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A Stress-Induced, Superoxide-Mediated Caspase-1 Activation Pathway Causes Plasma IL-18 Upregulation

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Summary

Psychological/physical stresses are known to cause relapses of autoimmune and inflammatory diseases. To reveal a mechanism by which noninflammatory stresses affect host defenses, responses to immobilization stress in mice were investigated, focusing on the role of a multifunctional cytokine, interleukin-18 (IL-18). In the adrenal cortex, the stress induced IL-18 precursor proteins (pro-IL-18) via adrenocorticotropic hormone (ACTH) and a superoxide-mediated caspase-1 activation pathway, resulting in conversion of pro-IL-18 to the mature form, which was released into plasma. Inhibitors of caspase-1, reactive oxygen species, and P38 mitogen-activated protein kinase (MAPK) suppressed stress-induced accumulation of plasma IL-18. These inhibitors also blocked stress-induced IL-6 expression. This, together with the observation that IL-6 was not induced in IL-18-deficient mice, showed that IL-6 induction by stress is dependent on IL-18. In stressed organisms, IL-18 may influence pathological and physiological processes. Controlling the caspase-1 activating pathway to suppress IL-18 levels may provide preventative means against stress-related disruption of host defenses.

Introduction

Psychological and/or physical stresses affect host defenses comprising neuronal, endocrine, and immune systems (Dugue et al., 1993; Kiecolt-Glaser et al., 2003). A variety of cytokines (e.g., IL-1 β, IL-6, and TNF-α) are upregulated by stresses (Dugue et al., 1993; Mekaouche et al., 1994; Zhou et al., 1993), suggesting that the cytokine may be involved in the interference with host defenses (Schubert et al., 1999; Walker et al., 1997; Zorzon et al., 2001). However, molecular mechanisms by which stresses induce cytokines that impair host defenses are not well understood.

IL-18 was originally discovered as an interferon-γ (IFN-γ)-inducing factor (Okamura et al., 1995; Ushio et al., 1996). Subsequent studies have demonstrated that IL-18 has multiple biological activities (for review see Dinarello et al. [1998], Nakanishi et al. [2001a], Nakanishi et al. [2001b], and Okamura et al. [2003]), including induction of Fas ligand, elevation of cytolytic activity of T cells (Nakanishi et al., 2001a), and production of Th2 cytokines (Hoshino et al., 1999; Yoshimoto et al., 2000). IL-18 also activates Toll-like receptor 2 (TLR2; Blease et al., 2001) and myeloid differentiation protein 88 (MyD88; Adachi et al., 1998), which are required for induction of IL-6 (Takeuchi et al., 2000), suggesting that IL-18 may play a role in IL-6 expression. Thus, IL-18 is involved in the production of both Th1 and Th2 cytokines (Hoshino et al., 1999; Dinarello et al., 1998; Nakanishi et al., 2001a; Nakanishi et al., 2001b; Okamura et al., 2003).

IL-18 is produced as a 24 kD precursor, which is processed to an 18 kD mature, bioactive form by caspase-1 (IL-1β-converting enzyme, ICE, [Ghayur et al., 1997; Gu et al., 1997]). Caspase-1 is induced in the form of an inactive precursor, procaspase-1, which is activated by caspase-11 (Wang et al., 1998). Caspase-11 is produced as inactive procaspase-11, which is activated by cathepsin B (Schotte et al., 2000). Expression of caspase-11 mRNA has been reported to require NF-κB transactivation into the nucleus (Li et al., 2002; Schauvliege et al., 2002), which is mediated by P38 MAPK (Vanden Berghe et al., 1998).

Conti et al. have shown that IL-18 mRNA is expressed in response to ACTH and cold stress in the adrenal gland (Conti et al., 1997). Sugama et al. have reported differential IL-18 promoter usage in the adrenal gland and immune cells with adrenal gland-specific expression of IL-18 mRNA by ACTH, suggesting that IL-18 may be induced in the adrenal gland during stress (Sugama et al., 2000). They have also detected pro-IL-18, but not mature IL-18 proteins, in the adrenal gland and in plasma in ACTH-administrated mice. On the other hand, plasma IL-18 levels have been reported to be elevated in psychiatric patients (Kokai et al., 2002). It is unclear whether psychological/physical stresses are responsible for high levels of mature IL-18 in plasma.

In this study, the expression of IL-18 in immobilization-stressed mice was investigated, focusing on the processing of pro-IL-18 and the release of the mature form into plasma. In addition, involvement of IL-18 in the regulation of another stress-related cytokine, IL-6 (Dugue et al., 1993; Kiecolt-Glaser et al., 2003), was examined. We found that the stress caused an accumulation of pro-IL-18 and induction of the P38 MAPK-caspase-11-caspase-1 cascade in the adrenal gland, leading to the processing of pro-IL-18 in the adrenal gland and the release of mature IL-18 into plasma. We

