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Extract of Reishi Polysaccharides Induces Cytokine Expression via TLR4-Modulated Protein Kinase Signaling Pathways

Hsien-Yeh Hsu,2* Kuo-Feng Hua,* Chun-Cheng Lin,†‡ Chun-Hung Lin,†§ Jason Hsu,¶ and Chi-Huey Wong†‡§

We have demonstrated that an extract of *Ganoderma lucidum* (Reishi or Ling-Zhi) polysaccharides (EORP) exerts immunomodulating activities by stimulating the expression of inflammatory cytokines from mouse spleen cells. Interestingly, via responding to LPS in genetic variation of murine macrophage HLeNC2 and GG2EE cell lines, and using TLR4 Ab blockade in human blood-derived monocyctic macrophages, we have found that the TLR4, but not complement receptor type 3, is a putative receptor of EORP, mediating the consequent immunomodulating events associated with IL-1 gene expression. Based on our studies of reactive oxygen species production, polymyxin B inhibition, and protein tyrosine kinase (PTK) activity, we ruled out the possibility of LPS contamination in EORP. We have found that EORP differentially modulates the protein kinase (PK)-mediated signal transduction pathways associated with inflammatory cytokine IL-1. In human macrophages and murine macrophage J774A.1 cells, EORP was found to up-regulate IL-1 secretion and pro-IL-1 (precursor of IL-1) as well as IL-1-converting enzyme expression. Specifically, EORP rapidly stimulates PTK-mediated phosphorylation, followed by induction of PKs and activation of MAPKs: ERK, JNK, and p38. Using PK inhibitors in the kinase activity assays, Western blot analyses and IL-1 ELISA, we have extensively examined and dissected the role of individual PK in the regulation of pro-IL-1 and IL-1. Our findings establish that EORP-mediated signaling pathways are involved in the pro-IL-1 and IL-1 regulation: PTK/protein kinase C/MEK1/ERK and PTK/Rac1/p21-activated kinase/p38. *The Journal of Immunology*, 2004, 173: 5989–5999.

In the oldest Chinese medical text, “Shen-Nong Ben Cao Jin,” *Ganoderma species* (a group of traditional fungal medicinal) is recorded as the king of herbs. *Ganoderma lucidum* (Reishi or Ling-Zhi) has been used as traditional Chinese medicine for promoting good health, perpetual youth, vitality, and longevity (1–3). In the animal studies, the crude or partially purified polysaccharides (PS)1 of *G. lucidum* were reported to exhibit antitumor activity as well as reduce tumor metastasis (4–8). The active constituents responsible for the antitumor and immunomodulating activities have been qualitatively described and the structure has been partially determined (9), but the molecular mechanisms of their actions have not been clearly elucidated.

The role of PS in stimulation of certain cells has been examined and discussed, although the results were diverse. The antitumor effect of PS on human myeloid leukemia cell lines, HL-60 and U937, has been studied (6), and the results support PS-mediated induction of IL-1, IL-6, and TNF-α from activated macrophages, and IFN-γ from activated T lymphocytes. Our previous finding showed that a glycoprotein fraction, isolated from water-soluble Reishi, could stimulate spleen cell proliferation and expression of cytokines, including IL-1, IL-2, and IFN-γ (1). The composition of this glycoprotein fraction has been determined, and the presence of fucose has been confirmed to be important for the activities (1).

Recent studies have established the central role of TLRs on macrophages in response to a wide variety of microbial pathogens with subsequent induction of cytokine activation. Mammalian cells contain TLRs with homology to *Drosophila* Toll and/or complement receptor type 3, NF-κB, and downstream MAPKs (12). This sequential signaling process stimulates the expression of genes that regulate the adaptive immune responses, such as IL-1, TNF-α, and IL-6, etc. Extensive investigations of TLR in response to bacteria have been replaced; however, much less is known with regard to fungi (13, 14). In addition to TLR signaling in response to fungal cell wall components (13), Kan and Bennett (15, 16) have shown that the β-glucan receptor and/or mannose/siucosyl receptors on human monocytes and mouse alveolar macrophages could recognize certain fungi.

There is little information about *G. lucidum* PS-mediated signal transduction, although β-(1→3)-d-glucans have been shown to

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1 Abbreviations used in this paper: PS, polysaccharide; ATF, activating transcription factor; CM-H 2DCFDA, 5(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate, acetyl ester; CR3, complement receptor type 3; EORP, extract of Reishi PS; ICE, IL-1-converting enzyme; LAL, Limulus amebocyte lysate; PAK, p21-activated kinase; PK, protein kinase; PLC, phospholipase C; PMA, phorbolmycin B; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species.

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*Address correspondence and reprint requests to Dr. Hsien-Yeh Hsu, Faculty of Medical Technology, Institute of Biotechnology in Medicine, National Yang-Ming University, 155 Li-Nong Street, Shih-Pai Taipei, Taiwan. E-mail address: hyh@ym.edu.tw

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2 Address correspondence and reprint requests to Dr. Hsien-Yeh Hsu, Faculty of Medical Technology, Institute of Biotechnology in Medicine, National Yang-Ming University, 155 Li-Nong Street, Shih-Pai Taipei, Taiwan. E-mail address: hyh@ym.edu.tw

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elicit the production of cytokines and NO (17–19). In addition, G. lucidum extract was shown to activate MAPKs and mediate the neuronal differentiation and neuroprotection of rat pheochromocytoma PC12 cells (20), perhaps via the Ras/ERK and CREB signal transduction pathways (20). The real carbohydrate epitope responsible for the antitumor activity and its receptor have not been identified, however, although the complement receptor type 3 (CR3) has been shown to bind β-glucan PS with undefined side chains (see Ref. 1 and sections below).

