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Suppression of LPS-induced Cyclooxygenase-2 (*cox-2*) Gene Expression in Mouse Macrophages by Excretory/Secretory Products of *Spirometra erinaceieuropaei* Plerocercoids

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The escape mechanism of parasites from inflammatory processes has been thought to be one of the most important tools for their survival in infected tissues. On the other hand, inducible cyclooxygenase-2 (cox-2) has been shown to play a major role in the process of inflammation. We investigated the effect of excretory/secretory (ES) products from the plerocercoids of *Spirometra erinaceieuropaei* on *cox-2* gene expression in a murine macrophage cell line (RAW 264.7). Preincubation of the macrophages with the ES products inhibited LPS-induced *cox-2* mRNA expression by 75% as well as its protein production. Dibutyryl cAMP enhances LPS-induced *cox-2* mRNA expression. The ES products also inhibited this enhanced expression by 46%. Inhibition of p38 mitogen-activated protein kinase (MAPK) with SB203580 or that of extracellular signal-regulated protein kinase with PD98059 reduced LPS-induced *cox-2* mRNA expression by 75% or 52%, respectively, along with its protein production. This evidence suggests that both MAPK pathways are crucial for full induction of *cox-2* gene expression. One experiment using a transcriptional inhibitor, actinomycin-D, showed that the ES products destabilized LPS-induced *cox-2* mRNA in RAW 264.7 macrophages. These results show that ES products from the plerocercoids of *Spirometra erinaceieuropaei* suppress LPS-induced *cox-2* mRNA expression in murine RAW 264.7 macrophages, and may attenuate inflammation around the plerocercoids of *S. erinaceieuropaei*.

Key words: *Spirometra erinaceieuropaei;* lipopolysaccharide; macrophage; cyclooxygenase-2; mitogen-activated protein kinase

Cyclooxygenase (cox) catalyzes the synthesis of prostaglandins (PGs) from arachidonic acid. Two isozymes, cox-1 and cox-2, have been identified, and they are encoded by separate genes. The cox-1 isozyme is constitutively present in most tissues and seems to catalyze the synthesis of PGs for normal physiological functions (O'Neill and Ford-Hutchinson, 1993). In contrast, cox-2 is not or is scarcely detectable in most tissues under basal conditions, but is dramatically induced by bacterial lipopolysaccharide (LPS), cytokines, growth factors or carcinogens (Kujubu et al., 1991).

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Abbreviations: cox, cyclooxygenase; dbcAMP, dibutyryl cAMP; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; ES, excretory/secretory; gapdh, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; PG, prostaglandin

The cox-2 isozyme is responsible for high levels of PG production, and secreted PGs are involved in various physiological events including progression of inflammation, immunomodulation, and transmission of pain (Vane and Booting, 1998; Kim et al., 2004). The central role of PGs in inflammation was firmly established after the discovery that the anti-inflammatory action of nonsteroidal anti-inflammatory drugs, such as acetylsalicylic acid, was mediated by the inhibition of cox. It has been widely accepted that macrophages play an important role in the regulation of inflammation and immune response. In inflammatory reactions, macrophages are the main producers of large quantities of PGE_2 (Giroux and Descoteaux, 2000).

Parasite survival may depend on the ability of the parasite to modulate host immune response by the release of immunomodulatory molecules that protect the organism (Maizels et al., 2001; Harnett et al., 2004). When larval plerocercoids of *S. erinaceieuropaei* are taken orally in many mammals including humans and rodents, these plerocercoids invade the peritoneal cavity from the intestine, and can survive in the tissues of hosts. In humans, infection with larval plerocercoids causes "sparganosis" in various tissues (Kudesia et al., 1998). Experimentally infected plerocercoids do not induce the strong inflammatory responses around the parasites and they can survive for long periods in the tissues of mice; hence, we hypothesized that larval plerocercoids of *S. erinaceieuropaei* secrete an immunosuppressive factor(s). We previously showed that excretory/secretory (ES) products from plerocercoids suppress LPS-induced expressions of chemokines KC and JE, the murine homologues of neutrophil chemoattractant growth related oncogene α (CXCL1/GRO-α) and monocyte chemoattractant protein-1 (CCL2/MCP-1), respectively (Fukumoto et al., 1997), tumor necrosis factor (TNF)-α (Miura et al., 2001; Dirgahayu et al., 2002), and IL-1β (Dirgahayu et al., 2004) in murine macrophages. We also found that these suppressions are caused by the reduction of phosphorylation of mitogen-activated protein kinase (MAPK), particularly extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAPK (Dirgahayu et al., 2002; Dirgahayu et al., 2004).