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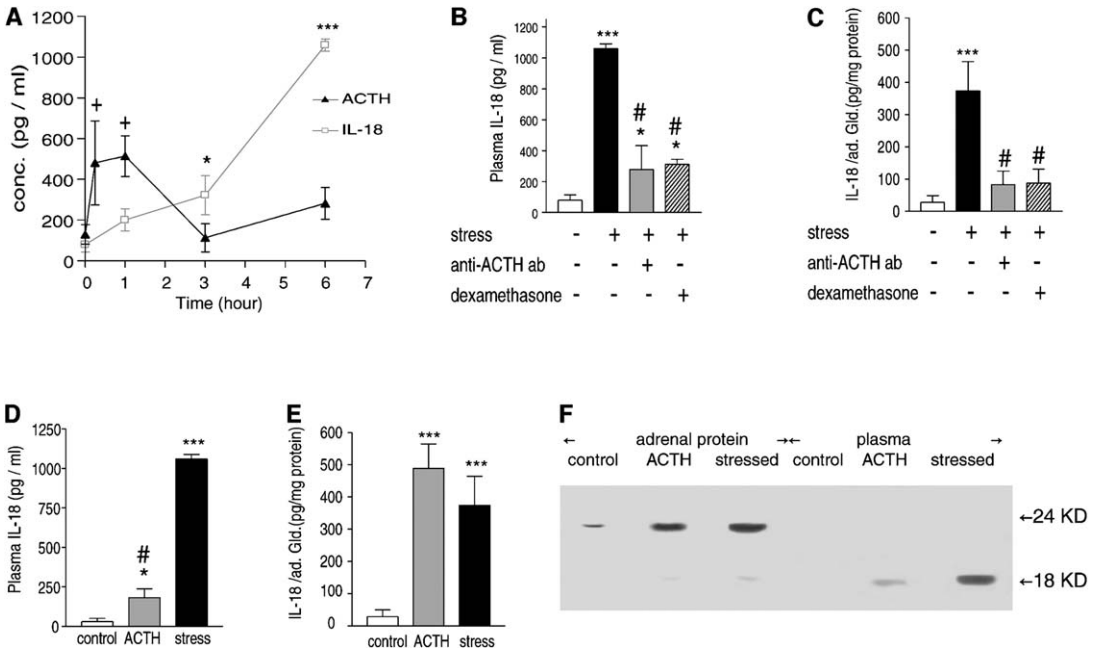

Figure 1. Immobilization Stress Elevates IL-18 Levels in Plasma and the Adrenal Gland

IL-18 and ACTH levels in plasma after stress (A). Effects of anti-ACTH antibody and dexamethasone on IL-18 in plasma (B) and in the adrenal gland (C) in stressed mice. Effects of ACTH and stress on IL-18 in plasma (D) and in the adrenal gland (E). IP-blot for IL-18 protein in the adrenal gland and plasma (F). *p < 0.05, **p < 0.01, and ***p < 0.001 to control; #p < 0.01 to stressed; n = 4–9. Data were presented as ± SD.

also show that IL-6 in plasma is elevated in stressed mice in an IL-18-dependent manner.

Results

Plasma IL-18 Protein Levels in Immobilization-Stressed Mice

Analysis of the effect of immobilization stress on IL-18 expression showed that there were increases in plasma IL-18 levels from 43 ± 34.3 pg/ml before the stress to 330 ± 64.3 and 1059 ± 30.022 pg/ml 3 and 6 hr after the stress, respectively (Figure 1A). ACTH in plasma increased more rapidly, reaching the maximal level (about 500 pg /ml) within 1 hr, but then decreased to the original level at 3 hr (Figure 1A). Thus, elevation of ACTH levels in plasma, which represents activation of the hypothalamus-pituitary-adrenal (HPA) axis, preceded the increase in IL-18 levels in plasma.

It has been reported that ACTH caused adrenal gland-specific expression of IL-18 mRNA (Sugama et al., 2000). We, therefore, studied whether ACTH is involved in the stress-induced elevation of plasma IL-18. Mice were treated with anti-ACTH antiserum or dexamethasone and analyzed for IL-18 levels in plasma and in the adrenal gland. We found that anti-ACTH antiserum and dexamethasone blocked stress-induced increases in IL-18 levels in both plasma (Figure 1B) and the adrenal gland (Figure 1C). In hemiadrenalectomized mice, plasma IL-18 levels after stress were lower than in sham-operated mice (data not shown). These results suggest that increases in IL-18 levels in plasma and the adrenal gland were caused by ACTH induced by the stress.

Next, effects of ACTH on IL-18 levels in plasma and in the adrenal gland were investigated. ACTH administration was found to cause elevation of plasma IL-18 levels but to a much lesser extent than the stress (Figure 1D). On the other hand, IL-18 in the adrenal gland in ACTH-treated mice was elevated as much as in stressed mice (Figure 1E). These results suggest that ACTH is primarily responsible for the elevation of IL-18 levels in the adrenal gland, but additional factors are required for elevating plasma IL-18 levels in stressed mice.

Western blot analysis showed that IL-18 was present in the form of 18 kD mature protein in plasma (Figure 1F, lane 1–3), whereas it was mainly in the form of 24 kD pro-IL-18 in the adrenal gland (Figure 1F, lane 4–6). These results suggest that pro-IL-18 in the adrenal gland is processed and released into plasma in stressed mice.

