardiomyopathy, and endometriosis (1). Therefore, to understand the role of IL-1 in a disease and further to regulate the IL-1 function in the therapeutic aspect, it is important to elucidate the regulatory mechanism of IL-1RI expression.

It was shown that cytokines, including IL-1, IL-4, IL-13, IFN, TGF-β, glucocorticoid (GC), and PGE₂ regulate the levels of IL-1R expression in vitro (9, 12–20). Previously, we have reported that IL-1 up-regulated IL-1RI via production of PGE₂ in human fibroblasts (12). Platelet-derived growth factor (PDGF) and GC are also reported to enhance IL-1R expression in fibroblasts (19, 21–23). IL-1 and IL-4 increase IL-1R in T cell line (13). TGF-β stimulates IL-1R expression in both positive and negative manners (16–18). Colotta et al. observed that IL-4, IL-13, and GC all increased IL-1 binding and mRNA for both IL-1RI and IL-1RII in neutrophils (9, 14, 20). It was also reported that IL-1RII expression in bone marrow cells was increased by IL-3, granulocyte-macrophage (GM)-CSF, and G-CSF treatment (24–26).

In the case of systemic inflammation, especially the LPS septic shock mouse model, a large amount of IL-1 is produced by a variety of cells, including macrophages/monocytes and endothelial cells (1, 27). Recently, we have reported that, by the RT-PCR method, a marked and sustained up-regulation of IL-1RI mRNA expression was observed in mouse hepatocytes by LPS administration in vivo (28). We also suggested that up-regulation of IL-1RI mRNA in response to LPS is mediated by the interaction of IL-1, IL-6, and GC (29). In this study we examined whether IL-1RI mRNA expression level in the hepatocytes reflects those of cell surface IL-1RI molecule and IL-1 signaling using primary cultured murine hepatocytes.

Materials and Methods

Reagents

Human recombinant IL-1α with a specific activity of 2 × 10⁷ U/mg was a gift of Dr. M. Yamada (Dainippon Pharmaceutical, Osaka, Japan). Human recombinant IL-6 with a sp. act. of 5 × 10⁷ U/mg was a gift of Dr. Y. Akiyama (Ajinomoto, Yokohama, Japan). Rabbit antiserum to IRAK was a kind gift from Dr. Z. Cao, Tulalik (South San Francisco, CA). Neutralizing Ab against murine type I IL-1R, M147, was kindly supplied by Immunex (Seattle, WA). Escherichia coli LPS (E. coli 026:B6) was purchased from Difco Laboratories (Detroit, MI). Dexamethasone 21-phosphate (Dex) was from Sigma (St. Louis, MO).

Mice

Female ICR mice (7 wk of age) were purchased from SLC (Hamamatsu, Shizuoka, Japan). Animals were maintained under specific pathogen-free conditions. Food and water were given ad libitum. They were used in experiments after 1 wk acclimation.
Drug treatment

Cytokines, Dex, or LPS were diluted in endotoxin-free PBS and stored at −20°C until they were used. The injected materials were determined previously to be free of endotoxin contamination by Limulus amoebocyte assay (sensitivity limit, 0.1 ng/ml). At time point zero mice were given a single i.p. injection of vehicle (PBS), Dex (1 mg/kg), IL-6 (2 mg/mouse), the combination of Dex (1 mg/kg) and IL-6 (2 μg/mouse), or LPS (10 μg/kg). Six hours after injection, mice were killed, and their livers were taken. The livers were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Mouse hepatocyte cultures

Mouse hepatocytes were isolated using a modification of the collagenase method (28). Briefly, the liver was perfused in situ with 0.0125% collagenase (Sigma) through the portal vein. The total cells isolated were centrifuged four times at 50 × g for 1 min at 4°C to remove nonparenchymal cells. The cells were finally suspended in Williams’ medium E containing 10% FBS, 100 U/ml streptomycin, 100 μg/ml penicillin, 0.25 μg/ml amphotericin B, and 10− M insulin at a density of 5 × 104 cells/ml. Two milliliters of the cell suspension were cultured in collagen-coated six-well dishes. The cell suspension contained >95% hepatocytes, and the viability of the cells was >80% by trypan blue exclusion. If the viability was <80%, the cells were not used. After incubation for 4 h at 37°C in 5% CO2 in air, nonadherent cells were removed, and fresh medium was added. After another 20 h incubation, the hepatocytes were washed twice and then stimulated with various reagents in fresh medium.