Studies using MAPK inhibitors, SB203580 and PD98059 revealed that MAPK is involved in LPS-induced *cox-2* transcription in RAW 264.7 macrophages (Chanmugam et al., 1995; Hwang et al., 1997). SB203580 inhibits the p38 MAPK pathway (Ajizian et al., 1999), and inhibits the promoter activities of *cox-2* (Ho et al., 2004), while PD98059 is a specific inhibitor for the ERK1/2 pathway (Alessi et al., 1995). In addition to the transcriptional effects of MAPK, AgC10, a mucin from *Trypanosoma cruzi* destabilized *cox-2* mRNA by inhibiting the activation of p38 MAPK (Alcaide and Fresno, 2004).

Besides MAPKs, the importance of NF-κB for the induction of *cox-2* in LPS-activated monocyte-macrophage lineage has been emphasized (D' Acquisto et al., 1998). However, we found that ES products do not affect the LPS-induced nuclear translocation of NF-κB (Dirgahayu et al., 2002).

Since ES products have the ability to suppress the phosphorylation of ERK1/2 and p38 MAPK in LPS-activated macrophages, we hypothesized that ES products could suppress LPS-induced *cox-2* gene expression in macrophages. We have shown in this paper that ES products suppress LPS-induced *cox-2* mRNA expression and cox-2 protein in RAW 264.7 cells, and ES products destabilize *cox-2* mRNA. This is the first report demonstrating that ES products from the parasitic helminth reduce *cox-2* mRNA and cox-2 protein in macrophages.

Materials and Methods

Plerocercoids of Spirometra erinaceieuropaei and their ES products

Plerocercoids of *Spirometra erinaceieuropaei* were collected from two species of snakes (*Elaphe quadrivirgata* and *Rhabdophis tigrinus*) in Ehime

Prefecture, Japan and stored for 6 to 10 months in the subcutaneous tissues of golden hamsters, which were housed and maintained according to the guidelines for proper treatment of animals at the Research Center for Bioscience and Technology, Tottori University, Japan. ES products were obtained as described previously (Miura et al., 2001). To obtain ES products, 25 plerocercoids aseptically removed from hamsters were incubated for 24 h in serum-free 25 mL Dulbecco's modified Eagle's medium (DMEM) in a 10 cm dish, and the incubation medium was dialyzed against distilled water and lyophilized. The protein concentration of this sample dissolved in DMEM was assayed using a protein assay kit (Bio-Rad, Melville, NY) and adjusted to 50 μg/mL. The average amount of total protein secreted from each plerocercoid for 24 h was approximately 30 μg in the present experiment.

Culture of macrophages

A murine macrophage cell line RAW 264.7 (RCB0535) was purchased from RIKEN Cell Bank (Wako, Japan). The cells were cultured in DMEM supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) containing 100 U/mL penicillin G (Banyu Pharmaceutical, Tokyo, Japan) and 100 μg/mL streptomycin (Meiji Seika, Tokyo, Japan), plated in tissue culture dishes (Greiner Bio-One, Tokyo, Japan) and finally incubated at 37˚C in an atmosphere of 5% CO_2 in a humidified incubator. In the present study, 5×10^6 cells were preincubated in a 10 cm culture dish for 3 h or 6 h in the presence or absence of ES products (5 μg/mL). The cells were washed with PBS and stimulated with LPS (100 ng/mL) in DMEM supplemented with 5% FBS. After 3 h of incubation with LPS, large amounts of *cox-2* mRNA were expressed in a time-course experiment. Then, the cells were incubated with LPS for 3 h in the present experiments. A concentration of 5 μg/mL ES products was used, because this concentration had been effective in TNF-α gene expression (Miura et al., 2001), and similar activation pathways such as NF-κB and MAPK are involved in LPS-induced *cox-2* and TNF-α gene expressions.