Induction of Caspase-1 by Stress

Western blot analysis of caspase-1 showed that procaspase-1 was induced by ACTH and the stress in the adrenal gland (Figure 2A). Caspase-1 activity in the adrenal gland was elevated 10-fold by the stress (Figure 2B). YVAD-CHO, a caspase-1 inhibitor, effectively inhibited the stress-induced increase in mature IL-18 levels in plasma (Figure 2C), but not in pro-IL-18 levels in the adrenal gland (Figure 2D). In caspase-1 knockout (KO) mice, no IL-18 was detectable in plasma before and after the stress, although pro-IL-18 levels were elevated after the stress (data not shown). These results indicated that the stress induced caspase-1 in the adrenal gland, which is responsible for the processing

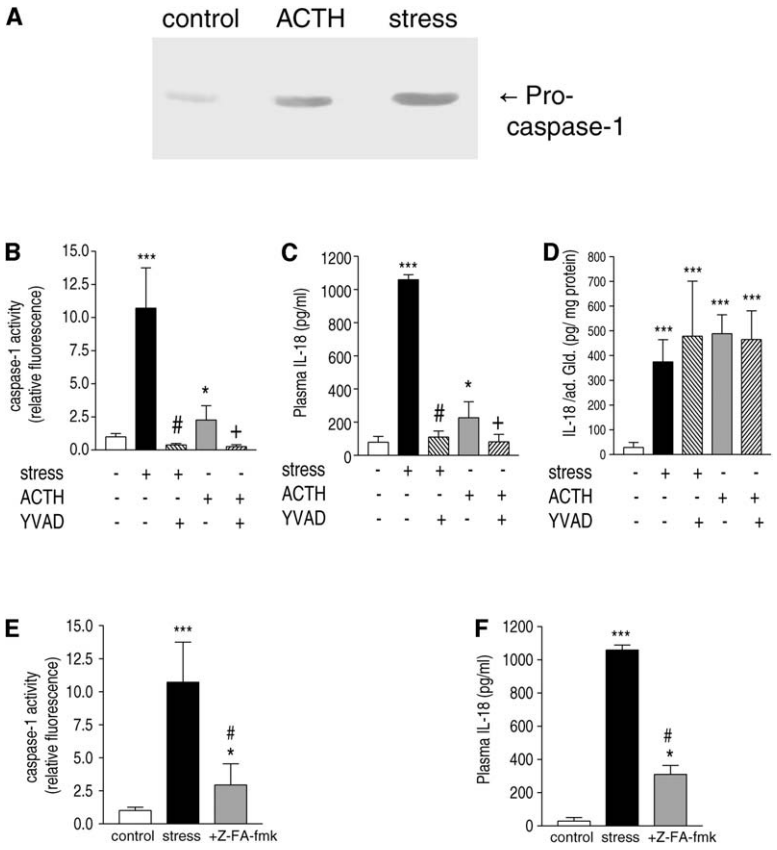

Figure 2. Caspase-1 Is Essential for the Stress-Induction of IL-18 in Plasma

Western blot of procaspase-1 in the adrenal gland (A). Effects of stress on caspase-1 activity in the adrenal gland (B). Effects of YVAD-CHO on IL-18 levels in plasma (C) and the adrenal gland (D). Effects of Z-FA-fmk on caspase-1 activity (E) and plasma IL-18 levels (F). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ to control; #$p < 0.01$ to stressed; +$p < 0.05$ to ACTH treated; n = 4–9. Data were presented as ± SD.

of pro-IL-18 in the adrenal gland and the elevation of IL-18 levels in plasma.

Effect of Caspase-11 Inhibition on the Caspase-1 Activation

Because caspase-1 has been reported to be activated by caspase-11 (Wang et al., 1998), and caspase-11 activation requires cathepsin-B (Schotte et al., 2000), the effect of the cathepsin-B inhibitor Z-FA-fmk and anti-caspase-11 antibody on caspase-1 activity in the adrenal gland and IL-18 levels in plasma was investigated. The results showed that both of them suppressed stress-induced increases in caspase-1 activity in the adrenal gland (Figure 2E) and IL-18 levels in plasma (Figure 2F), whereas controls (e.g., control IgG) did not, indicating that caspase-11 is involved in the stress-induced activation of caspase-1 in the adrenal gland.

Effect of P38 MAPK Inhibition on the Caspase-1 Activation

It has been shown that SB203580, a P38 MAPK inhibitor, suppresses induction of caspase-11 in C6 glioma cells in vitro (Hur et al., 2001). We examined whether caspase-11 induction in the adrenal gland is mediated by P38 MAPK by using another P38 MAPK inhibitor, SB202190, reported to be more specific than SB203580 and anti-phosphorylated P38 MAPK antibody. We found that SB202190 inhibited the stress-induced increase in procaspase-11 levels (Figure 3A), caspase-1 activity in the adrenal gland (Figure 3B), and IL-18 levels in plasma (Figure 3C). Similar inhibition was observed with anti-phosphorylated P38 MAPK antibody, whereas no inhibition was seen with IgG used as a control (Figures 3A–3C). PD 98059, a mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) inhibitor did not significantly affect P38 MAPK phosphorylation (data not shown) and caspase-11 induction (Figure 3A). Neither YVAD-CHO nor Z-FA-fmk affected phosphorylation of P38 MAPK in the adrenal gland (Figure 3D). Phosphorylation of P38 MAPK was induced by the stress in caspase-1 KO mice (Figure 3D, lane 5) as in wild-type (wt) mice (Figure 3D, lane 2), indicating that the phosphorylation of P38 MAPK was independent of caspase-1.

