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Suppression of inducible nitric oxide synthase and cyclooxygenase-2 gene expression by 22(R)-hydroxycholesterol requires *de novo* protein synthesis in activated macrophages.

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Abstract

Liver X receptors (LXRs) play an important role in lipid metabolism. Recently, a role for these proteins was identified in suppressing the inflammatory response. However it is not known whether the natural ligands of LXRs, e.g. 22(R)-hydroxycholesterol (22R-HC), can suppress the inflammatory response after the onset of inflammation. We demonstrate here that treatment of lipopolysaccharide-activated RAW 264.7 macrophages with 22R-HC markedly suppressed nitric oxide (NO) production and inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA expression. Additionally, 22R-HC did not affect the DNA binding activity of NF-κB, AP-1 and C/EBP(s), important transcriptional factors for iNOS and COX-2 genes expression. Furthermore iNOS and COX-2 mRNA suppression by 22R-HC was diminished by cellular treatment with cycloheximide. These results suggest that 22R-HC suppresses the expression of iNOS and COX-2 genes through *de novo* protein synthesis of an unidentified protein in LPS-activated macrophages.

Introduction

Liver X receptors (LXRs), a family of nuclear receptors, heterodimerize with retinoid X receptor (RXR) and bind specific elements (LXREs) characterized by direct repeats spaced by four nucleotides [1-4].The LXR signaling pathway is thought to play an important role in lipid metabolism [5-12]. Recent data suggests that the LXR pathway antagonizes the NF-κB signaling pathway and inihibits the expression of inflammatory genes downstream of NF-κB [13,14].

22(R)-hydroxycholesterol (22R-HC) is an intermediate produced during the biosynthesis of pregnenolone from cholesterol in cytochrome p450 C27 side chain cleavave [15]. As 22R-HC binds directly to LXRs and specifically activates LXR transcriptional activity [5, 6, 16]. In addition, when macrophages derived from wild-type mice were pretreated with 22R-HC, LPS-induced iNOS expression was inhibited. However, when macrophages derived from LXRαβ-/- mice were pretreated with 22R-HC, LPS-induced iNOS expression was not inhibited [13]. These results suggest that 22R-HC is one of natural

ligands for LXRs and might have an anti-inflammatory property through LXR pathway in macrophages.

In most clinical situation, inflammations likely exists at the time of therapy, and the possible clinical application of anti-inflammatory is limited if compounds cannot suppress pre-existing inflammation. It is not known whether 22R-HC can suppress the inflammatory response, even if the treatment is initiated shortly after the onset of inflammation.

Expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) is induced shortly after the onset of inflammation and these protein play critical roles in the tissue response to injury and infection [18-20]. Although the activity of iNOS and COX-2 is essential during states of infection and wound healing, aberrant or excessive expression of either iNOS or COX-2 has been implicated in the pathogenesis of many disease processes, such as septic shock, rheumatoid arthritis and atherosclerosis [21-23].

In this study, we examined the effect of 22R-HC treatment on iNOS and COX-2 mRNA expression

after the onset of inflammation using RAW264.7 macrophages that are fully activated by LPS. In this model, 22R-HC repressed NO production and iNOS mRNA expression even when macrophages were fully activated by LPS. In addition, we found that 22R-HC also repressed COX-2 mRNA expression and the inhibitory effect of 22R-HC on iNOS and COX-2 mRNA expression required *de novo* protein synthesis.

Materials and Methods

Reagent. 22(R)-hydroxycholesterol, dexamethasone, RU486, cycloheximide and lipopolysaccharide (LPS) from *Salmonella typhimurium* were purchased from Sigma (St. Louis, MO). [α-^{32}P]dCTP (~220TBq/mmol) was purchased from Amersham Biosciences Corp. (Piscataway, NJ).

Cell Culture. RAW264.7 cells, which are murine monocyte/macrophage cell line, were obtained from the American Type Culture Collection (Manassas, VA). RAW264.7 cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS; Biological Industries, Ashrat, Israel) plus penicillin (100 units/ml) and streptomycin (100 μg/ml). DMEM medium and Penicillin-Streptomycin were purchased from Gibco/Invitrogen corp. (Carlsbad, CA).

Nitrite Assays. At the indicated time points, the culture medium was collected for nitrite measurement, which was used as a measure of NO production. Culture medium (100 μl) was incubated with same volume of Griess reagent (0.1% *N*-1-(napthyl)ethylenediamine dihydrochloride, 1% sulfanilamide and

2.5% H_3PO_4), and the absorbance was measured at 550 nm [24]. The concentration of nitrite was calculated with sodium nitrite as a standard.

RNA analysis. Total RNA was extracted from RAW264.7 cells using Trizol reagent (Invitrogen, Tokyo, Japan). For Northern blots, total RNA (20 μg/lane) was separated on 1% formaldehyde agarose gels, transferred to Hybond XL nylon membranes (Amersham Biosciences). After UV cross-linking, the membranes were prehybridized, hybridized with a specific cDNA probe for iNOS, COX-2 or β-actin as below, washed according to the manufacture's instructions of Hybond HL and exposed to Hyperfirm MP (Amersham Biosciences). The levels of iNOS mRNA, COX-2 mRNA and β-actin mRNA were calculated on the basis of signals as measured by an image analyzer, BAS2000 (Fuji Photo Film. Co., Tokyo, Japan). The cDNAs for murine iNOS and murine β-actin mRNA were obtained from total RNA of mouse peritoneal macrophages by reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously [25], using the following primers: iNOS Fwd, 5'-cacatctggcagaatgagaagctg-3'; iNOS Rev,

5’-acacttcgcacaaagcagggcac-3’; β-actin Fwd, 5’-gagagggaaatcgtgcgtga-3’; β-actin Rev, 5’-acacttcgcacaaagcagggcac-3’. The cDNA fragment of murine iNOS or murine β-actin was subcloned in the pCRII-TOPO vector (Invitrogen) and their nucleotide sequences were confirmed by LI-COR DNA sequencer 4200 (LI-COR, Lincoln, NE) (iNOS: GenBank accession number U43428, β-actin: GenBank accession number M12481). For iNOS and β-actin cDNA probes, a 1.4-kb insert of iNOS in the pCRII-TOPO vector was digested with EcoRI, and a 0.45-kb insert of β-actin in the pCRII-TOPO vector was digested with EcoRI. For COX-2 cDNA probes, a 1.6-kb insert of COX-2 in the pBluescript KS vector was digested with HindIII (GenBank accession number M88242). The digested cDNA fragments of iNOS, COX-2 or β-actin were separated on 1x TAE agarose gels, purified by QIAquick gel extraction kit (Qiagen, Tokyo, Japan) and labeled with [α-^{32}P]dCTP using Rediprime II random primer labeling system (Amersham Biosciences).