Reverse transcription-PCR analysis

Total RNA was prepared using an ISOGEN Kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed, and PCR reactions were performed on a Perkin Elmer DNA Thermal Cycler (Takara Bio, Kusatsu, Japan). Primers used were as follows: *cox-2*: 517 bp, 5'-CTT CCT GAT CAA AAG AAG TGC TG-3' (sense) and 5'-ACT TGA GTT TGA AGT GGT AAC CGC-3' (antisense); and *β-actin*: 349 bp, 5'-TGG AAT CCT GTG GCA TCC ATG AA-3' (sense) and 5'-TAA AAC GCA GCT CAG TAA CAG TCC-3' (antisense). The PCR cycles consisted of denaturation at 94˚C for 30 s, annealing at 60˚C for 1 min and extension at 72˚C for 1 min. The number of cycles used was 29 for semi-quantitative analysis of *cox-2* or 25 for *β-actin*. A 15-μL aliquot of each PCR product was separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and photographed. For quantification, photographs showing PCR products were scanned and analyzed using the software Densitograph version 4.0 (ATTO, Tokyo, Japan). The amount of *cox-2* mRNA was normalized to that of *β-actin*.

Preparation of total RNA and Northern blot analysis

Northern hybridization analysis was performed as previously described (Fukumoto et al., 1997). Briefly, 10 μg of total RNA was separated on an 1% agarose gel containing 2.2 M formaldehyde, and subsequently blotted onto a MagnaGraph Nylon transfer membrane (Micron Separation, Westboro, MA) and then cross-linked to the membrane using an UV cross-linker (Funa-UV-Linker Fs 1500, Funakoshi, Tokyo, Japan). After prehybridization, the blots were hybridized with cDNA plasmid probes radiolabelled by [α-^{32}P]dCTP

(MP Biomedicals, Irvine, CA) using a Takara BcaBEST Labelling Kit (Takara Bio), and then autoradiography was performed. The murine-517 bp of the *cox-2* cDNA fragment was prepared by RT-PCR, followed by confirmation with sequencing. A plasmid encoding the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) cDNA, used as a control, was kindly provided by Dr. David Stern (Columbia University). Relative *cox-2* mRNA expressions were analyzed using a Molecular Imager, Model GS-535 (Bio-Rad) with reference to the expression of *gapdh* mRNA.

Preparation of cell lysate and Western blot

After the treatment, cell lysates were prepared as previously described (Dirgahayu et al., 2002). Briefly, the adherent cells were disrupted in cold lysis buffer (20 mM Tris, pH 7.5, 120 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 5 µg/mL aprotinin, 5 µg/mL pepstatin and 5 µg/mL leupeptin), homogenized for 5 s using a microtube homogenizer, and then kept on ice for 30 min. Insoluble material was removed by centrifugation at 4˚C, 12,500 rpm for 10 min and protein concentration in the clear supernatant was determined using a protein assay kit (Bio-Rad). The solubilized proteins (80 µg) were boiled in SDS-loading buffer, then separated on a 10% SDS-polyacrylamide gel with a running buffer containing 25 mM Tris, 192 mM glycine, pH 8.3, and 0.1% SDS. After electrophoresis, the separating gel was soaked in transfer buffer (25 mM Tris-HCl, 0.2 M glycine, 20% (v/v) methanol, pH 8.5) for 15 min and electrophoretically transferred to Hybond-ECL nitrocellulose (Amersham Biosciences, Piscataway, NJ) using a semidry blotting apparatus (Novablot, Amersham Biosciences), and then blocked overnight in Tris-buffered saline/tween-20 (0.05%) supplemented with 3% non-fat milk. The membranes were washed, incubated overnight with phospho-specific antibody recognizing cox-2 (1:300) detected with anti-rabbit horseradish peroxidase-conjugated antibodies (1:3,000) and subsequently visualized by an enhanced chemiluminescence reagent (Amersham Biosciences). The gel was stained with