We conducted immunohistochemical localization of IL-18, caspase-1, caspase-11, and activated P38 MAPK in the adrenal gland. In stressed mice, these proteins were colocalized in the zona reticularis of the adrenal cortex (Figure 3E), but in control mice, none of them was detected (data not shown). The inhibitors of caspase-1 (Figure 3F, panel 1), caspase-11 (Figure 3F, panel 2), and anti-caspase-11 antibody (data not shown) had no effect on the accumulation of phosphorylated P38 MAPK in the adrenal cortex. These results suggested that P38 MAPK is involved in stress-induced elevation of plasma IL-18 levels, possibly via induction of caspase-11, which in turn activates caspase-1.

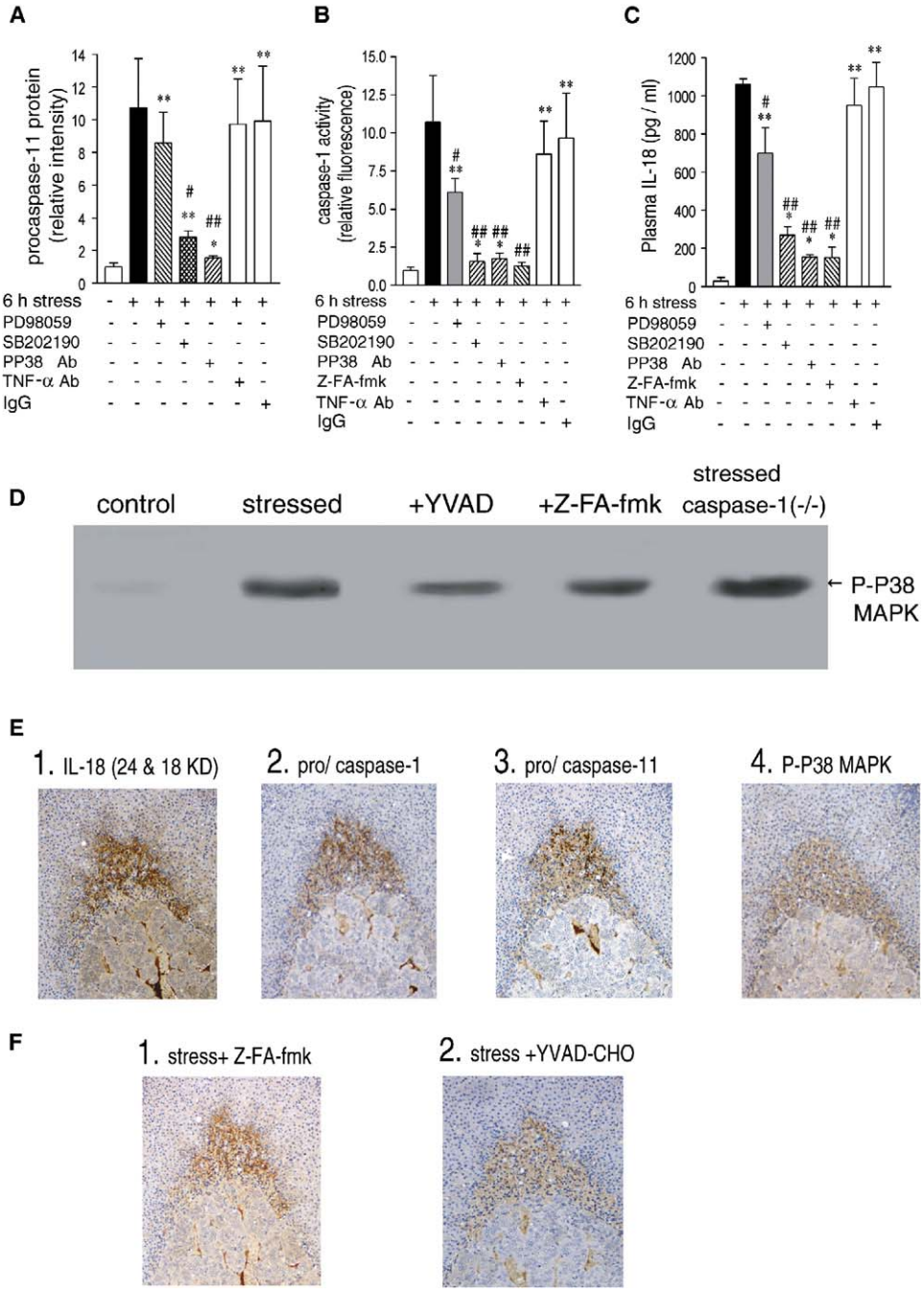

Figure 3. Caspase-1 Activation Is Suppressed by P38 MAPK Inhibitors

Effects of P38 MAPK inhibition on the expression of 43 kD caspase-11 precursor (A). Effect of P38 MAPK inhibition on caspase-1 activity (B) and plasma IL-18 levels (C). P38 MAPK inhibitors and Z-FA-fmk, a caspase-11 inhibitor, suppressed both levels. IP-blot analysis of activated P38 MAPK in the adrenal gland (D). Lane 1, no stress; lane 2, stressed; lane 3, +YVAD-CHO; lane 4, +Z-FA-fmk; and lane 5, stressed caspase-1 KO mice. Induction of IL-18 (panel 1), caspase-1 (panel 2), caspase-11 (panel 3), and activated P38 MAPK (4) in zona reticularis of the cortex of the adrenal gland in stressed mice (E). Expression of activated P38 MAPK in the presence of caspase inhibitors. Z-FA-fmk (panel 1) and YVAD-CHO (panel 2) in stressed mice (F). *$p < 0.05$ and **$p < 0.01$ compared to control; #$p < 0.01$ and ##$p < 0.001$ to stressed; n = 6. Data were presented as ± SD.