Preparation of nuclear protein extracts and electrophoretic mobility shift assays (EMSAs).

RAW264.7 cells were plated at a density of 2 X 10^6 cells in 10 cm diameter dishes, activated, harvested, and resuspended in hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Complete EDTA free; Roche Diagnostics, Tokyo, Japan)) for 15 min on ice, then vortexed for 10 s with 0.6% Nonidet P-40. Nuclei were separated from cytosol by centrifugation at 12,000 X *g* for 60 s and were resuspended in buffer C (10 mM HEPES, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail) and briefly sonicated on ice. Nuclear extracts were obtained by centrifugation at 12,000 X *g* for 20 min. Protein concentration was measured by the method of Bradford [26] by using the protein dye reagent (Bio-Rad Laboratories, Tokyo, Japan). For binding reactions, nuclear extracts (5 μg of protein) were incubated in 25 μl of total reaction volume containing 20 mM HEPES, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, 200 μg/ml bovine serum albumin, and 1.25 μg of poly(dI-dC) for 15 min at 4 ^{o}C. The ^{32}P-labeled oligonucleotide probe was

added to the reaction mixture and incubated for 20 min at room temperature. The reaction products were analyzed by electrophoresis in 8% polyacrylamide gel with 0.25 X TBE buffer. The gel was dried and analyzed by autoradiography. The ^{32}P-labeled oligonucleotide probe was prepared as described below. Double-stranded oligonucleotides were prepared by annealing the complementary single strands. Double-stranded oligonucleotides were radiolabeled with Klenow fragment of DNA polymerase I (Roche Diagnostics) and [α-^{32}P]dCTP (Amersham Biosciences) in a fill-in reaction for 5'-protruding ends. EMSAs were performed with ^{32}P-labelled double-stranded oligonucleotide probes representing the consensus sequences of the following transcriptional factors: NF-κB (5'-gatcAGTTGAGGGGACTTTCCCAGGC-3'), AP-1 (5'-gatcCGCTTGATGTCAGCCGGAA-3'), C/EBP (5'-gatcTGCAGATTGCGCAATCTGCA-3').

Statistical analysis. Results were expressed as mean ± S.E.M. When indicated, statistical significance was calculated by analyses of variance supported by the Scheffe multiple comparisons test. Differences

were considered significant at $p<0.05$.

Results

22R-HC inhibits NO production when administered after LPS activation of RAW264.7 macrophages.

We examined whether NO production in LPS-activated RAW264.7 macrophages was repressed following treatment with 22R-HC. 22R-HC was added to the cells 18 h before (-18 h), simultaneously (0 h) or 4 h after LPS activation (4 h), and cells were activated for 24 h with LPS in the absence or presence of 22R-HC. 22R-HC inhibited NO production by LPS-activated RAW264.7 cells under all conditions tested (Fig. 1A). Additionally, 22R-HC-mediated inhibition of NO production was dose dependent (Fig. 1B).

22R-HC suppresses iNOS mRNA expression in LPS-activated RAW264.7 cells.

22R-HC-mediated inhibition of NO production could arise from either a failure to upregulate iNOS expression or an inhibition of iNOS activity. To test the first hypothesis, we examined the effect of

22R-HC treatment on iNOS mRNA expression in LPS-activated macrophages by Northern blotting. LPS activation alone induced marked iNOS expression, and the addition of 22R-HC reduced the levels of iNOS mRNA expression (Fig 2A). Consistent with the inhibitory effect of 22R-HC on NO production, this effect was also dose dependent (Fig. 2A). Inhibition of iNOS expression did not become apparent until 4 h, but the difference in mRNA levels were marked by 8 h (Fig. 2B). The repression rate was 64% at 12 h (Fig. 2C), but this inhibition disappeared at 24 h (data not shown).

22R-HC has no inhibitory effect on DNA binding activity of NF-κB, AP-1 and C/EBP(s).

NF-κB is an important transcription factor regulating LPS-induced iNOS expression [27]. Therefore we examined whether 22R-HC inhibited the DNA binding activity of NF-κB, using an electrophoretic mobility shift assays. 22R-HC had no inhibitory effect on NF-κB DNA-binding activity at all time points (Fig. 3A). 22R-HC also had no effect on DNA binding activity of AP-1 and C/EBP(s), additional transcription factors capable of binding the iNOS promoter and facilitating iNOS genes expression [28]

(Fig. 3B and 3C).

22R-HC inhibition of iNOS mRNA expression requires *de novo* protein synthesis.

Because 22R-HC is thought to be a natural ligand for LXRs, we hypothesized that 22R-HC may induce *de novo* protein(s) synthesis through LXR pathway and the induced protein(s) may suppress the expression of iNOS gene. Using a protein-synthesis inhibitor cycloheximide, we examined by Northern blotting whether the inhibition of iNOS expression required *de novo* protein synthesis. By cellular treatment with cycloheximide, the suppression of iNOS mRNA expression by 22R-HC was diminished (Fig. 4A and 4B). Thus, *de novo* protein synthesis is reuquired for the inhibition of iNOS expression by 22R-HC.

22R-HC-mediated inhibition of NO production is independent of the glucocorticoid receptor pathway.

Glucocorticoid treatment is also known to inhibit NO production, and the glucocorticoid receptor

antagonist RU486 diminishes this effect (28)**.** Since 22R-HC is a biosynthetic precursor of glucocorticoid, 22R-HC might be converted to glucocorticoid in RAW264.7 cells. To rule out this possibility, we examined the effect of 22R-HC treatment on NO production in LPS-activated macrophages in the presence of the glucocorticoid receptor antagonist RU486. RU486 abolished the inhibitory effect of dexamethasone on NO production, but RU-486 did not block the inhibitory effect of 22R-HC on NO production (Fig. 5). Thus, 22R-HC inhibits NO production in LPS-activated RAW264.7 cells independently of glucocorticoid receptor pathway.

