Supplementary Data

Supplementary Methods

Reverse transcriptase-polymerase chain reaction for NQO1 expression

Total RNA was extracted from RAW 264.7 cells using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. cDNA was synthesized from 1 μg of RNA using *AccuPower*® RT PreMix of Bioneer (Daedeok-Gu, Daejeon, Korea) Premix. Polymerase chain reaction (PCR) amplification was carried out on cDNA equivalent to 100 ng of starting mRNA, using specific oligonucleotide primers for NQO1 (forward, 5′-CCATTCTGAAAGGCTGGTTTG-3′; reverse, 5′-CTAGCTTTGATCTGGTTGTC-3′) and for β-actin (forward, 5′-GACAACGGCTCCGGCATGT-3′; reverse, 5′-GCAACATAGCACAGCTTCT-3′). The reaction mixture was subjected to 35 cycles of PCR amplification as follows: denaturation at 94°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 1 min. The PCR products were electrophoresed on 1.8% ethidium-bromide-stained agarose gels and visualized using a gel documentation system (GelDoc XR; BioRad, Inc., Hercules, CA).

Western blot analysis

The method for western blot analysis was described in the main article in detail.

Antioxidant redox element-luciferase activity

Please see the main article for the method.

Macrophage collection from peritoneum of HO-1$^{-/-}$ mice

Thioglycollate (2.5 ml) was injected (i.p.) to HO-1$^{-/-}$ mice (9–10 weeks) 3 days before harvesting peritoneal macrophages as reported previously (1). In brief, mouse peritoneal macrophage cells were harvested by lavage with 6 ml of cold Hank's balanced salt solution (Wako Pure Chemical Industry, Osaka, Japan) from the peritoneal cavities. The cells were washed twice and suspended in the culture medium to give 10^6 cells/ml. Five hundred microliters of the cell suspension was dispensed into a 1.5-ml tube and incubated for 2 h at 37°C in 5% CO_2. The culture medium was removed gently by aspiration and changed to a fresh culture medium. For cell culture, RPMI-1640 (Wako Pure Chemical Industry) supplemented with 5% inactivated fetal bovine serum was used. All cells were suspended in the culture medium, plated onto a 24-well culture plate at a dose of 6×10^5 cells per well (final volume, 0.6 ml), and cultured in a humidified chamber at 37°C with 5% CO_2 and 95% air. The 5-h culture was washed three times with Hanks' balanced salt solution to remove non-adherent cells, and only the adherent cells were used for the macrophage culture.

Cecal ligation and puncture-induced sepsis

Please see the main article for the method.

Supplementary Results

Ethyl pyruvate augments lipopolysaccharide-induced heme oxygenase-1 expression in RAW 264.7 cells by activation of p38 mitogen-activated protein kinase

We found ethyl pyruvate (EP)-induced heme oxygenase-1 (HO-1) upregulation through p38 mitogen-activated protein kinase (MAPK) activation. However, previous studies have shown that EP inhibits the activation of p38 MAPK in RAW 264.7 cells incubated with lipopolysaccharide (LPS) (3). In addition, LPS also was shown to induce HO-1 mRNA and protein in RAW 264.7 cells, which were dependent on PI3K/Akt and p38 MAPK (2). The time course of HO-1 induction by EP in the presence of LPS was almost the same by EP alone (Supplementary Fig. S1A). As shown in Supplementary Figure S1B, LPS induced HO-1 expression, where EP further increased the expression of HO-1. Likewise, LPS increased phosphorylation of p38, in which addition of EP further increased the activation of p38 (Supplementary Fig. S1C).

EP intensifies LPS-activated NF-E2-related factor 2-antioxidant redox element-luciferase activity, which was dependent on p38 MAPK

As LPS activates NF-E2-related factor 2 (Nrf2)/HO-1 through p38 MAPK and PI3K/Akt signals (2) and EP also showed to activate p38 MAPK in the present study, it is necessary to check the antioxidant redox element (ARE)-luciferase activity by EP in the presence of LPS. We found that LPS increased ARE-luciferase activity, which was further increased by EP (Supplementary Fig. S2A). When pretreated with SB203580 (10 m*M*), the increased luciferase activity by EP in the presence of LPS was significantly inhibited (Supplementary Fig. S2B), indicating that p38 MAPK may play a crucial role for both LPS- and EP-activated HO-1 induction (through Nrf2-ARE-luciferase activity) in RAW 264.7 cells.