Involvement of Superoxide in the Upregulation of P38 MAPK

Reactive oxygen species have been shown to mediate P38 MAPK phosphorylation in lung fibroblasts (Han et al., 2003), neurtophils (Nick et al., 1997), and monocytes (Guha et al., 2000). We examined the effect of superoxide-dismutase (SOD) on P38 MAPK phosphorylation in stressed mice. SOD was found to inhibit P38 MAPK phosphorylation about 70% in the adrenal gland (Figure 4A).

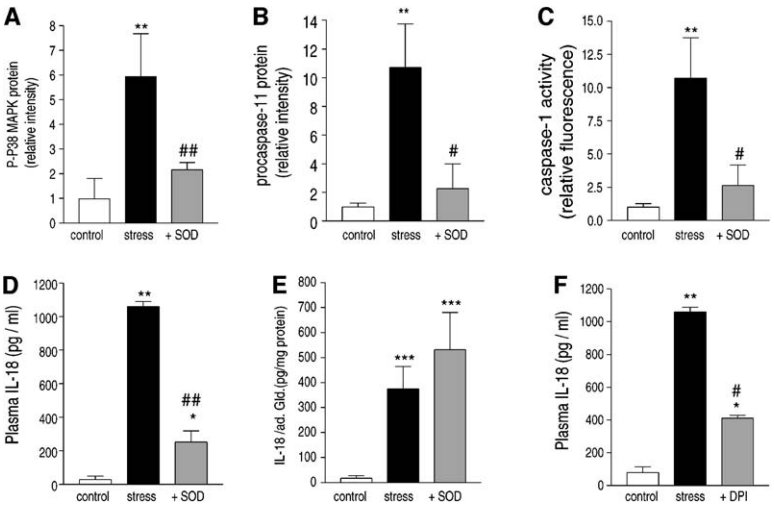

Figure 4. Superoxide Anion Is Involved in P38 MAPK Activation

Effect of SOD on levels of activated P38 MAPK protein (A), 43 kD procaspase-11 (B), caspase-1 activity (C), plasma IL-18 (D), and IL-18 precursor (E) in the adrenal gland. Effect of DPI, a NADPH oxidase inhibitor, on IL-18 levels in plasma (F). *p < 0.01 and **p < 0.001 to control; #p < 0.05 and ##p < 0.01 to stressed; n = 6. Data were presented as ± SD.

SOD also reduced levels of procaspase-11 (Figure 4B), caspase-1 activity (Figure 4C), and plasma IL-18 (Figure 4D), but not the adrenal pro-IL-18 (Figure 4E) in stressed mice. Stress induction of plasma IL-18 was inhibited by diphenyleneiodnium (DPI), a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor (Figure 4F), supporting the notion that superoxide anions are involved in stress induction of plasma IL-18 via P38 MAPK phosphorylation. Similar results were obtained with excised adrenal gland incubated with paraquat (data not shown).

Involvement of IL-18 in the Regulation of Plasma IL-6 Levels in Stressed Mice

The relationship between IL-18 and IL-6 was examined by using IL-18-deficient mice. Without the stress, IL-6 was undetectable in wt and IL-18 KO mice, but after 3 hr of the immobilization stress, IL-6 levels reached 230 pg/ml in wt mice and 25 pg/ml in IL-18 KO mice (Figure 5A). After release from the stress, plasma IL-6 remained at around 80 pg/ml in wt mice, whereas it decreased to 5 pg/ml in IL-18 KO mice (Figure 5A). Treatments with YVAD-CHO, SOD, anti-IL-18 antibody, or anti-IL-18 receptor chains antibodies resulted in 70% inhibition of stress-induced increases in plasma IL-6 levels in wt mice (Figure 5B). These results showed that the stress induces IL-6 in plasma in an IL-18-dependent manner.

Discussion

In this study, we showed that the acute immobilization stress caused increases in 24 kD pro-IL-18 levels in the adrenal gland and 18 kD mature IL-18 levels in plasma. These increases were preceded by elevation of ACTH levels in plasma, suggesting that ACTH is involved in the elevation of pro and mature IL-18. This was supported by the finding that administration of anti-ACTH antiserum or dexamethasone to block ACTH release resulted in suppression of the increases in both pro and mature IL-18 levels in stressed mice. These results indicated that the immobilization stress induces ACTH, which in turn induces pro-IL-18 in the adrenal gland.

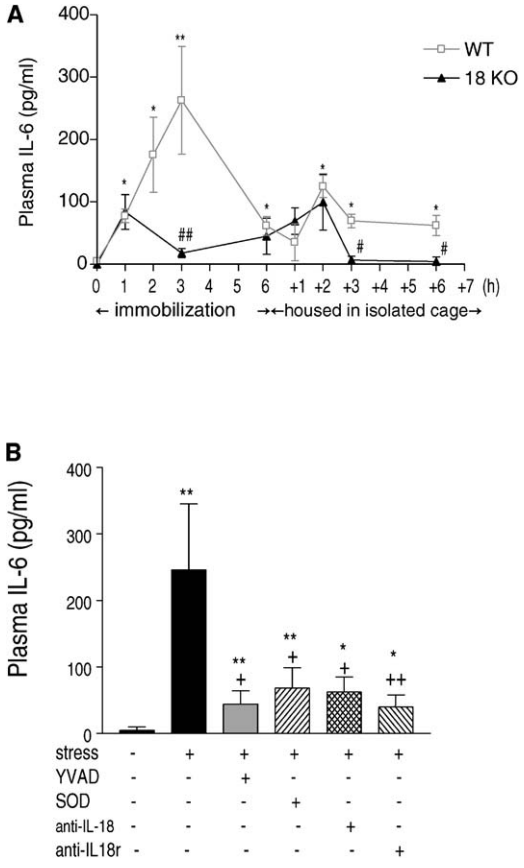

Figure 5. Stress Induction of IL-6 in Plasma Is IL-18 Dependent

(A) Plasma IL-6 levels in stressed wild-type (wt) and IL-18-deficient mice.

