

## Metformin inhibits expression of the proinflammatory biomarker inducible nitric oxide synthase in hepatocytes

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### ABSTRACT

**Background:** Metformin is used to treat patients with type II diabetes. However, there are few scientific reports on its anti-inflammatory effects. In the inflamed liver, proinflammatory cytokines stimulate liver cells, followed by inducible nitric oxide synthase (iNOS) expression. Excessive NO levels produced by iNOS have been implicated as a factor in liver injury. As a result, it is essential to inhibit iNOS induction to prevent liver injury.

**Objective:** This study aimed to investigate liver protective effects of metformin by examining interleukin (IL)-1 $\beta$ -stimulated hepatocytes.

**Methods:** Primary cultured rat hepatocytes were treated with interleukin (IL)-1 $\beta$  in the presence or absence of metformin. iNOS induction and its signaling pathway were analyzed.

**Results:** Metformin decreased iNOS protein and mRNA expression, resulting in the inhibition of hepatic NO production. Metformin also reduced tumor necrosis factor (TNF)- $\alpha$  and IL-6 mRNA expression. Metformin inhibited an essential signaling pathway for iNOS induction, type I IL-1 receptor upregulation. Transfection experiments revealed that metformin reduced iNOS mRNA levels through both promoter transactivation and mRNA stabilization. Delayed metformin administration after IL-1 $\beta$  addition also inhibited iNOS induction.

**Conclusion:** Metformin affects the induction of inflammatory mediators including iNOS and TNF- $\alpha$ , demonstrating its therapeutic potential for organ injuries, including the liver.

**Keywords:** metformin, inducible nitric oxide synthase, liver injury, primary cultured hepatocytes, type I interleukin-1 receptor, tumor necrosis factor- $\alpha$

## INTRODUCTION

Metformin is generally one of the most effective therapeutics for the treatment of type 2 diabetes because it specifically reduces hepatic gluconeogenesis without increasing insulin secretion, inducing weight gain, or posing a risk of hypoglycemia [1]. The anti-diabetic action of metformin depends on the activation of AMP-activated protein kinase (AMPK), which contributes to a reduction in hepatic gluconeogenesis and an increase in glucose uptake in skeletal muscles [2].

Insulin resistance is one of the major pathophysiological features which is implicated in non-alcoholic fatty liver disease (NAFLD) [3,4], contributing to both the initiation of the disease and progression to advanced forms of NAFLD. NAFLD is one of the most prevalent liver diseases world-wide and it is an increasingly frequent cause of cirrhosis [5 ,6]. The disease encompasses a wide spectrum of liver damage ranging from simple hepatic steatosis and non-alcoholic steatohepatitis (NASH) to liver cirrhosis.

In hepatic disorders, inflammatory cells such as macrophages gather around hepatic stellate (Kupffer) cells and discharge a variety of cytokines. During inflammation, proinflammatory cytokines and nitric oxide (NO), which is produced by inducible nitric oxide synthase (iNOS) in Kupffer cells and hepatocytes, play an important role as factors in liver injury [7]. iNOS expression was enhanced in the liver after feeding with a high fat diet [8, 9]. Given the critical role of iNOS in the development of liver fibrosis, long-term inhibition of iNOS may have therapeutic benefits in liver steatosis [10].

In animal liver injury models caused by various insults, such as ischemia–reperfusion, partial hepatectomy, and endotoxin shock, we discovered that drugs [11-15] demonstrating liver-protective effects inhibited the induction of iNOS and NO production, and reduced production of various inflammatory mediators, such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6,

and cytokine-induced neutrophil chemoattractant (CINC)-1 (human IL-8 analogue) [11-15]. Additionally, these clinical drugs [13,16,17] also inhibited the induction of iNOS and NO production in primary cultures of rat hepatocytes. Therefore, experiments using cultured hepatocytes revealed that preventing iNOS induction and NO production is an indicator of liver protection [10].

It has recently been demonstrated that metformin alleviates fulminant liver injury in lipopolysaccharide (LPS)/D-galactosamine-challenged mice [18]. Metformin-mediated amelioration of LPS/D-galactosamine induced hepatitis in mice, which correlated with reduced iNOS expression and NO production in macrophages [19, 20], although the mechanisms involved in the action of metformin are unclear.

In the present study, we examined whether metformin affects the induction of iNOS in IL-1 $\beta$ -stimulated hepatocytes and the mechanisms involved in the action of metformin.