In our previous report, a fucose-containing glycoprotein fraction from an extract of Reishi PS (EORP), i.e., fraction 3, stimulates spleen cell proliferation and cytokine expression (1). To further examine the EORP-mediated immunomodulating activities, we have focused on investigating the mechanism of EORP-induced protein kinase (PK) signaling in the regulation of cytokine expression. Specifically, we have studied the EORP-induced inflammatory cytokine IL-1 gene expression, and elucidated the molecular mechanism of EORP-induced signal transductions in the regulation of pro-IL-1/IL-1 expression. Furthermore, the effect of TLR4 as a candidate receptor for EORP-mediated signaling has been evaluated and examined.

Materials and Methods

Cell cultures

Using Histopaque-1077 method, human blood monocyte-derived macrophages (human macrophages) were isolated from the blood of healthy persons obtained from Taiwan Blood Center (Taipei, Taiwan). Murine macrophage J774A.1 cells (J774A.1 cells) were obtained from American Type Culture Collection (Manassas, VA). HeNC2 and GG2EE were from D. Radziioch (McGill University, Montreal, Canada). All cell cultures were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 2 mM l-glutamine (Invitrogen Life Technologies, Carlsbad, CA) and cultured in a 37°C, 5% CO2 incubator.

Materials

LPS (from Escherichia coli 0111:B4), Histopaque-1077, sodium orthovanadate, PMSF, BSA (fraction V), Limulus amebocyte lysate (LAL) assay, and polymyxin B were purchased from Sigma-Aldrich (St. Louis, MO). Immobilon polyvinylidene difluoride membrane was purchased from Millipore (Bedford, MA). DuPont nonradioactive Western blot chemiluminescence reagent, Renaissance, was purchased from DuPont NEN Research Product (Boston, MA). Mouse IL-1 ELISA kit was purchased from R&D Systems (Minneapolis, MN). RE5, RE6, and RE7 were from ProTech Technology (Taipei, Taiwan). GeneAmp RNA PCR kit for RT-PCR amplification was purchased from PerkinElmer (Branchburg, NJ). Abs: anti-IL-1α, 3ZD mAb (gift from National Institutes of Health, Bethesda, MD, to H.-Y. Hsu); anti-rabbit IgG-HRP and anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phosphotyrosine, clone 4G10 (mouse monoclonal IgG2b), was purchased from Upstate Biotechnology (Lake Placid, NY); monoclonal anti-β-actin Ab, monoclonal anti-p38 MAPK, activated (diphosphorylated p38) Ab, and monoclonal anti-actin Ab were purchased from Sigma-Aldrich; monoclonal anti-human TLR4 was from IMAGEGENE (Carlsbad, CA); and monoclonal anti-human CR3 was from Accurate Chemical & Scientific (Westbury, NY). The CaspACE assay system, Fluorometric, was purchased from Promega (Madison, WI). Kinase assay kits: p44/42 MAPK and p38 MAPK assay kits were purchased from Cell Signaling Technology (Beverly, MA). PK inhibitors: PD98059, Ro-31-8220, SB203580, SP600125, and LY294002 were purchased from Calbiochem-Novabiochem (San Diego, CA). Primers for pro-IL-1/IL-1 and GAPDH were from local MD Bio. (Taipei, Taiwan).

Preparation of EORP

Crude G. lucidum extract PS extract prepared via alkaline extraction with 0.1 N of Tris buffer (pH 7.0) as the eluent. The flow rate was set at 0.6 ml/min, and fractions were collected with 7.5 ml per tube. Five fractions were collected, and each was dialyzed to remove excessive salts and lyophilized to give fractions 1–5; each fraction was characterized, as described (1). In the following studies, the fucose-containing glycoprotein fraction (20–30% yield), i.e., F3 or EORP, was used.

Monitoring of LPS contamination in experiments

We were very aware of the problem of LPS contamination in the studies of EORP-mediated signaling. Specifically, from growth to harvest, the crude G. lucidum materials and PS extracts were prepared as GMP grade from Pharmaxen; the possible bacterial contamination was carefully monitored to meet the Food and Drug Administration standard. The reagents and utensils for preparation of EORP were either endotoxin-free grade or washed with PBS containing 50 μg/ml polymyxin B (PMB), then rinsed with PBS. EORP contained <1 ng of LPS/25 μg, as measured by LAL assay (Sigma-Aldrich). In addition, certain reagents were routinely checked by LAL for examination of LPS contamination.

Measurement of EORP- and LPS-induced intracellular reactive oxygen species (ROS)

Intracellular ROS stimulated by EORP and/or LPS were measured, as described previously (12), by detecting the fluorescent intensity of 5(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Molecular Probes, Eugene, OR).