(B) Effect of YVAD-CHO, a caspase-1 inhibitor, SOD, anti-IL-18 antibody, and anti-IL-18 receptor chains antibodies mixture (10 μg each) on plasma IL-6 levels in stressed wt mice. *p < 0.05 and **p < 0.01 to control; #p < 0.05 and ##p < 0.01 to wt at each time point; and +p < 0.01 and ++p < 0.001 to stressed wt; n = 6.

Data were presented as ± SD.

It has been reported that caspase-1 precursor is induced in the adrenal gland by lipopolysaccharide (LPS) (Tingsborg et al., 1997). In this study, we showed that the immobilization stress also induces casapse-1 precursor and active caspase-1. YVAD-CHO blocked the elevation of mature IL-18 in plasma, but not the induction of pro-IL-18 in the adrenal gland. In stressed caspase-1 KO mice, plasma IL-18 was not detectable, although pro-IL-18 protein was upregulated in the adrenal gland (data not shown). These results indicated that stress-induced caspase-1 is responsible for the processing of pro-IL-18 in the adrenal gland and the accumulation of mature IL-18 in plasma.

Caspase-11 has been shown to activate caspase-1 (Wang et al., 1998). In the present study, procaspase-11 was induced in the adrenal gland by the stress, and the inhibitor of caspase-11 Z-FA-fmk and anti-caspase-11 antibody blocked the activation of caspase-1 by the stress, indicating the involvement of caspase-11 in caspase-1 activation in stressed mice. In LPS-injected mice, IL-18 and IFN-γ induction by LPS was suppressed by caspase-11 inhibition (data not shown). Together, it is suggested that caspase-11 is involved in IL-18 conversion (activation) in stress as well as in endotoxemia.

We found that SB202190, a P38 MAPK inhibitor and anti-phosphorylated-P38 MAPK antibody blocked the stress-induced increases in procaspase-11 levels, caspase-1 activity, and plasma IL-18 levels. PD98059, an MEK inhibitor, showed no affection on the caspase-11 induction and lesser suppression on caspase-1 activity and IL-18 level than P38 MAPK inhibitors. YVAD-CHO, the caspase-1 inhibitor, and Z-FA-fmk, the caspase-11 inhibitor, suppressed stress-induced elevation of plasma IL-18 levels without affecting phosphorylation of P38 MAPK. Stress-induced phosphorylation of P38 MAPK occurred in caspase-1 KO mice as well as in wt mice. P38 MAPK, caspase-11, caspase-1, and pro-IL-18 proteins were accumulated in an overlapping manner in zona reticularis in the adrenal cortex, possibly forming a caspase-1 activating pathway to affect conversion of pro-IL-18 in the adrenal gland to the mature form to be released into plasma in stressed mice. P38 MAPK involvement in caspase-1 activation via caspase-11 is demonstrated. On the other hand, PD 98059, a MEK inhibitor, showed lesser suppression on the caspase-1 activation and IL-18 rise than P38 MAPK inhibitors. Combined treatment with Z-FA-fmk resulted in further downregulation, suggesting an ERK1/2-mediated, caspase-11-independent pathway for the stress-induced caspase-1 activation. Recently, Guo et al. reported that PD 98059 inhibited caspase-1 activation by Fas ligation in dendritic cells, discussing the involvement of ERK1/2 (Guo et al., 2003). Together, it is suggested that stress activates caspase-1 mainly via P38 MAPK/caspase-11 and subsidiary via ERK1/2 pathways.

It has been reported that reactive oxygen species participate in P38 MAPK activation (Guha et al., 2000; Han et al., 2003; Nick et al., 1997). In the present study, SOD inhibited the activation of P38 MAPK (Figure 4A), induction of caspase-11, activation of caspase-1 in the adrenal gland, and the elevation of IL-18 levels in plasma (Figures 4B–4D). However, induction of pro-IL-18 in the adrenal gland was not affected by SOD (Figure 4E). These results suggest that the stress induces superoxide anions, which activate the P38 MAPK cascade.

In our preliminary experiment using paraquat on the adrenal gland excised from ACTH administrated mice, superoxide-dependent P38 MAPK activation, procaspase-11 induction, upregulation of caspase-1 activity, and IL-18 conversion were observed. P38 MAPK inhibitors, but not PD98059, suppressed the procaspase-11 induction and subsequent events, and Z-FA-fmk inhibited the upregulation of caspase-1 activation, but not P38 MAPK phosphorylation, suggesting that the superoxide-induced caspase-1 activation is mainly mediated by P38 MAPK and caspase-11 and that P38 MAPK is involved in caspase-11 induction.