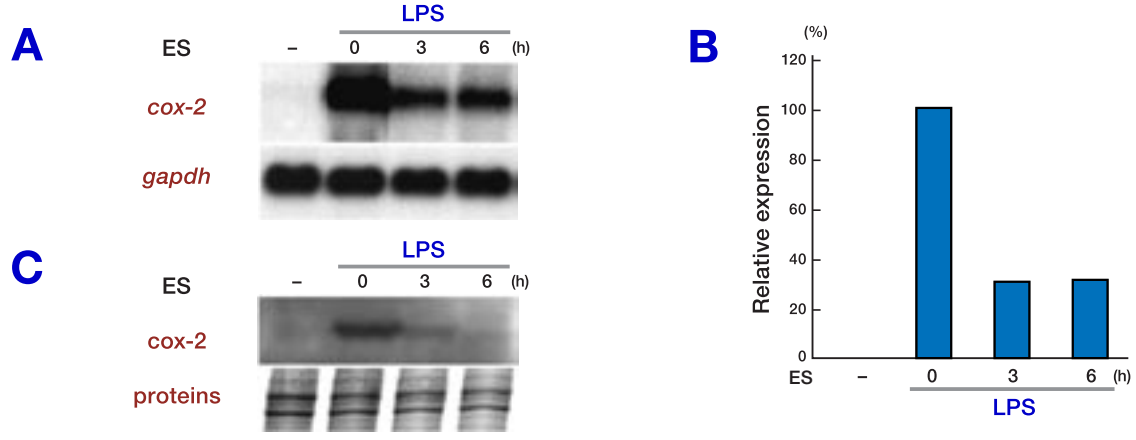

Fig. 1. Suppression of LPS-induced *cox-2* mRNA expression in RAW 264.7 macrophages by ES products. RAW 264.7 cells were left untreated or treated with ES products (5 µg/mL) for 3 h or 6 h, and the medium was replaced prior to stimulation with LPS (100 ng/mL) for 3 h.

A: Total RNA was obtained from the cells, and the expression of *cox-2* and *gapdh* mRNA was assessed by Northern blot analysis.

B: The *cox-2* mRNA level was quantified and normalized to *gapdh*. The relative *cox-2* mRNA levels are presented as percentages of the LPS-induced control in the absence of ES products.

C: The cox-2 protein in each cell lysate was analyzed by Western blot analysis. The same volume of each cell lysate was resolved by SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue to show that total protein was equally loaded. One representative of 2 independent experiments is shown.

Coomassie Brilliant Blue to demonstrate that an equal amount of proteins was loaded in each lane.

Results

Suppression of LPS-induced cox-2 mRNA expression by ES products

No appreciable band was observed in controls without LPS stimulation. Firstly, the effect of ES products on *cox-2* mRNA expression and protein production were investigated. RAW 264.7 cells were either left untreated or preincubated with ES products (5 μg/mL) for 3 h or 6 h. The cells were stimulated with 100 ng/mL LPS for 3 h. Total RNA or total protein was subjected to Northern blot or Western blot, respectively. The result shows that ES products strongly suppressed the levels of LPS-induced *cox-2* mRNA (Figs. 1A and B) and cox-2 protein (Fig. 1C) to a similar degree. The level of constitutively expressed *gapdh* mRNA was unaltered by exposure to ES products. Since preincubation for 3 h or 6 h showed no significant differences on *cox-2* mRNA expression or protein production, a 6 h pretreatment was used for the following experiments.

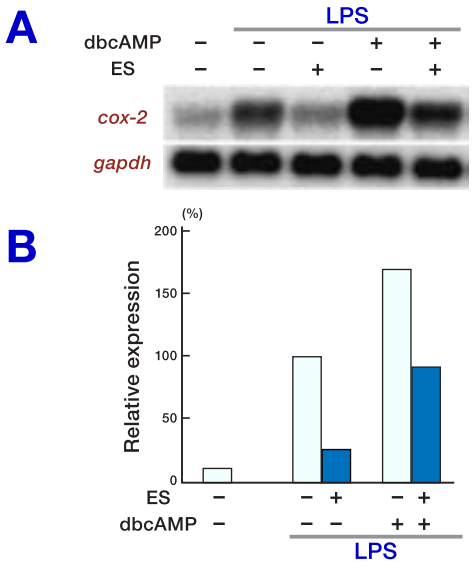

Fig. 2. The suppressive effect of ES products on *cox-2* mRNA expression induced by dbcAMP and LPS. RAW 264.7 cells were left untreated or preincubated with ES products for 6 h prior to medium change. The cells were then either left untreated or treated with dbcAMP (0.1 mM) for 15 min and were then stimulated with LPS (100 ng/mL) for 3 h. Total RNA was obtained from the cells and *cox-2* and *gapdh* mRNA expression was assessed by Northern blot analysis (**A**). The *cox-2* mRNA level was quantified and was normalized to *gapdh*. The relative *cox-2* mRNA levels are presented as percentages of the LPS-induced control in the absence of ES products and dbcAMP (100%) (**B**). The figure is representative of 3 independent experiments.