RNA isolation, RT-PCR for pro-IL-1/IL-1, Bio-Rad (Hercules, CA) protein assay, Western blot analysis, and ELISA for IL-1

Methods were conducted, as described previously (12, 21).

Assays of ERK, JNK, and p38 MAPK (p38) in EORP-treated cells with or without PK inhibitors

Methods for the assay of these PKs, including cell lysate preparation, immunoprecipitation of PK, in vitro PK reaction, analysis of PK activity, and quantification of PK activity, were as described previously (12, 21).

Statistical analysis

Statistical differences between the experimental groups were examined by ANOVA, and statistical significance was determined at p < 0.05. The experiments were conducted three times or as indicated; all data are expressed as mean ± SE.

Results

TLR4 functions as a receptor for EORP and mediates EORP-induced transducing signals

The role of TLRs, including TLR4, in LPS-mediated signaling has been exposed and studied (11, 12); however, the role of TLRs in recognition of fungal PS is less studied (13, 14). To examine whether TLR4 participates in the EORP-mediated signal transductions, we first chose two genetically specific murine macrophage cell lines, HeNC2 (peritoneal macrophages from wild-type C3H/HeN mice with functional TLR4) and GG2EE (peritoneal macrophages from C3H/HeJ mice lacking functional TLR4) (22–25). As can be seen, HeNC2 exhibited pro-IL-1 production upon EORP stimulation (25 μg/ml) (Fig. 1A, sample 3), whereas no detectable pro-IL-1 was found in EORP-treated GG2EE (Fig. 1A, sample 3 vs 6). Similarly, in the LPS test, we detected pro-IL-1 production from HeNC2 with LPS stimulation (1 μg/ml), but no response in GG2EE (Fig. 1A, sample 2 vs 5). These results suggest that TLR4 is involved in EORP-regulated pro-IL-1 expression.

Next, to investigate the potential interaction of TLR4 and EORP leading to the induction of pro-IL-1 expression, experiments were conducted via preincubation of human blood monocyte-derived macrophages with the mouse anti-human TLR4 mAb (HTA125), which was known to specifically inhibit LPS-induced signalings through TLR4 (26). As expected, HTA125 mAb inhibited LPS-induced TLR4-mediated induction of pro-IL-1 expression (Fig. 1B, sample 3) and EORP-mediated pro-IL-1 expression (Fig. 1B, sample 5). Alternatively, using mouse anti-human CR3 mAb, we
Similar results were obtained in three separate experiments. The production of ROS in murine macrophage J774A.1 cells (Fig. 2A, sample 4 vs 7), indicating that cells pretreated with EORP significantly reduced the LPS-induced IL-1 was ~40 pg/ml (6 h), 130 pg/ml (12 h), and 160 pg/ml (24 h), respectively, and then decreased after 36 h.

The molecular mechanism of IL-1 secretion was then investigated; first, EORP-induced pro-IL-1 expression was detected by Western blot analysis (Fig. 3B). Interestingly, an additive effect of EORP and LPS on pro-IL-1 induction was found in sample 4 (EORP (12.5 µg/ml) plus LPS (0.5 µg/ml)) and sample 7 (EORP (25 µg/ml) plus LPS (1 µg/ml)); each induced more pro-IL-1 than either EORP (samples 3 and 6) or LPS (samples 2 and 5) alone (Fig. 3B). Next, in the time course study, EORP-induced expression of pro-IL-1 in J774A.1 cells was detected between 3 and 12 h, peaked at 6 h, and started to decrease after 12 h; it then gradually returned to the basal level after 24 h (Fig. 3C). A similar mechanism was found in EORP-induced pro-IL-1 expression in human macrophages. As can be seen in Fig. 3D, pro-IL-1 expression increased with increase of EORP concentration, in a dosage-dependent manner (~6–50 µg/ml). Moreover, using RT-PCR, we found that incubation with EORP for 1 h induced pro-IL-1 mRNA expression as compared with the control, and the level reached its maximum between 4 and 8 h (Fig. 3E); it then began to decline after 12 h and remained higher than that of control cells. After 24 h, the pro-IL-1 message nearly returned to the basal level. A similar up-regulation of pro-IL-1 mRNA was also detected by Northern analyses (data not shown).

Second, we tested, in the presence and absence of PMB, the effect of EORP and LPS on the induction of pro-IL-1 in J774A.1 cells. As shown in Fig. 2B, with or without PMB (10 µg/ml) pretreatment of cells, EORP at concentrations of 25–200 µg/ml stimulates the activation of pro-IL-1 in a dose-dependent fashion. In contrast, PMB effectively inhibits LPS (0.01–1 µg/ml)-induced pro-IL-1 in cells. These results clearly demonstrated that PMB effectively inhibited the activity of LPS, but not the activity of EORP.