Preparation of iodinated IL-1

Human recombinant IL-1α was labeled with 125I using the Bolton-Hunter reagent (2766 mCi/mmol, ICN Pharmaceuticals, Costa Mesa, CA) for 1 h on ice as previously described (19). After stopping the reaction with 0.5 ml of 0.5 M glycine in 0.1 M borate buffer, pH 8.5, [125I]-IL-1α was separated from free iodine by ultrafiltration with Microcon-3 (Amicon, Beverly, MA). The labeling efficiency of IL-1α was 28.8 mCi/mg protein.

Receptor binding assay

After treatment with various reagents, mouse hepatocytes seeded in six-well dishes were washed twice with ice-cold binding buffer (RPMI 1640 supplemented with 1% BSA) and incubated in 1 ml of the same buffer containing 2.5 ng [125I]-IL-1α in the presence or absence of 400-fold unlabeled IL-1α. After 1 h incubation at 4°C, the cells were rinsed four times with ice-cold PBS and solubilized in 3 ml of 0.1N NaOH containing 2% NaNO3 and 1% SDS. The total cell-associated radioactivity was determined by a gamma counter (model ARC-380, Aloka, Tokyo, Japan).

RNA extraction and Northern blot analysis

Total RNA was extracted from hepatocytes seeded in 100-mm plates or 100 mg of livers following the guanidinium thiocyanate method (30). Twenty micrograms of total RNA was separated in a 1% agarose gel containing 2% formaldehyde and transferred onto a nitrocellulose filter, Hy-bond-C extra (Amersham Life Science, Buckinghamshire, U.K.) with 20 × SSC. The filters were prehybridized, and the levels of mRNAs were determined with specific labeled probes. A 559-bp murine IL-1RI fragment and a 392-bp murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) fragment were obtained by RT-PCR using EL-4 6.1 cell-derived mRNA as a template and oligonucleotides, based on published sequences (7, 31). Primers used for IL-1RI were 5′-ATAATGAGAGATCCGAGGTCCGAGTG-3′ and 5′-AGGGCTCTTCATGTCAGGCAAGGCAGG-3′, and for GAPDH were 5′-TGGTTGACAGCATGCTG-3′ and 5′-TGGATGCGAGCTTGGCAGGACC-3′. Both fragments were respectively cloned into the BamHI site of pGEM-3Z vectors. Probes were labeled with [α-32P]dCTP by random priming (Nippon Gene, Tokyo, Japan). After hybridization, the filters were washed in 2× SSC and 0.1% SDS at room temperature for 5 min, followed by twice washing in 0.2× SSC and 0.1% SDS at 65°C for 30 min. Filters were autoradiographed using a Bioimage analyzer, BAS 2000 (Fuji Film, Tokyo, Japan).

Preparation of cell extracts

After primary cultured hepatocytes were pretreated for 6 h with or without the combination of Dex (10−6 M) and IL-6 (1000 U/ml) on 100-mm dishes, IL-1 (1000 U/ml) was added to the medium. After incubation at 37°C for 5 min, the medium was removed, the cells were washed three times with ice-cold PBS containing 1 mM EDTA, and the plates were chilled on ice immediately. An amount equal to 0.8 ml of ice-cold lysis buffer (50 nM HEPES, pH 7.6, 250 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1% Tween 20, 10% (v/v) glycerol, 10 mM β-glycerophosphate, 5 mM p-nitrophenyl phosphatase, 1 mM sodium orthovanadate, 1 mM benzamidin, 0.4 mM PMSF, 1 mM sodium metabisulfite, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) was added into the plates, and stirred for 5 min with a rubber policeman. The cell lysates were collected and stored at −80°C until they were used. After thawing on ice, the cellular lysates were sonicated three times for 3 s. The cell debris was pelleted by 20 min centrifugation in a microcentrifuge, and the supernatants were collected.