EP activates NQO1, *another Nrf2 target gene*

Because EP induced HO-1 through Nrf2 activation, we investigated to confirm that Nrf2 is a target for EP by checking the expression of another Nrf2 target gene, *NQO1*. As shown in Supplementary Figure S3, EP increased *NQO1* mRNA expression starting from 2 h and reached a peak at 4 h and maintained until 12 h (Supplementary Fig. S3A). The increased expression of *NQO1* was significantly diminished by siNrf2 transfection (Supplementary Fig. S3B). The control siRNA and siNrf2 RNA did not influence the expression of *NQO1*.

EP did not inhibit inducible nitric oxide synthase and high-mobility group box 1 expression in LPS-activated peritoneal macrophages from HO-1$^{-/-}$ mice

So far, we showed that the inhibitory effect on LPS-induced inducible nitric oxide synthase (iNOS) and high-mobility group box 1 (HMGB1) expression by EP depends on HO-1

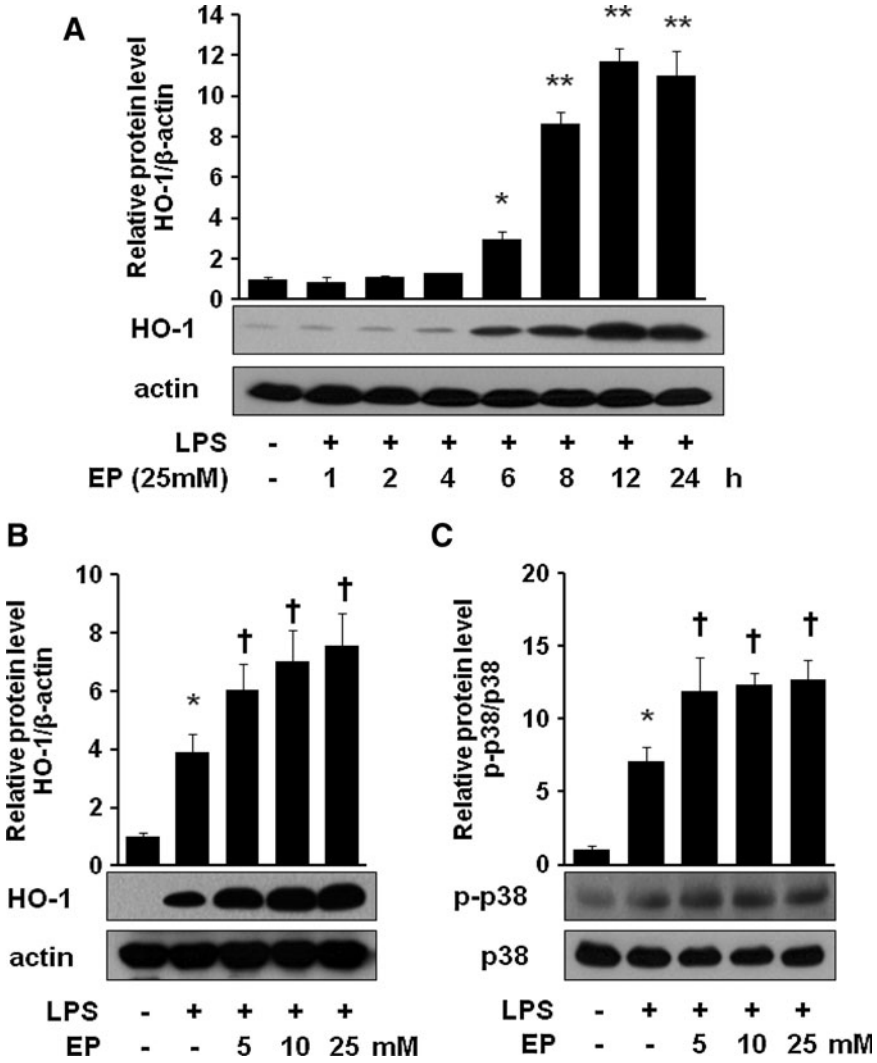
induction; therefore, we were curious whether EP inhibits LPS-activated iNOS and HMGB1 expression in macrophages from HO-1 gene–deleted mice. Supplementary Figure S4 clearly shows that LPS significantly increased expression of iNOS and HMGB1, where addition of EP failed to reduce the expression of iNOS and HMGB1, reinforcing the importance of HO-1 for anti-inflammatory action of EP.