As incubation of macrophages with LPS induced the appearance of many phosphotyrosyl proteins (12), we were interested in comparison of PTP between EORP and LPS in human blood monocyte-derived macrophages. Thus, cells were stimulated with or without EORP (25 µg/ml) or LPS (1 µg/ml) for 15 and 60 min, respectively, followed by SDS-PAGE and Western blot analysis with monoclonal anti-phosphotyrosine IgG (12). As can be seen in Fig. 2C, incubation of human macrophages with EORP induced the appearance of many phosphotyrosyl proteins compared with that with LPS or medium alone. For example, upon 15- and 60-min stimulation with EORP or LPS, some tyrosine-phosphorylated proteins of molecular mass at 38, 42–44, and ~52 kDa (Fig. 2C, sample 1 vs 2, 3, 4, and 5) were identified and immunoreacted with anti-p38 IgG (38 kDa), anti-ERK IgG (42–44 kDa), and anti-JNK IgG (~52 kDa), respectively (data not shown); however, additional phosphotyrosyl proteins were observed with EORP treatment (Fig. 2C, indicated as +), suggesting that EORP-induced signaling is different from that of LPS (see following sections) and is LPS independent.

EORP up-regulates IL-1 secretion, pro-IL-1 protein, and message expression, and stimulates the activity of IL-1-converting enzyme (ICE or caspase 1) in human macrophages and J774A.1 cells

To detect the effect of EORP on IL-1 secretion, we used ELISA to quantitate mature IL-1 secretion in the conditioned medium of J774A.1 cells. As shown in Fig. 3A, ~60, 140, and 280 pg/ml secreted IL-1 protein was detected in the conditioned medium of 6-, 12-, and 24-h EORP-treated cells, respectively, compared with that of control cells (below a detectable level), and continued to increase to 300 pg/ml after 36 h. Relatively, the concentration of LPS-induced IL-1 was ~40 pg/ml (6 h), 130 pg/ml (12 h), and 160 pg/ml (24 h), respectively, and then decreased after 36 h.

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FIGURE 1. TLR4 functions as a binding site/receptor for EORP and mediates EORP-regulated signal transduction. A, Pro-IL-1 production in response to EORP depends on functional TLR4. Two genetically specific murine macrophage lines, HeNC2 (functional TLR4 normal) and GG2EE (functional TLR4 deficient), were used to examine pro-IL-1 production. HeNC2 and GG2EE were treated with EORP (25 µg/ml) and LPS (1 µg/ml) for 6 h, respectively. Whole cell lysates were analyzed by Western blot with anti-IL-1 antisera, as described in Materials and Methods. The pro-IL-1 and actin (as an internal control) are indicated as arrows on the right. Similar results were obtained in three separate experiments.

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Human primary macrophages

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Also tested the involvement of CR3 in EORP-mediated signal transductions. As can be seen, CR3 mAb failed to block EORP-induced pro-IL-1 production (Fig. 1B, sample 4 vs 7), indicating that EORP is not recognized by CR3. All of these results indicate that TLR4 functions as a receptor for EORP.

EORP is LPS free and different from LPS in ROS production, in stimulation of pro-IL-1 with PMB, and in protein tyrosine phosphorylation (PTP)

To rule out the possibility of LPS contamination in EORP, we conducted a series of experiments, which showed different results between EORP and LPS regarding biochemical reactions and signalings. First, we have demonstrated that LPS quickly stimulates the production of ROS in murine macrophage J774A.1 cells (Fig. 2A or Ref. 12); in contrast, EORP induces much less ROS in a short or long period of time (Fig. 2A). Moreover, we have found that cells pretreated with EORP significantly reduced the LPS-stimulated ROS production (Fig. 2A). The molecular mechanism of EORP reduction of LPS-induced ROS is under investigation; however, the results indicated that it is unlikely that EORP was contaminated with LPS.

EORP up-regulates IL-1 secretion, pro-IL-1 protein, and message expression, and stimulates the activity of IL-1-converting enzyme (ICE or caspase 1) in human macrophages and J774A.1 cells
Posttranscriptional regulation and processing of the pro-IL-1 protein into IL-1 secretion via ICE have been well reported in various cells, including macrophages with LPS (12). Because incubation of macrophages with EORP induced the expression of pro-IL-1 and increased IL-1 secretion in a time-dependent fashion (Fig. 3, C vs A), we examined whether EORP would stimulate ICE activity during IL-1 secretion. As shown in Fig. 3F, EORP increased ICE activity up to 1.5-fold between 6 and 12 h, and gradually reached to 2-fold at 24 h, compared with the control cells or the LPS-treated cells for 18 h. This result led us to the study of EORP-mediated signal transductions, including PKs, in the regulation of pro-IL-1/IL-1.
Our previous results demonstrated that LPS (12) or PS fucoidan (21) transduced the PK-regulated signaling pathways involved in the up-regulation of IL-1 expression. To examine EORP-mediated signal transduction pathways in the regulation of IL-1 gene expression, first we tested whether EORP stimulates MAPKs. Using Western blot analyses with anti-phospho-ERK Ab, we detected the activated, Thr202/Tyr204-phosphorylated form of ERK. Upon EORP stimulation, phosphorylated ERK in J774A.1 cells was detected in 10 min, and the level became 3-fold higher when it reached the maximum in 20 min; after 60 min, ERK phosphorylation decreased.