Immunoprecipitation and immunoblotting analysis

One microliter of rabbit antisera against IRAK was added to the cell extracts and incubated at 4°C for 2 h, after which protein A beads were added for an additional 2 h. Beads were washed three times with the lysis buffer and suspended in 20 μl of SDS-sample buffer. After being boiled for 5 min, proteins were separated by 8% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to PVDF-membrane, Immobilon (Millipore, Bedford, MA). Membranes were incubated with the antisera to IRAK at 1:1000 dilution and then with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA). The reactive proteins were detected with enhanced chemiluminescence reagents (Amersham) and analyzed by a chemiluminescence image analyzer, LAS-1000 (Fuji Film).

Measurement of serum level of SAA

An ELISA was established for the determination of SAA content in mouse serum. Serum and standard murine SAA (Calbiochem-Novabiochem, La Jolla, CA) was serially diluted with coating buffer (50 ml Tris-HCl, pH 8.0, 0.1% BSA, 5 mM EDTA, 0.1 M NaCl, 0.1% Tween 20). For coating, 0.1 ml of coating solution was distributed into each well of a 96-well plate (Nunc-ImmuNoPlate, MaxiSorp, Roskilde, Denmark) and incubated at room temperature for 1 h. The coated plate was blocked for 1 h at room temperature with 0.3 ml/well of blocking buffer (PBS, 0.1% BSA). After blocking, rat mAb IgG against human SAA (0.25 μg/ml in blocking buffer) that cross-reacts with murine SAA (Biosource International, Camarillo, CA) were added at a volume of 0.1 ml/well, and the plate was incubated at room temperature for 1 h. Thereafter, 0.1 ml of 1/5000 diluted horseradish peroxidase-conjugated goat anti-rat IgG (1:5000 in blocking buffer) (Santa Cruz Biotechnology) were added into each well, and the plate was incubated for 1 h at room temperature. Three times washing with washing buffer (PBS, 0.05% Tween 20) were conducted between each step to completely remove reagents not bound to the solid phase. Finally, 0.1 ml of O-phenylenediamine diamin substrate solution was added into each well, and the plate was incubated at room temperature for 10 min to allow yellowish color to develop before terminating the enzymatic reaction by the addition of 0.1 ml/well of 2 N H2SO4. The amount of SAA protein in each sample was measured as absorbance at 490 nm using an ELISA plate reader (Bio-Rad, Richmond, CA).

Results

GC and IL-6 synergistically up-regulate liver and hepatocyte IL-1RI mRNA expression in vivo and in vitro

We have previously shown that in vitro Dex and IL-6 synergistically up-regulated IL-1RI mRNA expression in mouse hepatocytes as determined by the RT-PCR method (28). To confirm the observation, we determined the level of IL-1RI mRNA of primary cultured mouse hepatocytes treated with Dex, IL-6, or the combination of Dex and IL-6, for 6 h by Northern blot analysis using a specific probe for murine IL-1RI (Fig. 1A). Although the expression level of IL-1RI mRNA in control hepatocytes was below the limit of detection, a weak, but significant, band was detected in Dex- or IL-6-treated hepatocytes. Treatment with the combination of Dex and IL-6, however, markedly increased IL-1RI mRNA expression. These results are consistent with our previous observation obtained by RT-PCR analysis (28). The synergistic effect of Dex and IL-6 was not due to an improvement of culture condition because cell viability was not different from control cells. To examine whether Dex and IL-6 also increase IL-1RI mRNA expression in vivo, mice were injected ip. with Dex, IL-6, or the combination of Dex and IL-6, and IL-1RI mRNA expression in the liver was analyzed (Fig. 1B). IL-1RI mRNA expression was also up-regulated in the similar manner as the in vitro treatment. LPS

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used as a positive control induced more up-regulation of IL-1RI mRNA expression.

Augmenting effect of Dex and IL-6 on IL-1 binding to hepatocytes

To determine whether Dex and IL-6 increase cell surface IL-1R on primary cultured mouse hepatocytes, binding assay using [125I]IL-1 was performed (Fig. 2). In control hepatocytes, specific [125I]IL-1 binding activity was very low; it was close to the limit of detection. After incubation with Dex or IL-6 alone for 24 h, the binding activity was slightly increased. In contrast, the binding activity was markedly increased by the treatment with the combination of Dex and IL-6. The same level of increase in IL-1 binding was observed in the hepatocytes treated with the combination for 6 h (data not shown). Scatchard plot analysis was performed using the hepatocytes treated with or without the combination of Dex and IL-6. As the specific binding of [125I]IL-1 to control hepatocytes was quite low, it was difficult to perform the exact analysis.