Thus, the process in which IL-18 is generated in plasma in stressed mice may be summarized as follows: stress activates the HPA axis to induce ACTH, which induces pro-IL-18 and procaspase-1 in the adrenal cortex. Stress also generates superoxide anions that induce P38 MAPK phosphorylation (Thr180/Tyr182), which induces caspase-11, which in turn activates caspase-1. Independently, a ERK1/2 pathway subsidiary participates in caspase-1 activation. Caspase-1, thus activated, converts pro-IL-18 in the adrenal gland into the mature form, which is secreted into plasma.

IL-18 activates TLR2 (Blease et al., 2001) and MyD88 (Adachi et al., 1998), which are required for IL-6 induction (Takeuchi et al., 2000), suggesting that IL-18 might induce IL-6. In this study, this possibility was supported by the following results: (1) stress elevated plasma IL-6 levels in wt mice, but not in IL-18 KO mice, (2) YVAD-CHO, a caspase-1 inhibitor, or SOD blocked the stress-induced elevation of IL-18 and IL-6 in plasma, and (3) anti-IL-18 antibody suppressed IL-6 levels in stressed mice. In contrast, IL-18-deficient mice showed 10% reduction in IL-6 induction by LPS (data not shown). Together, our results demonstrated that immobilization stress elevates IL-6 levels in plasma in an IL-18-dependent pathway.

Glucocorticoids (GCs) have negative and positive effects with a dynamic and bidirectional spectrum of activities on immune response. Production of IFN-α and -γ, IL-12, and TNF-α is suppressed but that of IL-4, IL-10, and IL-13 is upregulated by GCs (for review, see Elenkov [2004]). GCs have also been reported to modulate genes involved in the priming of the innate immunity response (for review, see Franchimont [2004]). In the present study, dexamethasone inhibited the induction of pro-IL-18 via the suppression of ACTH release (Figures 1B and 1C). Immobilization of mice resulted in elevation of corticosterone levels in plasma to about 400 pg/ml (data not shown), whereas IL-18 conversion via the caspase-1 pathway was maintained in the adrenal gland, indicating that GCs do not inhibit the caspase-1 activating pathway. When mice were released from the restrainer, corticosterone levels declined, but IL-18 levels were sustained (data not shown), likely due to a longer half-life of IL-18 than that of GCs. It is suggested that IL-18 may act on cells primed by GCs and is involved in allo- and/or homeostatic responses.

It has been reported that plasma IL-18 levels in patients with autoimmune and inflammatory diseases (Nakanishi et al., 2001a; Nakanishi et al., 2001b; Oka-

mura et al., 2003), including rheumatoid arthritis (RA) (Walker et al., 1997), systemic lupus erythematosus (Schubert et al., 1999), and multiple sclerosis (Zorzon et al., 2001), are elevated after psychological and/or physical stresses. In RA patients, neutrophil has been shown to produce several cytokines, including TNF-α, in response to IL-18 (Leung et al., 2001), suggesting that IL-18 may activate inflammatory processes in autoimmune patients. Bossu et al. have reported that IL-18 cDNA vaccination ameliorates spontaneous lupus-like nephritis in MRL/lpr mice (Bossu et al., 2003), indicating that IL-18 is involved in autoimmune pathology. This raises the possibility that stress-induced exacerbation of inflammatory and autoimmune diseases may be suppressed by regulating levels of mature IL-18 and superoxide anions. Thus, controlling the caspase-1 cascade may serve as a means to prevent host-defense disruption caused by psychological/physical stressors.

Experimental Procedures

Animals

Male C57B6 mice were purchased from Seac Yoshitomi (Japan). IL-18 KO mice (C57/B6 background) (Takeda et al., 1998), and caspase-1 KO mice (Balb/C background) (Kuida et al., 1995) were kindly provided by Dr. K. Takeda and Dr K. Kuida, respectively. Mice were housed one per cage at 20°C. Experiments involving mice were conducted starting at 10 a.m.

Chemicals and Reagents

Anti-ACTH antiserum was obtained from Progen Biotechnik, Heidelberg, Germany; anti-mouse IL-18 monoclonal antibody from MBL, Japan; anti-mouse caspase-1 antibody (sc-515) from Santa Cruz, USA; anti-mouse/rat caspase-11 antibody from BIOMOL, USA; anti-phosphorylated (Thr180/Tyr182) P38 MAPK antibody from Cell Signaling Technology, USA; anti-TNF-α antibody from Alexis, USA; and normal rabbit IgG from Vector, UK. Affinity-purified polyclonal IgGs against mouse IL-18 receptor α and β chains were raised in rabbits inoculated with synthetic peptides of hydrophilic sequences near the putative transmembrane domains of IL-18 receptor α chains (Parnet et al., 1996) and β chains (Born et al., 1998), respectively. Synthetic ACTH (1–24 amino terminus) was purchased from Peninsula Laboratories Inc. (USA). Ac-YVAD-CHO and Ac-YVAD-MCA were purchased from Peptide Institute (Japan), Z-FA-fmk and SB202190 from Calbiochem-Novabiochem (San Diego, USA), and PD98059 (IC50 values are 1–20 μM for MAPK/ERK kinase or MEK) from TOCRIS (UK). Diphenyleneiodonium chrolide (DPI), a NADPH oxidase inhibitor and SOD were purchased from Sigma (St. Louis, USA). Z-FA-fmk, which has been reported to show affection on cathepsin B (Schotte et al., 2000), but not on caspases (Sillence and Allan, 1997; McColl et al., 1998; Gregoli and Bondurant, 1999), was used as a cathepsin-B inhibitor, which in turn results in selective suppression on caspase-11 (Schotte et al., 2000). SB202190 was employed as a P38 MAPK inhibitor, which binds with the ATP pocket of the active kinase to inhibit the P38 α and β isoforms selectively (IC50 values are 50 and 100 nM for p38α/SAPK2α and p38β2/SAPK2β, respectively [Frantz et al., 1998; Manthey et al., 1998]).