FIGURE 3. Effect of EORP on IL-1 secretion and pro-IL-1 and pro-IL-1 mRNA expression in J774A.1 cells and in human macrophages. A, EORP up-regulates IL-1 secretion. Cells were treated with EORP (25 µg/ml) or LPS (1 µg/ml); conditioned medium was harvested at the time indicated within 24 h. Concentrated medium was assayed for IL-1 concentration using IL-1-specific ELISA; one of four experiments is presented (n = 4). B, Western blot analysis of pro-IL-1 expression in EORP-treated cells. Cells were treated with EORP (12.5 and 25 µg/ml) or LPS (0.5 and 1 µg/ml) for 6 h; whole cell lysates were then analyzed by Western blot with anti-IL-1 antiserum, as described in Materials and Methods. The position of pro-IL-1 and actin (as an internal control) is indicated as arrows on the right side; one of four experiments is presented (n = 4). C, Time course, and D, dose response of Western blot analyses of pro-IL-1 expression in EORP-treated cells. Cells were treated with EORP for various times, as indicated; whole cell lysates were then analyzed by Western blot with anti-IL-1 antiserum, as described in Materials and Methods. The position of pro-IL-1 and actin (as an internal control) is indicated as arrows on the right side; one of four experiments is presented (n = 4). E, RT-PCR analysis of pro-IL-1 mRNA in cells. Total RNA was isolated from cells treated with EORP between 1 and 24 h. Ethidium bromide-stained agarose gel with amplified pro-IL-1 mRNA at 563 bp and normalized by comparison with RT-PCR of GAPDH mRNA (450 bp) is indicated with arrows for pro-IL-1 and GAPDH. One of four experiments is presented. F, Time-dependent EORP-induced ICE activation. Cells were treated with EORP for various times or with LPS for 18 h; cell extracts (90 µg of protein) were then incubated in the presence of the fluorescence ICE substrate Ac-YVAD-AMC (50 µM) for 1 h at 30°C. The ICE activity was measured fluorometrically with excitation at 360 nm and emission at 460 nm, as previously described (12, 21). The figure is representative of three such independent experiments.

EORP activates MAPK phosphorylation and activity in J774A.1 cells and human macrophages

Our previous results demonstrated that LPS (12) or PS fucoidan (21) transduced the PK-regulated signaling pathways involved in the up-regulation of IL-1 expression. To examine EORP-mediated signal transduction pathways in the regulation of IL-1 gene expression, first we tested whether EORP stimulates MAPKs. Using Western blot analyses with anti-phospho-ERK Ab, we detected the activated, Thr202/Tyr204-phosphorylated form of ERK. Upon EORP stimulation, phosphorylated ERK in J774A.1 cells was detected in ~10 min, and the level became 3-fold higher when it reached the maximum in 20 min; after 60 min, ERK phosphorylation decreased.
gradually returned to the basal level. Interestingly, ERK was phosphorylated again in ~120 min (Fig. 4, A and D). Further studies on human macrophages showed a similar trend in the phosphorylation of ERK (data not shown). In addition, EORP stimulation of ERK activity was demonstrated by the phosphorylation of Elk, a downstream substrate of ERK (Fig. 4, A and E).

The inflammatory response of human macrophages or J774A.1 cells to EORP-induced IL-1 expression prompted us to investigate the possibility of EORP activation of stress-related JNK pathway. Cells incubated with EORP showed JNK phosphorylation, as determined by Western blot analysis, with anti-phospho-JNK Ab that recognizes the activated, Thr183/Tyr185-phosphorylated form of JNK. JNK phosphorylation gradually increased in 10 min, and reached the maximal level (8-fold increase) at ~20 min; after 120 min, the induced JNK phosphorylation returned to 4-fold (Fig. 4, B and D).

To explore EORP-mediated additional signal transduction pathways, we further examined whether EORP would induce p38 phosphorylation, another important stress-related MAPK member. Upon EORP stimulation, p38 phosphorylation gradually increased as detected by Western blot analyses with anti-phospho-p38, an Ab that specifically recognizes the activated, Thr180/Tyr182-phosphorylated form of p38. The time course study of EORP-induced p38 phosphorylation indicated that after 20 min, p38 showed a 7-fold increase in phosphorylation compared with that of control cells; after 120 min, it returned to the basal level (Fig. 4, C and D). A similar trend was observed in p38 phosphorylation in human macrophages (data not shown). To further dissect the effect of EORP-induced p38 activity, an in vitro kinase assay was conducted. Upon EORP stimulation, phosphorylation of activating transcription factor-2 (ATF-2), a downstream substrate of p38, was detected in 10 min, and reached the maximal level (>15-fold) in 60 min; then it slightly decreased after 120 min (Fig. 4, C and E).