However, the analysis showed that control cells exhibit 4.9 × 10^3 binding sites/cell with a K_d of 1.3 × 10^{-11} M. After treatment with the combination of Dex and IL-6, the number of binding sites was increased to 7.7 × 10^3 /cell with a K_d of 0.79 × 10^{-11} M.

Increased expression of type I IL-1R by the treatment with Dex and IL-6

We determined which type of IL-1R expression, type I or II, is augmented by the treatment with Dex and IL-6. The increased binding was completely reduced to the level of untreated cells by anti-murine IL-1RI Ab, M147, which blocks IL-1 binding to murine IL-1RI (Fig. 4). These results (Figs. 2 to 4) indicate that the treatment with the combination of Dex and IL-6 increased the
number of IL-1RI on mouse hepatocytes through up-regulation of mRNA level.

**Pretreatment with Dex and IL-6 augments the IL-1-induced IRAK activation**

To determine whether the up-regulation of IL-1RI augments IL-1 signaling, we examined IL-1-induced IRAK activation. After 6 h pretreatment with or without Dex and IL-6, primary cultured mouse hepatocytes were stimulated with IL-1 for 5 min; IRAK was isolated and detected by immunoprecipitation followed by immunoblotting (Fig. 5). It is known that activation of IRAK causes its own phosphorylation, which retards its mobility in an SDS gel (32). In each sample, an 80-kDa form of IRAK was detected that corresponds to unphosphorylated IRAK. The 80-kDa band was not detected when normal rabbit serum was used for immunoprecipitation (data not shown). The density of the band was markedly decreased by the treatment with IL-1, after pretreatment with the combination of Dex and IL-6, when it was compared with the pretreatment with medium followed by IL-1. We were unable to detect the phosphorylated form of IRAK; perhaps it was soon degraded by proteasomes (33). Since it is reported that, once IRAK is highly phosphorylated, the unphosphorylated form is decreased by IL-1 treatment (33), our results demonstrate that the up-regulation of IL-1RI by pretreatment with the combination of Dex and IL-6 augments IL-1 signaling.

**In vivo treatment of mice with Dex and IL-6 increases the response to IL-1 in production of SAA**

It is known that hepatocytes produce SAA in response to IL-1 and IL-6, alone or in synergy (34). We attempted to determine whether Dex and IL-6 treatment augments the production of SAA by hepatocytes. In the primary cultured mouse hepatocytes, however, the level of SAA in the culture supernatants was below the detection limit even after stimulation with these stimuli (data not shown). We then administered IL-6 or Dex, alone or in combination, into mice. After 6 h, IL-1 was injected, and then IL-1-inducible serum level of SAA was determined after 5-h injection of IL-1. SAA was not induced by IL-6 or Dex alone, and some by the combination of them without IL-1 administration (Fig. 6). IL-1 augmented the production of SAA, especially after pretreatment with the combination of Dex and IL-6.

**Discussion**

This study demonstrated that Dex and IL-6 increased the number of cell surface IL-1RI in murine hepatocytes as well as IL-1 signaling through up-regulation of IL-1RI mRNA level. We have reported that, by RT-PCR method, Dex and IL-6 up-regulated the expression of IL-1RI mRNA in murine hepatocytes in vitro (29). Since it is often argued against the quantification of the RT-PCR method, we performed the Northern blot analysis. In either in vitro treatment of primary cultured hepatocytes or in vivo administration, Dex and IL-6 synergistically augmented the expression of IL-1RI mRNA, confirming our previous observation with the RT-PCR method. Using primary cultured hepatocytes, we also showed that IL-6 and Dex synergistically increased the binding of [125I]IL-1 to the cells. Scatchard analysis indicated that the treatment increased the number of cell surface IL-1R without the change of its affinity. There has been no solid evidence whether hepatocytes express type I or type II IL-1R. In this study, the augmented binding level of [125I]IL-1 was completely inhibited by the Ab against IL-1RI, indicating that Dex and IL-6 up-regulate IL-1RI. This was consistent with the IL-1RI mRNA expression level. In our study, we cannot detect type II IL-1R mRNA by the RT-PCR method in either unstimulated and stimulated hepatocytes with the combination of Dex and IL-6 (data not shown). However, we do not rule out the possibility that the basal minimum binding reflects type II IL-1R because the binding was not inhibited by the Ab against IL-1RI. It is reported that IL-4, IL-13, and GC all increased IL-1 binding and mRNA for both IL-1RI and IL-1RII in neutrophils (9, 14, 20). Since the IL-1RI expression level is higher than IL-1RI in both protein and mRNA levels, it was suggested that they suppress signal transduction for IL-1 in neutrophils. However, as demonstrated in this study, the increased expression of IL-1R on hepatocytes was due to IL-1RI.