22R-HC inhibits COX-2 mRNA expression in LPS-activated RAW264.7 cells.

We examined the effect of 22R-HC on COX-2 mRNA expression in LPS-activated RAW264.7 cells in a manner similar to that used for iNOS. Like iNOS expression, COX-2 mRNA expression was also suppressed by 22R-HC, but, dose response and time course of the repression differed. 22R-HC inhibited COX-2 mRNA expression at 3 μM (Fig. 6A), and COX-2 mRNA expression was suppressed 4 h after

22R-HC administration. This effect was maximal at 8 h (Fig. 6B and 6C). Additionally, COX-2 mRNA expression was also diminished by cellular treatment with cycloheximide even in the presence of 22R-HC (Fig 4A and 4C).

Discussion

In the present study, we clearly demonstrated that 22R-HC inhibits NO production and iNOS and COX-2 mRNA expression in LPS-activated RAW264.7 macrophages. These results suggest that 22R-HC can negatively regulate excess NO and inflammatory prostaglandin production during an inflammatory response, even after the onset of inflammation. This is especially important for the clinical application of anti-inflammatory therapies because, in most cases, treatment is not initiated until after the initiation of the inflammatory response. Sepsis is systemic inflammatory response to infection associated with coagulopathy, multi organ failure, and death [23, 30]. Bacterial toxins induce host cells to release NO, prostaglandins and cytokines, and all of these compounds are likely related to the pathogenesis of sepsis. Although medical treatment is typically initiated after the onset of sepsis, few compounds substantially improve motality after sepsis has begun. However, lysophosphatidylcholine and nicotine are effective even when treatment occur after the induction of peritonitis [31, 32]. Based on the current study, 22R-HC

may also be effective post hoc treatment for sepsis, and this should be investigated using models of sepsis such as cecal ligation and puncture.

Joseph et al. showed that a synthetic LXR agonist repressed the transcriptional activity of NF-κB, but not that of AP-1 in the presence of LXR, using a luciferase reporter assay [13]. It has been shown that the LXR agonist inhibits NF-κB-induced MMP-9 gene expression through LXR activation without affecting NF-κB DNA binding activity [17]. We also observed that 22R-HC did not affect the DNA binding activities of NF-κB, AP-1 and C/EBP. These results suggest that LXR agonists did not affect the DNA binding activity of NF-κB but lowered mRNA levels of two genes regulated by this transcription factor. Castrillo et al. provided evidence that Toll-like receptor (TLR) and LXR signaling pathway may compete for limiting amounts of transcriptional co-activators such as p300 and CBP [33]. It is possible to speculate that suppression of iNOS and COX-2 mRNA expression by 22R-HC may result from the competition of p300 and CBP between TLR and LXR signal pathway. However, since only this molecular mechanism

can not simply explain why the treatment of cycloheximide diminishes the suppression of iNOS and COX-2 mRNA expression, other mechanisms may exist.

22R-HC had no suppressive effect on iNOS and COX-2 mRNA expression in cycloheximide-treated cells. These results suggest that suppression of iNOS and COX-2 mRNA expression by 22R-HC requires *de novo* protein synthesis. It is thought that there are at least two hypotheses about how 22R-HC represses these genes expression thorough *de novo* protein synthesis. As the first hypothesis, 22R-HC might induce unidentified protein(s) that modulate the stability of iNOS and COX-2 mRNA. It has been reported that dexamethasone also inhibits iNOS mRNA expression in LPS-activated RAW264.7 cells [29]. Although the molecular mechanism remains uncertain, dexamethasone appears to reduce iNOS mRNA stability and suppress the accumulation of iNOS mRNA. 22R-HC may also reduce iNOS and COX-2 mRNA stability. As we do not determine whether 22R-HC reduces iNOS and COX-2 mRNA stability, it has two possibilities, transcriptional or posttranscriptional regulation. More investigations will be needed. As the

second hypothesis, 22R-HC might induce unidentified protein(s) that repress transcriptional activity of NF-κB. This repressor(s) might be LXR-induced gene product. Although LXR pathway suppress TLR 3/4 signal pathway, the mechanism remains uncertain. If we could identify this repressor(s), we would be able to understand the crosstalk between LXR and TLR signal pathway.

Recently, roles for nuclear receptor corepressor (N-CoR) and the closely related silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) as the overall regulators of NF-κB transcription activity have been identified [34, 35]. N-CoR- and SMRT-containing complexes is known to interact with Sin 3 and recruit the histone deacetylases that lead to hypoacetylation of histones and transrepression of target transcription factors. It is possible that these complexes may be related to 22R-HC suppression of iNOS and COX-2 mRNA expression. , and this possibility needs to be investigated.

In summary, our results demonstrate that 22R-HC inhibits NO production and iNOS and COX-2 mRNA expression, even when administered after macrophage activation. This inhibitory effect requires *de novo*

protein synthesis through the glucocorticoid receptor independent pathway.

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Figure Legends

Fig. 1

22R-HC inhibits NO production in LPS-activated RAW 264.7 macrophages.

(A) RAW264.7 cells were activated for 24 h with LPS (100 ng/ml) in absence or presence of 22R-HC (12 μM). 22R-HC was added to the cells 18 h before (-18 h), simultaneously (0 h) or 4 h after LPS activation (4 h). (B) RAW264.7 cells were treated with the indicated concentration of 22R-HC (0, 3, 6, 12 μM) for 24 h after being incubated with LPS (100 ng/ml) for 4 h. NO was detected as nitrite in the culture medium. Results were expressed as mean ± S.E.M. (n=9). *$P<0.05$ significantly lower than the samples with LPS activation only.

Fig. 2

22R-HC inhibits iNOS mRNA expression in LPS-activated RAW264.7 macrophages.