Role of EORP-induced PK in the regulation of pro-IL-1 expression

To elucidate further the role of various EORP-induced PK-mediated signaling pathways in the regulation of pro-IL-1 protein expression, we used certain specific pharmacological antagonists, such as Ro-31-8220, PD98059, SP600125, SB203580, and LY294002, which inhibit the activation of protein kinase C (PKC), MEK1, JNK, p38, and PI3K, respectively. The dose response for specific PK inhibitors was monitored by directly assaying individual kinase activity, and the effective concentrations of PK inhibitors were determined (data not shown). Initially, we examined whether the PKC/MEK1/ERK pathway is one of EORP-induced PK downstream signaling cascades in the regulation of pro-IL-1. J774A.1 cells were exposed to concentrations of inhibitors, as indicated, followed by incubation with EORP. Western blot analysis showed that Ro-31-8220 (Fig. 5A) and PD98059 (Fig. 5B) block EORP-induced pro-IL-1 expression at 5 and 50 μM, respectively. These results indicate that the PKC/MEK1/ERK pathway is involved in the EORP-induced pro-IL-1 expression. Next, to investigate the role of EORP-induced JNK activity, we examined SP600125 in the regulation of pro-IL-1 expression. As shown in Fig. 5C, SP600125 (at concentration 1~20 μM) had no effect on EORP-induced pro-IL-1 expression. Moreover, using a specific p38 inhibitor, SB203580, we further examined the potential role of EORP-induced p38 activation in the regulation of pro-IL-1. Western blot analyses were conducted to study the dose response of SB203580 (concentrations at 0.1, 0.5, 1, and 2 μM) in EORP-induced pro-IL-1 expression in J774A.1 cells. SB203580 completely inhibits EORP-induced pro-IL-1 at concentration above 0.5 μM compared with control cells (Fig. 5D, sample 2 vs 4), but less inhibition below 0.1 μM (sample 3).

Effect of LY294002 (an inhibitor of PI3K) on EORP-induced JNK and p38 activity as well as on EORP regulation of pro-IL-1 and IL-1 secretion

As demonstrated above, the PKC/MEK1/ERK pathway plays an important role in the EORP-mediated regulation of pro-IL-1 expression. To explore other EORP-mediated signal transduction pathways associated with pro-IL-1 expression, we used LY294002, a specific inhibitor of PI3K, to examine the potential role of PI3K in EORP-mediated activation of JNK and p38. J774A.1 cells were preincubated with or without LY294002 for 60 min, followed by EORP treatment for additional 20 min; the phosphorylation of JNK and p38 was then examined. In the absence of LY294002, EORP induced the phosphorylation of JNK and p38 by ~13-fold (Fig. 6A, sample 2 vs 1) and 10-fold (Fig. 6A, sample 2 vs 1), respectively, compared with the control (sample 1). Surprisingly, cells preincubated with LY294002, at the concentration as high as 100 μM, followed by treatment with EORP, showed no alteration of JNK and p38 phosphorylation (Fig. 6A, samples 3, 4, 5, and 6 vs 2), indicating that PI3K was less or not associated with EORP activation of JNK and p38. The roles of PI3K in the EORP-mediated regulation of pro-IL-1 and IL-1 secretion were further examined. Preincubation of cells with LY294002 (50 μM) for 60 min, followed by exposure of cells to EORP for additional 6 h, pro-IL-1 in cell lysates was detected by Western blot analysis. The result showed that preincubation of LY294002 and the EORP-treated cells significantly decreased pro-IL-1 production (Fig. 6B, sample 2 vs 4) compared with EORP-treated cells. Furthermore, the effect of LY294002 on EORP-induced IL-1 secretion was detected by ELISA. Cells preincubated with LY294002 before EORP treatment showed a significant reduction in the secreted IL-1 to 5 pg/ml (6 h), 14 pg/ml (12 h), and 18 pg/ml (24 h), respectively, in the conditioned medium (Fig. 3A), which was much lower than the corresponding EORP-treated control cells.

Discussion

The role of TLR4 in Gram-negative bacterial LPS-mediated signalings has been studied extensively (11, 12, 27); however, the role of TLR4 in response to fungal PS is less studied and unclear. Recent reports of TLR-mediated production of the inflammation cytokine TNF, following stimulation by Aspergillus fumigatus (13, 28), prompted us to study TLR4 as the candidate receptor/binding site for EORP. Using a genetic approach, the relationship between LPS and TLR4 has been reported (22–25). Similarly, we used two mouse macrophage strains: HeNC2 (peritoneal macrophages with functional TLR4) and GG2EE (peritoneal macrophages lacking functional TLR4) to investigate the relationship between EORP and TLR4. Our data indicated that HeNC2 exhibited pro-IL-1 expression upon EORP stimulation; in contrast, no apparent pro-IL-1 expression in GG2EE was observed when incubated with EORP (Fig. 1A, sample 3 vs 6). In addition, we further demonstrated that HTA125, a mouse anti-human TLR4 mAb, inhibited/reduced EORP- and LPS-induced pro-IL-1 expression, respectively (Fig. 1B, sample 4 vs 5 and sample 2 vs 3). These results indicate that binding (interaction) of EORP to TLR4 triggers the downstream signaling of PK activity and related immunomodulating activities, including expression of the inflammatory cytokine IL-1 (Fig. 7, and the following discussion) and TNF (data not shown).