Recent studies revealed, that once IL-1 binds to IL-1RI, the complex associates with the IL-1R accessory protein (IL-1RacP) and initiates a signaling cascade leading to NF-κB activation (35, 36). MyD88 and then IRAK are recruited to the receptor complex, where IRAK is highly phosphorylated (37, 38). IRAK then leaves the receptor complex and interacts with TRAF6 (39); subsequently, the phosphorylated IRAK is degraded by proteasomes (36). Recently a kinase complex consisting of the NF-κB-inducing kinase (NIK) and two IκB kinases (IκBα and -β) have been found (40–45). NF-κB-inducing kinase interacts with TRAF6, and IκB
kinases phosphorylate IkB proteins, which is required for the induction of ubiquitination of IkB, and then activation of NF-κB. Therefore, activation (phosphorylation) of IRAK is a hallmark in activation of IL-1 signaling cascade. After pretreatment of the primary cultured hepatocytes with Dex and IL-6, the IL-1-induced activation of IRAK was augmented. Therefore, Dex and IL-6 appeared to augment the expression of functional IL-1RI. It is of note that this study is the first to demonstrate that IRAK activation is induced by IL-1 in murine hepatocytes and that the up-regulation of IL-1RI by physiological stimuli leads to the augmented activation of IRAK.

IL-1, alone or in synergy with Dex and IL-6, induces production of acute phase proteins from hepatocytes (46–48). SAA is the representative in mice, the production of which is induced mainly by IL-1 (46, 48). In mouse, two major acute phase SAA genes (SAA1 and SAA2), a minor acute phase SAA gene (SAA3), and an SAA pseudogene (SAA4) have been described (46). Similarly, two acute phase SAA genes (SAA1 and SAA2), an SAA pseudogene (SAA3), and a constitutively activated gene (SAA4) have been found in humans. We first attempted to determine whether IL-1 induces SAA production from primary cultured hepatocytes. However, we were unable to detect SAA in the culture supernatants; perhaps the production level was quite low in vitro. Since IL-1RI mRNA expression level in the mouse liver in vivo was comparable to that of in vitro cultured hepatocytes, we administered IL-6 or Dex, alone or in combination, into mice, and then IL-1 inducible serum level of SAA was determined. Dex or IL-6 administration without IL-1 treatment did not induce SAA. Only the combination of Dex and IL-6 induced the production of SAA, and the level was markedly elevated after stimulation with IL-1. It is of note that the SAA induction level was comparable to that of the IL-1-binding ability of hepatocytes. SAA gene activation, however, is regulated not only by IL-1 but also by IL-6 and Dex. It is reported that NF-κB and C/EBPα-enhancer-binding proteins (C/EBPs) regulate SAA gene transcription in mouse, rabbit, rat, and humans; the activation of them is induced by IL-1 and IL-6, respectively (46–52). In mouse SAA1 and SAA2 gene, however, there are no functional NF-κB binding sites. Therefore, IL-1 may regulate the gene activation through other transcription factors, such as SAA activating sequence binding factor (SAF) and specificity protein 1 (SP-1) (53), or other mechanisms. In rat, SAA gene is negatively regulated by the transcription factor YY1 (54). Not only in gene activation, but also posttranscriptional regulation, mRNA stability is involved in the induction of SAA (55).

It is known that IL-1 induces GC directly or indirectly from adrenal cortex (56, 57), while IL-1 decreases GC receptor in hepatocytes (58). In contrast, GC inhibits the production and function of IL-1 through inhibiting NF-κB activation (59–61). IL-1 induces IL-6 production from many cell types (62), and GC up-regulates the expression of IL-6 receptor in hepatocytes (63). Therefore, these cytokines and GC form a complex network, and our study demonstrated a novel regulatory mechanism between IL-1, IL-6, and GC through regulation of IL-1RI expression.

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References