(A) RAW264.7 cells were activated with LPS (100 ng/ml) for 4 h and treated with the indicated

concentration of 22R-HC (0, 3, 6 or 12 μM) for 12 h. Total RNA was isolated, and total RNA (20 μg per lane) was analyzed for iNOS mRNA expression levels by Northern blotting. The relative amount of iNOS mRNA was measured by an image analyzer BAS2000 after normalization with that of β-actin. Similar results were obtained in two additional experiments. ND; not detect. NC; negative control. (B) RAW264.7 cells were activated with LPS (100 ng/ml) for 4 h and treated with or without 22R-HC (12 μM) for the indicated time. Total RNA was isolated, and total RNA (20 μg per lane) was analyzed for iNOS mRNA expression levels by Northern blotting. The relative amount of iNOS mRNA was measured by an image analyzer BAS2000 after normalization with that of β-actin. Similar results were obtained in two additional experiments. ND; not detect. NC; negative control. (C) Percent (%) Repression represents the ratio of iNOS mRNA level of RAW264.7 macrophages treated with 22R-HC to that of RAW264.7 macrophages treated without 22R-HC at each time point.

Fig. 3

22R-HC has no effect on DNA-binding activity of NF-κB or AP-1 or C/EBP(s) in LPS-activated RAW264.7 macrophages.

RAW 264.7 cells were activated with LPS (100 ng/ml) for 4 h and then treated with 22R-HC (12μM) for the indicated time. Nuclear extracts were incubated with ^{32}P-labeled oligonucleotides containing consensus binding site for NF-κB or AP-1 or C/EBP and analyzed by electrophoretic mobility shift assay as described in materials and methods. The gels are representatives of three separate experiments with similar result. (A) NF-κB, (B) AP-1, (C) C/EBP(s). NS; non specific band.

Fig. 4

22R-HC inhibition of iNOS mRNA expression requires *de novo* protein synthesis.

RAW264.7 cells were activated with LPS (100 ng/ml) for 4 h and treated with or without 22R-HC (12 μM) or cycloheximide (3.6 μM) for the indicated time. Total RNA was isolated, and total RNA (20 μg per lane) was analyzed for iNOS mRNA expression levels by Northern blotting. The relative amount of iNOS

mRNA was measured by an image analyzer BAS2000 after normalization with that of β-actin. Similar results were obtained in two additional experiments. ND; not detect. NC; negative control.

Fig. 5

22R-HC mediated inhibition of NO production is independent of the glucocorticoid receptor pathway.

RAW264.7 cells were activated with LPS (100ng/ml) and treated with or without RU486 (1μM) for 30min prior to the addition of 22R-HC (12μM) or dexamethasone (1μM) and the cells were further incubated for an additional 24 h. NO was detected as nitrite in the culture medium. Results were expressed as mean ± S.E.M. (n=6). *P<0.05 significantly lower than the samples with LPS activation only. DEX; dexamethasone.

Fig. 6

22R-HC inhibits COX-2 mRNA expression in LPS-activated RAW264.7 macrophages.

RAW264.7 cells were activated with LPS (100 ng/ml) for 4 h and treated with the indicated

concentration of 22R-HC (0, 3, 6 or 12 μM) for 12 h. Total RNA was isolated, and total RNA (20 μg per lane) was analyzed for COX-2 mRNA expression levels by Northern blotting. The relative amount of COX-2 mRNA was measured by an image analyzer BAS2000 after normalization with that of β-actin. Similar results were obtained in two additional experiments. ND; not detect. NC; negative control. (B) RAW264.7 cells were activated with LPS (100 ng/ml) for 4 h and treated with or without 22R-HC (12 μM) for the indicated time. Total RNA was isolated, and total RNA (20 μg per lane) was analyzed for COX-2 mRNA expression levels by Northern blotting. The relative amount of COX-2 mRNA was measured by an image analyzer BAS2000 after normalization with that of β-actin. Similar results were obtained in two additional experiments. ND; not detect. NC; negative control. (C) Percent (%) Repression represents the ratio of iNOS mRNA level of RAW264.7 macrophages treated with 22R-HC to that of RAW264.7 macrophages treated without 22R-HC at each time point.