In contrast, the enhancement of oxidative burst response, phagocytosis, and antimicrobial activity in neutrophils by β-glucans has been demonstrated (29, 30). In addition, the β-glucan-binding receptors located on leukocytes have been reported, and the potential functions have been characterized (31–33). The active constituents...
FIGURE 4. Time course of EORP-induced MAPK phosphorylation and activity. Analysis of time course of ERK1/2 (A), JNK1/2 (B), and p38 (C) phosphorylation and activity in EORP-treated J774A.1 cells. Cells were stimulated with EORP (25 µg/ml), and the cell lysates were collected at different periods of time. Cell lysates were analyzed by Western blot with anti-diphosphorylated p44/42, anti-diphosphorylated JNK1/2, or anti-diphosphorylated p38 mAb, as described in Materials and Methods. For in vitro kinase assay, J774A.1 cells were treated, as described above. The activated ERK and p38 were immunoprecipitated from cell lysates using specific Abs. Recombinant Elk-1 fusion protein and ATF-2 fusion protein were used as substrates for ERK and p38, respectively. EORP-induced ERK (A) and p38 (C) activities were monitored by phosphorylation of substrate. These were measured by quantitative immunoblotting with phospho-Elk-1 (Ser383) Ab and phospho-ATF-2 (Thr71) Ab, respectively. The experiments were conducted three times, and a representative result is shown in the figure. D. Histogram represents quantification by PhosphorImager of EORP-stimulated phospho-ERK1/2, phospho-JNK1/2, and phospho-p38 in J774A.1 cell sample using ImageQuaNT software from Amersham Pharmacia Biotech (Sunnyvale, CA). E. Histogram represents quantification by PhosphorImager of EORP-stimulated ERK1/2 and p38 activity in J774A.1 cell sample using ImageQuaNT software from Amersham Pharmacia Biotech. All data of relative activity are expressed as comparison with untreated cells (t = 0; phosphorylation of control cells defined as 1). Similar experiments were repeated three times, and a representative result is shown in the figure.
of EORP (i.e., F3) responsible for the activities described in this study were known to contain either a PS backbone with \( \beta-(1\rightarrow3) \) linkages or a polymannose backbone with \( \beta-(1\rightarrow4) \) linkages, both with side chains of unknown structure (1). The interaction of CR3 (or CD11b/CD18) with \( \beta-(1\rightarrow3) \)-glucan PS has been studied (31–33). In this study, we found that mouse anti-human CR3 mAb failed to block EORP-induced pro-IL-1 production (Fig. 1B, sample 4 vs 7), excluding the involvement of CR3 in EORP-mediated signalings and activities.

The PS fractions from \( G. \) lucidum induce cytokine expression, including IL-1, IL-6, and TNF in macrophages, and IFN in T lymphocytes (3, 7, and this study). Moreover, a fucose-containing glycoprotein fraction isolated from the water-soluble extract of \( G. \) lucidum has been shown to stimulate spleen cell proliferation and cytokine expression (1). In this study, we analyzed the EORP-induced inflammatory cytokine IL-1 gene expression. Based on ELISA, we have demonstrated that neither IL-1 nor pro-IL-1 preexists in quiescent human blood monocyte-derived macrophages and murine macrophage J774A.1 cell. However, upon EORP stimulation, IL-1 can be detected after 6 h, and within 12–24 h EORP induces \( >150 \) pg/ml IL-1 secretion. Similarly, a significant TNF production was detected by ELISA in the cells treated with EORP (data not shown). IL-1 secretion is consistent with the sequential synthesis of pro-IL-1 mRNA and pro-IL-1 as well as induced ICE activity after EORP stimulation. The PS fractions from \( G. \) lucidum...
were also reported to induce signal transduction pathways in certain cells (7, 20); for example, in PC12 neuron cells, PS induce Ras/ERK signaling, mediate the neuronal differentiation, and prevent nerve growth factor-dependent apoptosis (20). Our current results demonstrate that incubation of macrophages with EORP quickly activates protein tyrosine kinases (PTKs) (Fig. 2C), leading to activation of various mitogen-like signaling molecules and PKs, including ERK, JNK, and p38 and stimulation of downstream pro-IL-1/IL-1 expression (Fig. 7). These responses are comparable with cells treated with certain mitogens, cytokines (34), fucoidan (21), or LPS (12). Importantly, various experiments (Fig. 2. A–C) were conducted for EORP and LPS, including the difference in ROS production and EORP suppression of LPS-induced ROS releasing, the PMB inhibitory and PTK activity studies, as well as the negative results in toxicity test of EORP in mice survival study (data not shown), and the results have ruled out the LPS contamination possibility in EORP.

Either EORP (Reishi) or LPS transduces part of signalings via the TLR4. Although we could not totally rule out the involvement of other receptors in the signal transductions, the biological outcomes are quite different. EORP has been used to promote health, but concentrated LPS of contaminated bacteria causes high mortality in vitro or in vivo. The molecular mechanisms for EORP or LPS in the differential immunity outcomes are not clear; our current results indicate that: 1) LPS quickly stimulates macrophage production of ROS, but not in EORP treatment. Moreover, cells pretreated with EORP significantly reduce the LPS-stimulated ROS production. 2) Production of IL-1 is 40, 50, and 80% through the signaling pathways of PTK→(phospholipase C (PLC)-γ1)→PKC→MEK1→ERK, PTK→Rac1→p21-activated kinase

FIGURE 7. The proposed EORP-mediated signal transduction pathways in the regulation of pro-IL-1 expression and IL-1 secretion.
IFN-CD80/86 expression than that of LPS; however, LPS stimulates mechanism for up-regulation is unclear. 5) In addition, our unpublished stimulation of pro-IL-1/IL-1 (data not shown); the molecular discussion). 4) The down-regulation of macrophage pro-IL-1/IL-1 production in response to subsequent LPS challenge is typical LPS tolerance. In contrast, pre-exposure to EORP up-regulates LPS stimulation of pro-IL-1/IL-1 (data not shown); the molecular mechanism for up-regulation is unclear. 5) In addition, our unpublished results show that Reishi is more potent in the stimulation of CD80/86 expression than that of LPS; however, LPS stimulates IFN-γ and IL-10 production in human mononuclear cells, but Reishi does not (data not shown).