Cecal ligation and puncture by itself increases p38 phosphorylation in lung tissues, which was further augmented by administration of EP

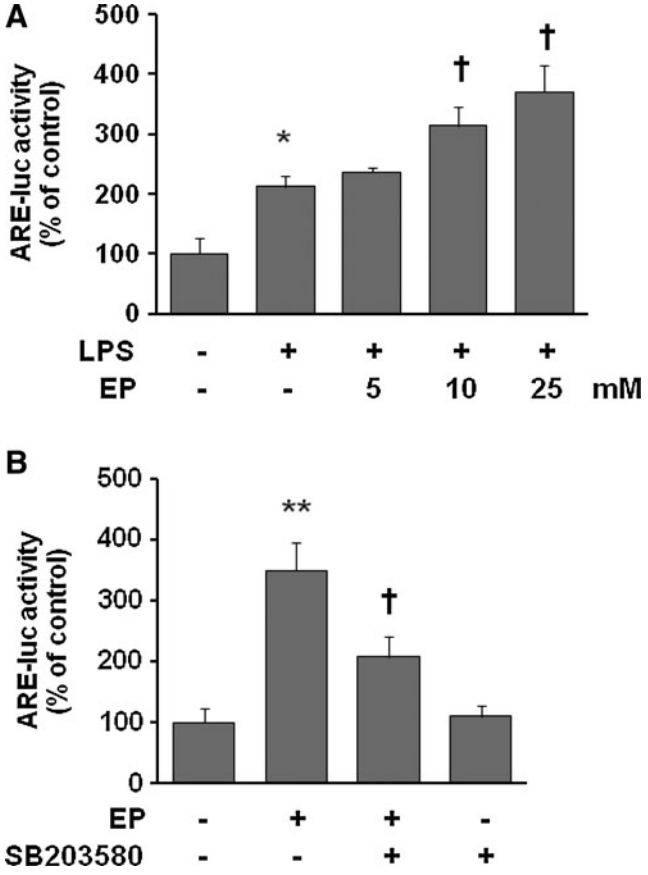
So far, we showed EP further augmented LPS-induced p-p38 expression in RAW 264.7 cells *in vitro*; therefore, we investigated whether administration of EP also increases p-p38 in lung tissues of cecal ligation and puncture (CLP)-induced septic mice. As shown in Supplementary Figure S5, CLP increased p-p38, which was further increased by EP treatment.