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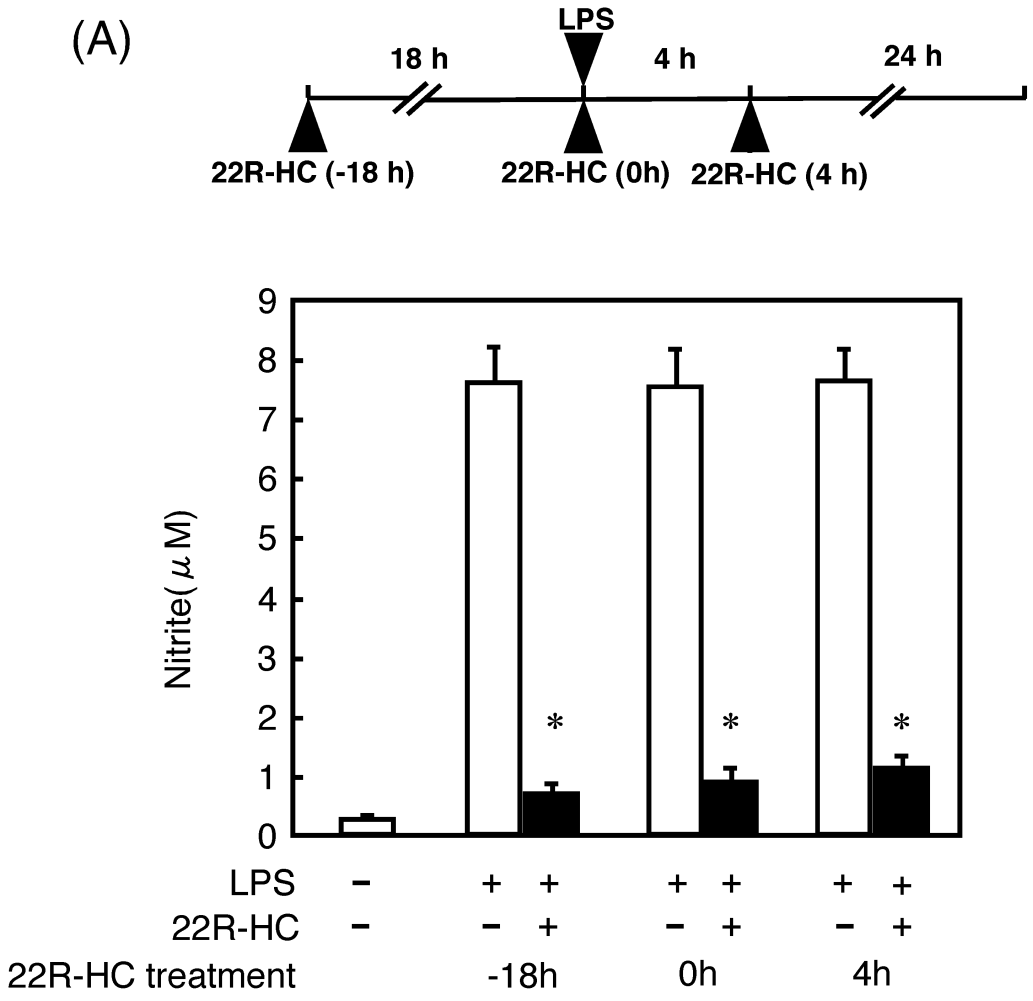
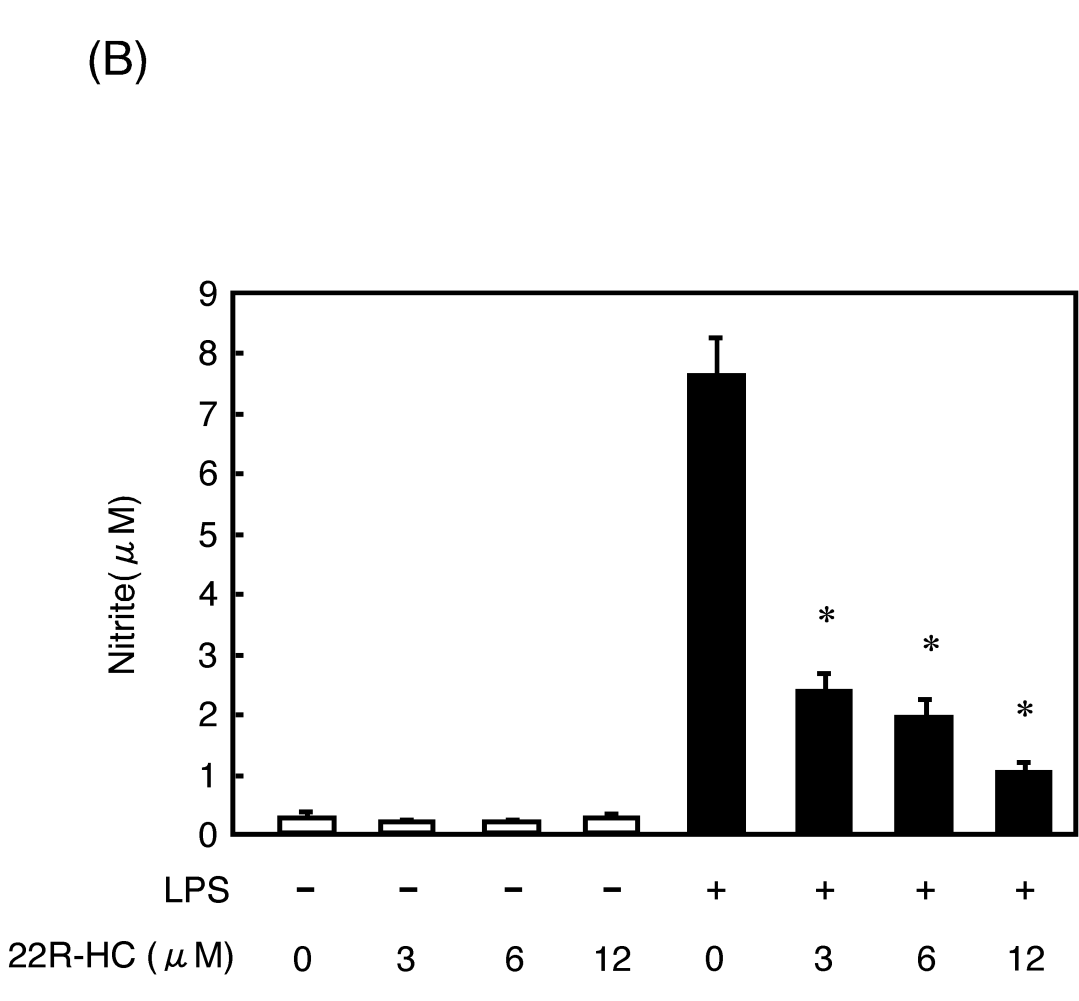
Fig.1


(A)
LPS
18 h
4 h
24 h
22R-HC (-18 h)
22R-HC (0h)
22R-HC (4 h)
Nitrite(μM)
9
8
7
6
5
4
3
2
1
0
*
*
*
LPS - + + + + + +
22R-HC - - + - + - +
22R-HC treatment
-18h
0h
4h


(B)
Nitrite(μM)
9
8
7
6
5
4
3
2
1
0
*
*
*
LPS - - - - + + + +
22R-HC (μM) 0 3 6 12 0 3 6 12