Suppressive effect of ES products on cox-2 mRNA induced by dbcAMP and LPS

The plerocercoids release PGE_2 (Fukushima et al., 1993; Gao et al., 1998), which enhances the induction of LPS-induced *cox-2* mRNA by an adenyl cyclase/cAMP-dependent pathway (Hinz et al., 2000). We tested the possibility of ES products on LPS-induced *cox-2* mRNA expression in the presence of dibutyryl cAMP (dbcAMP). As seen in Fig. 2, LPS-induced *cox-2* mRNA expression (Lane 2) was further enhanced by treatment with dbcAMP 1.7 times (Lane 4). Pretreatment of macrophages with ES products prior to stimulation with LPS (Lane 3) or with LPS and dbcAMP (Lane 5) strongly suppressed *cox-2* mRNA expression by 75% and 46%, respectively. No appreciable band was detected in the untreated control (Lane 1), nor in cells treated with dbcAMP or ES products without LPS-induction (data not shown).

Involvement of MAPK in LPS-induced cox-2 mRNA

We examined the effects of MAPK inhibitors for the ERK1/2 pathway (PD98059) and for p38 MAPK (SB203580) in LPS-induced *cox-2* mRNA expression. The macrophages were pretreated for 1 h with SB203580 (10 μM), PD98059 (50 μM) or both, and these cells were then stimulated with LPS for 3 h. The total RNA or protein was sub-

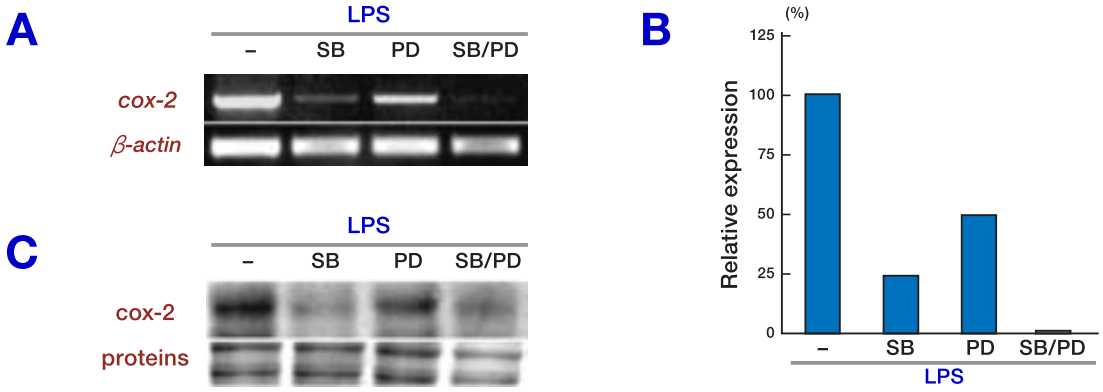

Fig. 3. Effect of MAPK inhibitors on LPS-induced *cox-2* mRNA expression. RAW 264.7 cells were treated with PD98059 (50 μM), SB203580 (10 μM) or a combination of both for 1 h and were stimulated with LPS (100 ng/mL) for 3 h. Total RNA and total protein were obtained from the cells and *cox-2* and *β-actin* mRNA expressions were assessed by semi-quantitative RT-PCR (**A**). The *cox-2* mRNA level was quantified and was normalized to *β-actin*. The relative *cox-2* mRNA levels are presented as percentages of the LPS-induced control in the absence of MAPK inhibitor (**B**). The cox-2 protein expression was assessed by Western blot analysis (**C**). One representative of 2 independent experiments is shown.