Supplementary References

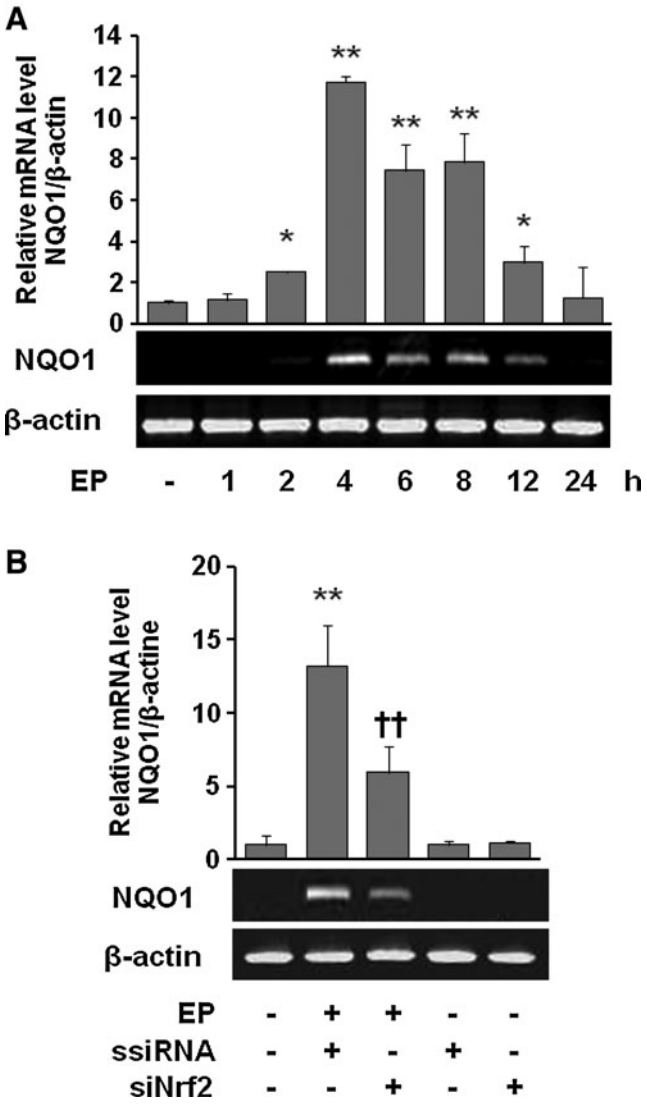
1. Jun CD, Choi BM, Hoon R, Um JY, Kwak HJ, Lee BS, Paik SG, Kim HM, and Chung HT. Synergistic cooperation between phorbol ester and IFN-gamma for induction of nitric oxide synthesis in murine peritoneal macrophages. *J Immunol* 153: 3684–3690, 1994.
2. Pischke SE, Zhou Z, Song R, Ning W, Alam J, Ryter SW, and Choi AM. Phosphatidylinositol 3-kinase/Akt pathway mediates heme oxygenase-1 regulation by lipopolysaccharide. *Cell Mol Biol (Noisy-le-grand)* 51: 461–470, 2005.
3. Ulloa L, Ochani M, Yang H, Tanovic M, Halperin D, Yang R, Czura CJ, Fink MP, and Tracey KJ. Ethyl pyruvate prevents lethality in mice with established lethal sepsis and systemic inflammation. *Proc Natl Acad Sci U S A* 99: 12351–12356, 2002.



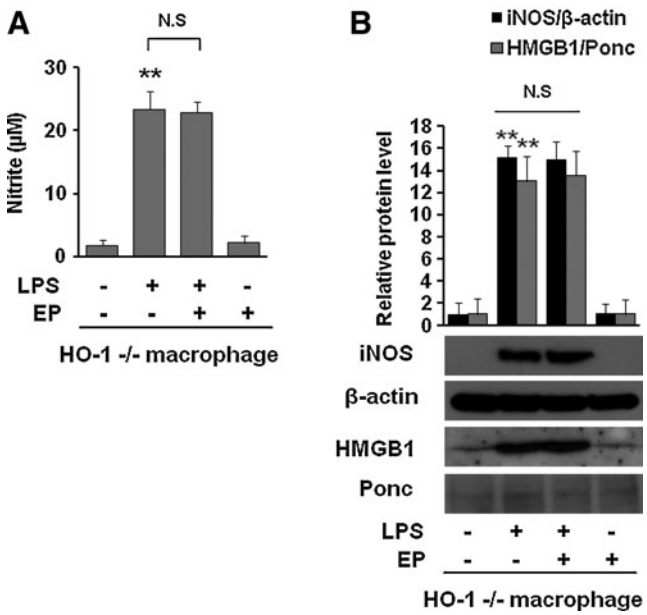
SUPPLEMENTARY FIG. S1. EP reinforces LPS-induced HO-1 expression and phosphorylation of p38 MAPK. Cells were treated with EP (5, 10, and 25 m*M*) in the presence or absence of LPS. To see the time course of HO-1 induction by EP in the presence of LPS, cells were incubated with 25 m*M* EP along with LPS (1 μg/ml) for 1, 2, 4, 6, 8, 12, and 24 h **(A)**. After incubation with 8 h **(**HO-1 induction, for **B)** or 6 h **(**p-p38, for **C)**, cells were harvested and subjected to western blot as described in the Materials and Methods section. Data shown are representative blots with similar results of three independent experiments. Data are represented as mean ± SD of three independent experiments. Significance compared to control, $^{*}p < 0.05$ and $^{**}p < 0.01$; significance compared to LPS, $^{\dagger}p < 0.05$. EP, ethyl pyruvate; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase.