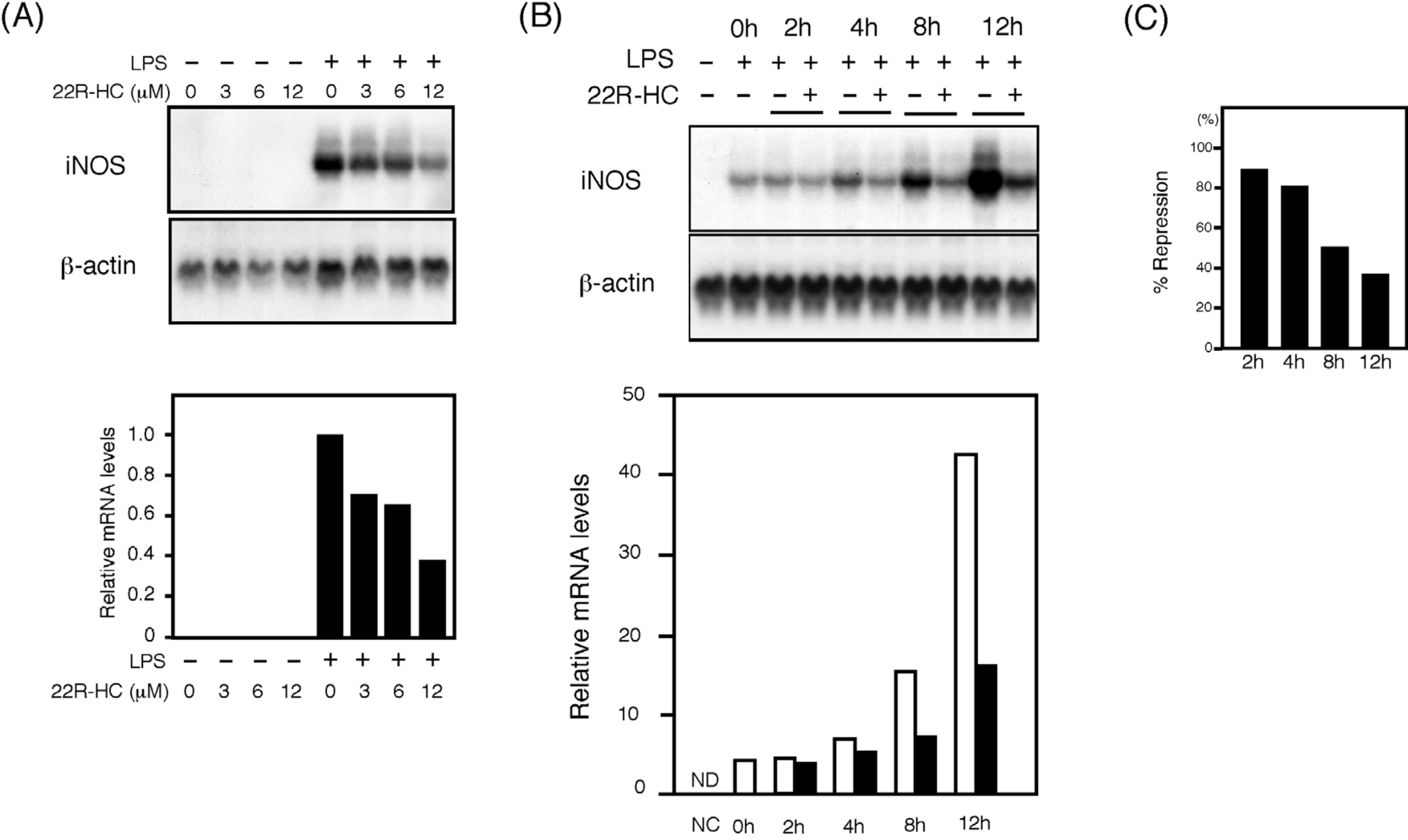

(A)
LPS – – – – + + + +
22R-HC (μM) 0 3 6 12 0 3 6 12
iNOS
β-actin
Relative mRNA levels
1.0
0.8
0.6
0.4
0.2
0
LPS – – – – + + + +
22R-HC (μM) 0 3 6 12 0 3 6 12
(B)
0h 2h 4h 8h 12h
LPS – + + + + + + + + +
22R-HC – – – + – + – + – +
iNOS
β-actin
Relative mRNA levels
50
40
30
20
10
0
ND
NC 0h 2h 4h 8h 12h
(C)
% Repression
(%)
100
80
60
40
20
0
2h 4h 8h 12h

Fig.2

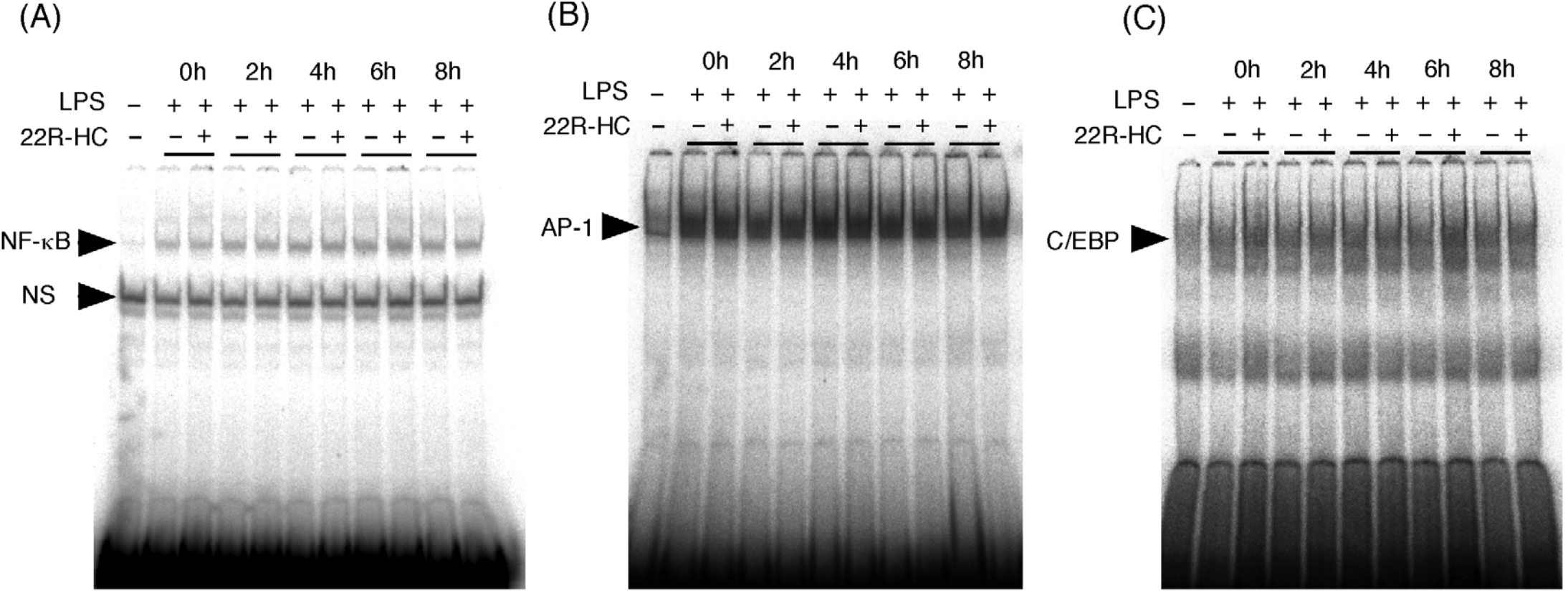

(A)
0h 2h 4h 6h 8h
LPS – + + + + + + + + +
22R-HC – – + – + – + – + – +
NF-κB
NS
(B)
0h 2h 4h 6h 8h
LPS – + + + + + + + + +
22R-HC – – + – + – + – + – +
AP-1
(C)
0h 2h 4h 6h 8h
LPS – + + + + + + + + +
22R-HC – – + – + – + – + – +
C/EBP