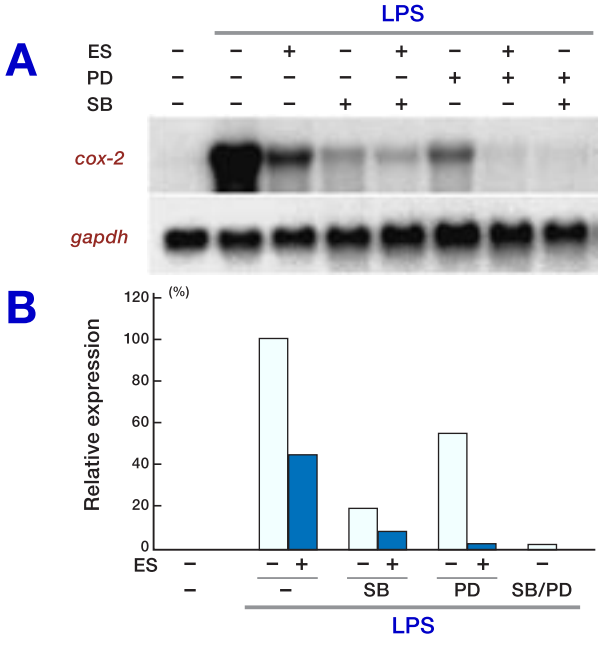

Fig. 4. Additive suppression of LPS-induced *cox-2* mRNA expression by a combination of MAPK inhibitor and ES products. RAW 264.7 cells were left untreated or preincubated with ES products (5 μg/mL) for 6 h prior to medium change. The cells were then left untreated or treated either with PD98059, SB203580 or a combination of both for 1 h and were then stimulated with LPS (100 ng/mL) for 3 h. Total RNA was obtained from the cells and was subjected to Northern blot analysis (**A**). The *cox-2* mRNA level was quantified and was normalized to *gapdh*. The relative *cox-2* mRNA levels are presented as percentages of the LPS-induced control in the absence of ES products and MAPK inhibitor (100%) (**B**). The figure is representative of the 3 independent experiments.

jected to semi-quantitative RT-PCR or Western blot analysis, respectively. SB203580 (Fig. 3A, Lane 2) and PD98059 (Lane 3) suppressed *cox-2* mRNA expression by 75% or 52%, respectively, and *cox-2* protein production (Fig. 3C, Lanes 2, 3, respectively). The combination of both inhibitors reduced the *cox-2* mRNA (Fig. 3A, Lane 4) and cox-2 protein (Fig. 3C, Lane 4) to nearly null. Figure 3B shows the quantitative relations.

Next we examined the effect of ES products in combination with MAPK inhibitor(s) on *cox-2* mRNA expression by Northern blot analysis. ES products or MAPK inhibitors reduced LPS-induced *cox-2* mRNA expression, and the combination with ES products and each MAPK inhibitor additively suppressed *cox-2* mRNA expression (Fig. 4).

Destabilization of LPS-induced cox-2 mRNA by ES products

The level of *cox-2* mRNA is regulated both transcriptionally and post-transcriptionally (Wadleigh et al., 2000; Alcaide and Fresno, 2004). Therefore, we examined the effect of ES products on *cox-2* mRNA stability in LPS-stimulated macrophages using a transcriptional inhibitor,

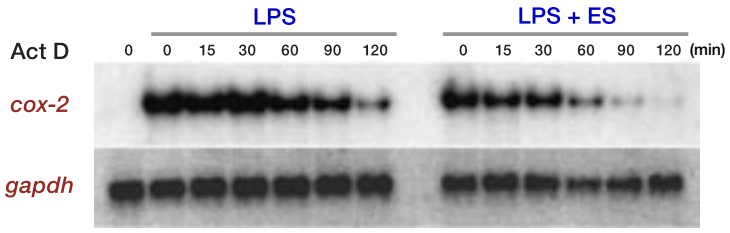

Fig. 5. Destabilization of LPS-induced *cox-2* mRNA by ES products. RAW 264.7 cells were left untreated or pre-incubted with ES products (5 μg/mL) prior to medium change. The cells were then stimulated with LPS (100 ng/mL) for 3 h and treated with actinomycin D (10 μg/mL). Total RNA was obtained at different time points (0, 15, 30, 60, 90 and 120 min) after the addition of actinomycin D. Total RNA was obtained from the cells and was subjected to Northern blot analysis. The *cox-2* mRNA expression was quantified and was normalized to *gapdh*. One representative experiment of three is shown. Act D, actinomycin D.