To further understand the molecular mechanisms of EORP binding to TLR4 and the subsequent signaling cascade in the regulation of cytokine pro-IL-1/IL-1 expression, we used specific pharmacological antagonists such as Ro-31-8220, PD98059, LY294002, SP600125, and SB203580, to inhibit the phosphorylation of PKC (35), MEK1 (36), PI3K (37), JNK (38), and p38 (39), respectively. We have demonstrated the differences in the activation time and the extent of activity for EORP-stimulated MAPKs (ERK, JNK, and p38) as well as PKC and MEK1. Both PKC and MEK1 are the upstream signaling components during the induction of ERK activity (40), and incubation of cells with EORP induces ERK activity, which is inhibited by Ro-31-8220 and PD98059; hence, pro-IL-1 production and IL-1 secretion were reduced (Fig. 5A and B). These results indicate that EORP-induced PKC/MEK1/ERK pathway activation involves regulation of pro-IL-1/IL-1 expression, although we could not rule out the possible existence of cross-interaction between MEK kinase members and ERK (see the following discussion). The activation of JNK by the upstream signaling molecules Rac1 and/or Cdc42 in cells including macrophages has been reported (12), and most likely p38 activity is activated in a similar way (12, 41). In addition, the PKA family of PK has been recognized as one of the main targets to interact with the GTPases of Rac1/Cdc42 (42), and generates downstream signaling networks in the stimulated macrophages (12). We have observed that EORP first stimulates PKA activation (data not shown), then the activation of JNK and p38, followed by the induction of pro-IL-1. The current results of EORP-induced PKA, JNK, and p38 activation are comparable with our recent demonstration of Rac1 activation of PKA-mediated signalings by either LPS (12) or fucoidan (a principal PS sulfate ester in brown seaweeds, Phaeophyceae species) (21), leading to the activation of JNK and p38, respectively. Together, our results support that Rac1 and PKA are at the upstream of JNK and p38. In addition, we have demonstrated that EORP-mediated signaling of PKA/Rac1/PKA/JNK and of PTK/Rac1/PK/SNFK is associated with pro-IL-1/IL-1 expression.

To further examine and dissect the relationship of PI3K-mediated signalings of JNK and p38 and their roles in the regulation of pro-IL-1 expression (Fig. 7), a specific inhibitor of PI3K, LY294002, was used (12, 21). Our data showed that inhibition of PI3K by LY294002 did not alter EORP-induced JNK and p38 phosphorylation (Fig. 6A), but inhibited pro-IL-1 and IL-1 secretion (Fig. 6B). These results suggest that neither endogenous PI3K nor PI3K-related downstream signalings involve EORP-mediated activation of JNK and p38. However, it is likely that PI3K-mediated other pathway(s) participates in EORP-induced pro-IL-1/IL-1 expression (Fig. 7). Interestingly, LPS-mediated activation of PI3K and the mediated downstream signalings of JNK and p38 are completely different from those of EORP (12). Although LPS stimulation of a single MAPK (i.e., p38) produces only a modest effect on pro-IL-1, activation of each MAPK pathway is important for a complete induction of IL-1 gene expression (12). Our current results illustrate that the two MAPK pathways are simultaneously activated, and a dramatic induction of IL-1 gene expression is observed, suggesting a cooperative effect on the EORP-mediated regulation of IL-1 among these kinases. Furthermore, we have demonstrated that EORP induces cytokine production in human blood monocyte-derived macrophages, and the activation of ERK, JNK, and p38 by EORP in human macrophages and J774A.1 cells indicates that the effect of EORP on macrophages is species independent. Nevertheless, that EORP stimulates the activation of transcription factors, e.g., AP-1, NF-κB, and ATF-2, etc., needs further investigation.

In summary, we have used a macrophage model to study in vitro the immunomodulating effect of G. lucidum (Reishi or Ling-Zhi) PS, i.e., EORP. The results suggest that TLR4, not CR3, is a putative receptor for EORP. We have found that binding of EORP to TLR4 activates PKC, MEK1, PAK, and MAPKs, and transduces diverse signaling cascades that lead to different biochemical reactions, such as multiple cytokine productions. We have further systematically dissected the molecular mechanisms of EORP-mediated signalings in regulation of cytokine IL-1 expression. Specifically, we have established the signal transduction pathways of PTK→PLC-y1→PKC→MEK1→ERK and of PTK→Rac1→PAK→p38, but less of PTK→Rac1→PAK→JNK in the cooperative regulation of pro-IL-1 protein and IL-1 secretion (Fig. 7). EORP-induced ICE activation results in the alternative between degradation of pro-IL-1 and increase of IL-1 secretion with time, indicating that EORP mediate transcriptional, posttranscriptional, and posttranslational regulation of IL-1 gene expression. Taken together, the current finding that TLR4 is a receptor for EORP-mediated signal transduction in regulation of pro-IL-1/IL-1 expression will contribute to our understanding of G. lucidum-mediated immunomodulation activities.

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