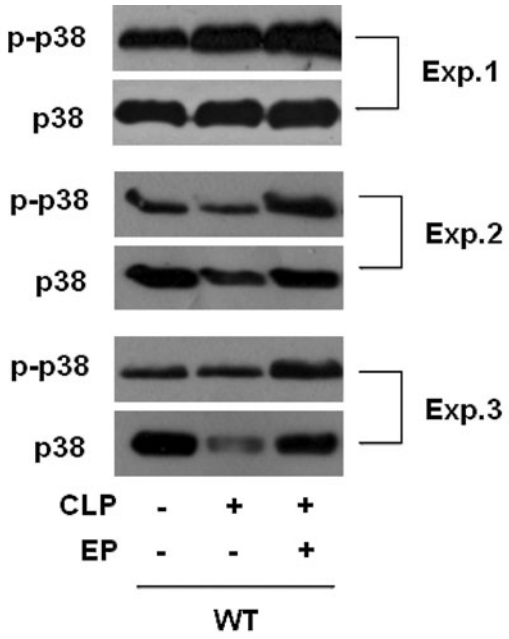
SUPPLEMENTARY FIG. S2. EP intensifies LPS-activated Nrf2-ARE-luciferase activity by a p38 MAPK-dependent manner. Cells were transiently transfected with the ARE-luc vector, and transfected cells were treated with different concentrations of EP (0, 5, 10, and 25 m*M*) in the presence or absence of LPS. To see the effect of the p38 MAPK inhibitor, transfected cells were pretreated 1 h before addition of LPS (1 μg/ml) + EP (25 m*M*) **(A)**. To see whether SB203580 by itself affects the ARE-luciferase activity, cells were pretreated with or without SB203580 for 1 h. After that, cells were further incubated for 1 h by addition of EP (25 m*M*) or not depending on experimental purposes **(B)**. Then, cells were harvested and subjected to luciferase assay as described in the Materials and Methods section. Data are represented as mean ± SD of three independent experiments. Significance compared to control, $^{*}p < 0.05$ and $^{**}p < 0.01$; significance compared to LPS or EP, $^{\dagger}p < 0.05$. Nrf2, NF-E2-related factor 2; ARE, antioxidant redox element.



SUPPLEMENTARY FIG. S3. Nrf2 is a target gene for EP. For RNA experiments, cells were treated with EP at the indicated time points **(A)**. Cells were transfected with control RNA (scramble) or siNrf2 RNA, and treated with EP for 4 h **(B)**. After incubation, total RNA was extracted and subjected to reverse transcriptase-polymerase chain reaction analysis for the measurement of *NQO1* mRNA expression. Data shown are representative blots with similar results of three independent experiments. Data are represented as mean ± SD of three independent experiments. Significance compared to control, $^{*}p < 0.05$ and $^{**}p < 0.01$; significance compared to EP + ssiRNA, $^{\dagger\dagger}p < 0.01$.



SUPPLEMENTARY FIG. S4. EP fails to inhibit iNOS and HMGB1 expression in peritoneal macrophages from HO-1$^{-/-}$ mice in response to LPS. Peritoneal macrophages were pretreated with or without EP (25 m*M*) for 1 h. The cells were stimulated with LPS (1 μg/ml) for 16 h. The culture medium was collected for nitrite concentration **(A)** or HMGB1 **(B)**. Then, the cells were lysed and harvested and subjected to western blot for iNOS detection **(B)**. Data shown are representative blots with similar results of two independent experiments. Data are represented as mean ± SD of two independent experiments. $^{**}p < 0.01$ compared to control. N.S., not significant; iNOS, inducible nitric oxide synthase; HMGB1, high-mobility group box 1.



SUPPLEMENTARY FIG. S5. Administration of EP increases p38 phosphorylation in lung tissues of CLP-induced septic mice. Balb/c mice were treated with saline (i.p., $n = 5$) or EP (40 mg/kg i.p., $n = 5$) at 0 and 12 h after onset of sepsis (CLP). Twenty-four h later, mice were killed under anesthesia and vital organs, including lung tissues, were isolated. Shown are p-p38 western blot examples of three independent experiments (Exp. 1, 2, and 3) of lung tissues of sham control and CLP-induced septic mice treated with or without EP. CLP operation by itself tended to increase p-p38 expression, where EP treatment further increased p-p38 expression. There are variations in the level of p-p38 expression depending on each mouse, but it is clear that administration of EP further augmented the phosphorylation of p38. CLP, cecal ligation and puncture.