Immobilization Stress

For the immobilization stress, mice were placed in a restrainer (a rounded plastic tube 27 mm in diameter) for 1, 3, or 6 hr, and IL-18 levels were analyzed. For analysis of IL-6, mice were immobilized as described above and then housed in the original cage for 6 hr prior to assaying IL-6.

Assays of Cytokines

After the immobilization, mice were anesthetized with diethyl ether, and blood was taken into EDTA-containing tubes. Plasma was separated by centrifugation at 10,000 × g for 10 min. at 4°C and stored at –80°C. IL-18 and IL-6 were analyzed by ELISA using Quantikine immunoassay kit (R&D Systems) and OptEIA (BD-Pharmingen, CA, USA), respectively.

Treatment with Anti-ACTH Antiserum, Dexamethasone, ACTH, and Anti-IL-18 Antibody

Mice were intraperitonealy injected with anti-ACTH antiserum (50 μl, 1:1) or dexamethasone (1 ng/50μl PBS[–]) or anti-IL-18 antibody (50 μl, 1:1) and 10 min later placed in the immobilization tube for 6 hr. Synthetic ACTH (250 μg/50 μl PBS[–]) was injected to the left limb, and 6 hr later, mice were subjected to experiments.

Treatment with Caspase-1 Inhibitor, Caspase-11 Inhibitor, P38 MAPK Inhibitors, Anti-Phosphorylated-P38 MAPK Antibody, MEK Inhibitors, SOD, and DPI

Mice were placed in the restrainer for 1 hr and injected with Ac-YVAD-CHO (200 μM/50 μl PBS[–]), Z-FA-fmk (100 μM/50 μl PBS[–]), and SB202190 (2 μg/50 μl PBS[–] per mouse), PD98059 (20 μg/50 μl PBS[–] per mouse), anti-phosphorylated-P38 MAPK antibody (2 μl/50 μl PBS[–] per mouse), anti-TNF-α antibody (4 μl/50 μl PBS[–] per mouse), or normal rabbit IgG (4 μl/50 μl PBS[–] per mouse) intraperitonealy. SOD (20 U) and DPI (30 μg) were injected into mice intraperitoneally 1 and 3 hr after the start of immobilization.

Immunoprecipitation and Western Blotting

For Western blot analysis of IL-18 and phosphorylated P38 MAPK in the adrenal tissue, the tissue was homogenized by a Polytron homogenizer in immunoprecipitation (IP) buffer (50 mM Tris, [pH 7.4], 150 mM NaCl, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate) with a protease inhibitor mixture (Roche Diagnostics Japan). The homogenate (100 μg of proteins) was precleared by protein A/G agarose (Exalpha Biologicals, Inc., USA), incubated with anti-mouse IL-18 monoclonal antibody (MBL) or anti-phosphorylated (Thr180/Tyr182) P38 MAPK antibody (Cell Signaling Technology) with the protein A/G agarose (50 μl), and centrifugated (13,000 × g for 25 min., 4°C). The pellet was suspended in loading buffer and boiled to retrieve adrenal proteins, electrophoresed in a 10%–20% SDS-polyacrylamide gradient gel, and electrotransferred on Hybond ECL (Amersham) nitrocellulose membrane. Membranes were incubated with antibody against IL-18 (MBL), phosphorylated (Thr180/Tyr182) P38 MAPK (Cell Signaling Technology), caspase-1 (Santa Cruz, USA), or caspase-11 (BIOMOL, USA), and reacted proteins were detected by ECL plus system (Amershan biosciences). Procaspase-11 and activated P38 MAPK were quantified by using the Science Lab 99 image-analysis system (Fujifilm, Japan).

Immunohistochemistry

Adrenal glands were formalin fixed, paraffin embedded, and cut to 4 μm sections. They were incubated with antibodies to P38 MAPK, caspase-11, caspase-1 (1:200 in all cases), or IL-18 (1:400). Horseradish peroxidase-conjugated secondary antibody (Santa Cruz, USA) and diaminobenzidine were used to develop color. Sections without primary antibody and sections from no-stressed mice were employed as control.

Analysis of Caspase-1 Activity

Caspase-1 activity in the adrenal gland was measured by using a caspase assay kit (BD-Pharmingen, CA, USA). In brief, adrenal tissue homogenates were incubated with a caspase-1 fluorogenic substrate, Ac-YVAD-AMC, and AMC liberated from Ac-YVAD-AMC was measured by using a fluorometric plate reader with an excitation wavelength of 380 nm and an emission wavelength of 420–460 nm.

Statistical Analysis

All data were presented as the mean ± SD from at least three independent experiments. Statistical comparisons between different treatments were done by Student's t test using the GraphPad Prism program (GraphPad Software Inc., USA). p values less than 0.05 were considered statistically significant.

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