actinomycin-D. As shown previously, the macrophages were treated with ES products for 6 h, washed, and stimulated with LPS for 3 h. Then the cells were further treated with actinomycin D (10 μg/mL) at varying intervals. Total RNA was subjected to Northern blot hybridization to examine the remaining *cox-2* mRNA. As seen in Fig. 5, *cox-2* mRNA was degraded rapidly in macrophages treated with ES products, in comparison to cells without treatment of ES products. The *cox-2* mRNA levels were normalized to *gapdh* mRNA levels. The remaining *cox-2* mRNA level at 90 min was 43% in the absence of ES products or 17% in the presence of ES products. The level at 120 min was 25% in the absence of ES products or 3% in the presence of ES products. ES products reduced the stability of *cox-2* mRNA.

Discussion

Some parasites such as filarial nematodes secrete immunomodulatory molecules that protect the organism (Maizels et al., 2001; Harnett e al., 2004). The cox-2 isozyme has been known as one of the potent inflammatory factors of host inflammation through PGs (Vane and Booting, 1998; Kim et al., 2004). We found that ES products from plerocercoids of *S. erinaceieuropaei* inhibit *cox-2* gene expression in LPS-activated macrophages. Although AgC10, a mucin from *Trypanosoma cruzi*, a protozoan parasite, is reported to destabilize *cox-2* mRNA (Alcaide and Fresno, 2004), this is the first report that shows that the parasitic helminth suppresses *cox-2* gene expression.

It is well known that PGE_2 potentiates LPS-induced *cox-2* mRNA expression via an adenyryl cyclase/cAMP-dependent pathway (Hinz et al., 2000). As the plerocercoids released PGE_2 in the supernatants of the in vitro culture (Fukushima et al., 1993; Gao et al., 1998), we also examined the effect of ES products on *cox-2* mRNA expression stimulated by the cAMP-dependent pathway. Stimulation by dbcAMP, a cell-permeable cAMP analogue, could not induce *cox-2* gene expression, but enhanced the induction of *cox-2* mRNA by LPS, and the ES products suppressed the enhanced *cox-2* mRNA expression. These results suggest that ES products may inhibit the autocrine feed-forward loop of PGE_2 in LPS-stimulated macrophages around the plerocercoids of *S. erinaceieuropaei*.

In agreement with other studies (Chanmugam et al., 1995; Hwang et al., 1997), we showed that ERK1/2 and p38 MAPK pathways are crucial for *cox-2* gene expression and protein production in LPS-activated RAW 264.7 macrophages. Of note, PD98059 (an inhibitor for the ERK1/2 pathway) and SB203580 (an inhibitor of the p38 MAPK pathway) additively suppressed gene expression (Figs. 3 and 4). The ES products and both of MAPKs inhibitors also additively affected *cox-2*

gene expression (Fig. 4). Recently, we reported that ES products suppress TNF-α mRNA expression by inhibiting ERK1/2 and p38 MAPK pathways in RAW 264.7 macrophages stimulated with LPS (Dirgahayu et al., 2002). We also showed that ES products do not affect LPS-induced nuclear translocation of NF-κB by electrophoretic mobility shift assay (Dirgahayu et al., 2002), while the activation of NF-κB is important for the induction of *cox-2* mRNA in the LPS-activated monocyte-macrophage lineage (D'Acquisto et al., 1998). Although the mechanism of suppression by ES products needs to be investigated further, these lines of data suggest the possibility that ES products suppress LPS-induced *cox-2* gene expression by inhibiting ERK1/2 and p38 MAPK pathways.

Recently, p38 MAP kinase has been reported to play a major role in mRNA stabilization through AU-rich elements in the 3'-untranslated regions (Winzen et al., 1999; Lasa et al., 2000 and 2001). The 3'-untranslated region of *cox-2* contains 22 AUUUA motifs that are recognized to be important determinants of mRNA instability (Chen and Shyu, 1995; Zubiaga et al., 1995). We observed that ES products reduce *cox-2* mRNA stability (Fig. 5), and suppose that ES products destabilize *cox-2* mRNA by inhibiting the phosphorylation of p38 MAPK.

The present investigation showed that ES products suppress *cox-2* mRNA expression and cox-2 protein production, which may attenuate inflammation around the plerocercoids.

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