Fig.3

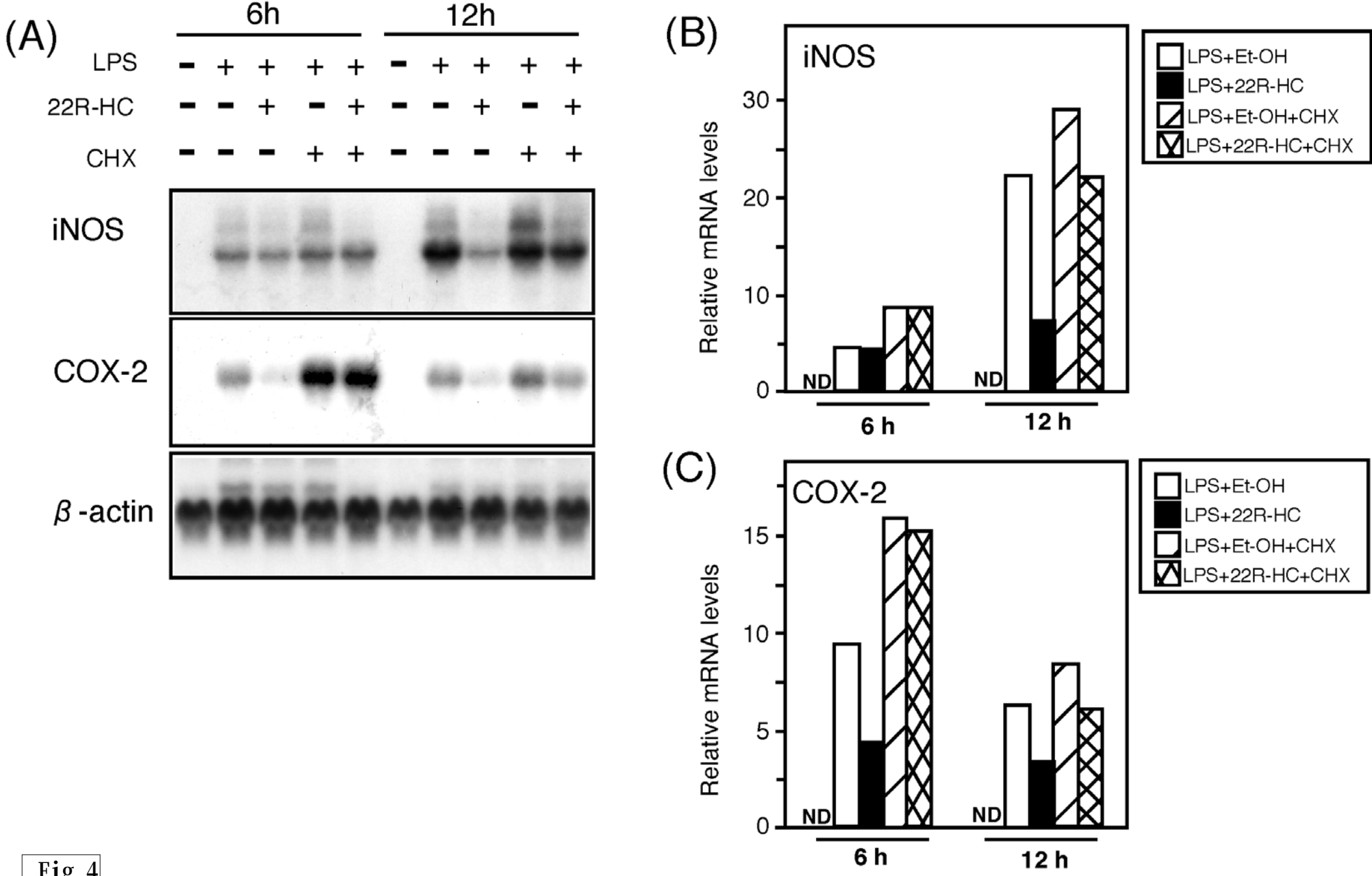
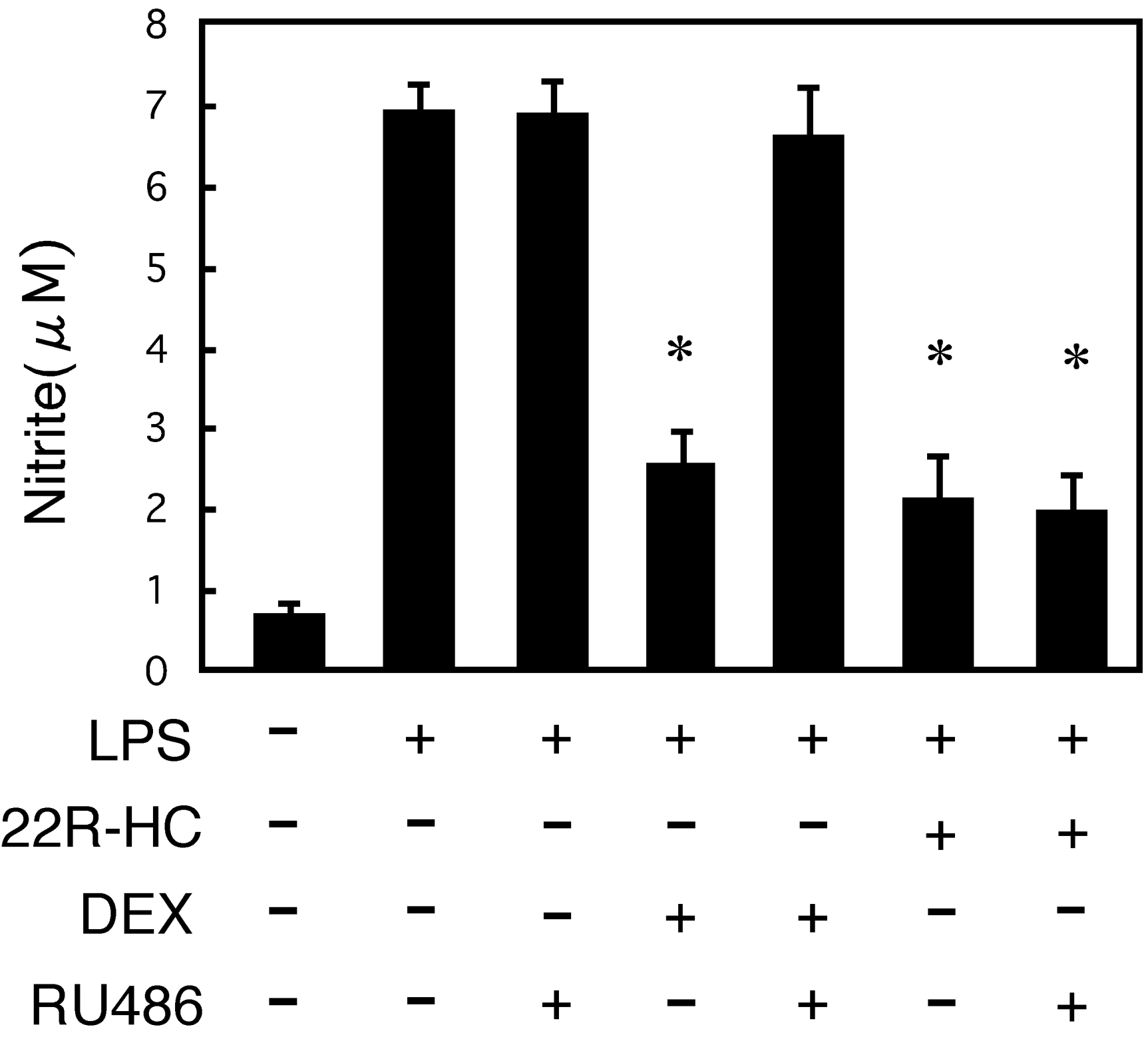

(A)
6h
12h
LPS
22R-HC
CHX
iNOS
COX-2
β -actin
(B)
iNOS
Relative mRNA levels
30
20
10
0
ND
ND
6 h
12 h
LPS+Et-OH
LPS+22R-HC
LPS+Et-OH+CHX
LPS+22R-HC+CHX
(C)
COX-2
Relative mRNA levels
15
10
5
0
ND
ND
6 h
12 h
LPS+Et-OH
LPS+22R-HC
LPS+Et-OH+CHX
LPS+22R-HC+CHX

Fig.4

Fig.5



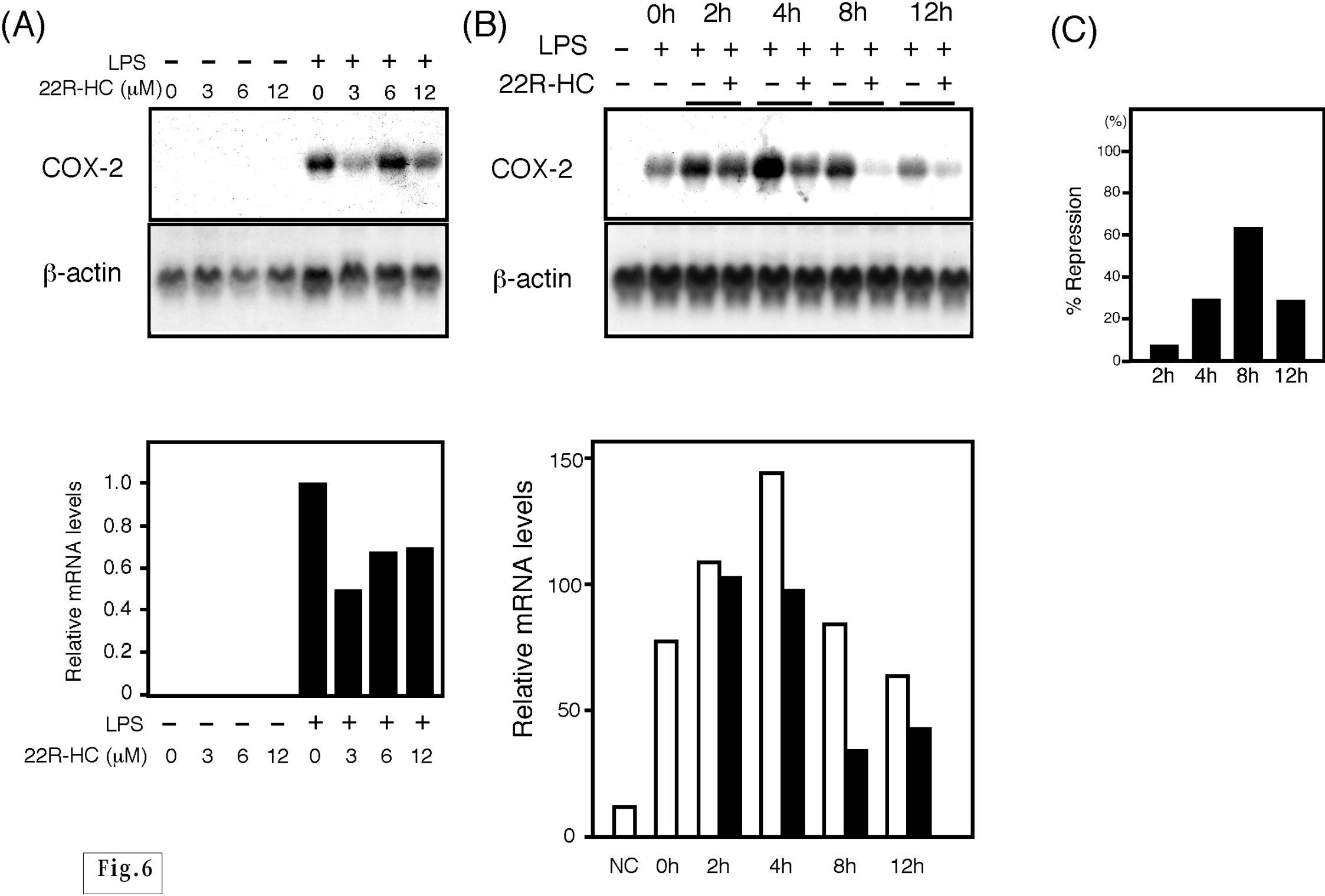

(A)
LPS
22R-HC (μM)
COX-2
β-actin
Relative mRNA levels
(B)
0h 2h 4h 8h 12h
LPS
22R-HC
COX-2
β-actin
Relative mRNA levels
NC 0h 2h 4h 8h 12h
(C)
% Repression
(%)
2h 4h 8h 12h

Fig.6