## MATERIALS AND METHODS

### *Materials*

Metformin (Wako Pure Chemicals, Osaka, Japan) was dissolved in Williams' medium E (WE) and vortexed for 10 min at room temperature. The supernatant was filter-sterilized with a 0.45- $\mu$ m membrane filter (Millipore, Billerica, MA, USA) before use in experiments. Recombinant human IL-1 $\beta$  ( $2 \times 10^7$  U/mg protein) was purchased from My Bio Source (San Diego, CA, USA). Male Wistar rats (200–250 g and 6–7 weeks old) were purchased from Charles River (Tokyo, Japan), kept at 22°C under a 12:12 h light:dark cycle, and received food and water *ad libitum*. All experiments on rats were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. The experiments were approved by the Animal Care Committee of Kansai Medical University.

### *Primary hepatocyte culture*

Hepatocytes were isolated from rats by perfusion with collagenase (Wako Pure Chemicals) [21]. Isolated hepatocytes were suspended in culture medium at  $6 \times 10^5$  cells/mL, seeded into 35-mm plastic dishes (2 mL/dish; Falcon Plastic, Oxnard, CA, USA), and cultured at 37°C in a CO<sub>2</sub> incubator under a humidified atmosphere of 5% CO<sub>2</sub> in air. The culture medium was WE supplemented with 10% newborn calf serum, 10 nM insulin, and 10 nM dexamethasone. After 5 h, the medium was replaced with fresh serum and hormone-free WE, and the cells were cultured overnight before use in experiments. The number of cells attached to the dishes was calculated by counting the number of nuclei [22] and using a ratio of  $1.37 \pm 0.04$  nuclei/cell (mean  $\pm$  SE, n = 7 experiments).

### ***Treatment of cells with metformin***

On day 1, the cells were washed with fresh serum- and hormone-free WE, and incubated with IL-1 $\beta$  (1 nM) in the same medium in the presence or absence of metformin. The doses of metformin used are indicated in the appropriate Figures and their legends.

### ***Determination of NO production and lactate dehydrogenase activity***

The culture medium was used to measure nitrite (a stable metabolite of NO) levels, which reflects NO production, using the Griess method [23]. Culture medium was also used for measurements of lactate dehydrogenase (LDH) activity to reflect cell viability using a commercial kit (Roche Diagnostics, Mannheim, Germany).

### ***Western blot analysis***

Total cell lysates were obtained from cultured cells as described previously, with minor modifications [16]. Briefly, cells ( $1 \times 10^6$  cells/35 mm dish) were lysed with sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (final: 125 mM Tris-HCl, pH 6.8; containing 5% glycerol, 2% SDS, and 1% 2-mercaptoethanol), subjected to SDS-PAGE, and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Immunostaining was performed using primary antibodies against mouse iNOS (Affinity BioReagents, Golden, CO, USA), human I $\kappa$ B $\alpha$ , mouse type I IL-1 receptor (IL-1RI) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rat  $\beta$ -tubulin (internal control; Clone TUB2.1; Sigma Chemical Co., St. Louis, MO, USA). This was followed by visualization with an enhanced chemiluminescence blotting detection reagent (GE Healthcare Biosciences Corp., Piscataway, NJ, USA).

For Akt, total cell lysates prepared from 100-mm dishes ( $5 \times 10^6$  cells/dish) were pre-cleared with protein A (Sigma Chemical Co.) and then mixed with a mouse monoclonal antibody against human Akt1 (Akt5G3; Cell Signaling, Beverly, MA, USA) and protein G-Sepharose (Pharmacia LKB Biotech, Uppsala, Sweden). After incubation overnight at 4°C, immunocomplexes were centrifuged ( $16,000 \times g$  for 5 min). The beads were washed with solubilizing buffer, dissolved in SDS-PAGE sample buffer, and analyzed by western blotting using rabbit polyclonal antibodies against human Akt and phospho-Akt (Ser473) (Cell Signaling) as primary antibodies. For p65, nuclear extracts were immunoprecipitated with an anti-p65 antibody (H286; Santa Cruz Biotechnology). The bands were analyzed by western blotting using an antibody against human NF- $\kappa$ B p65 (BD Transduction Laboratories, Lexington, KY, USA).

### ***Reverse transcriptase-polymerase chain reaction (RT-PCR)***

Total RNA was extracted from cultured hepatocytes using the guanidinium-phenol-chloroform method [24]. For strand-specific RT-PCR analysis, cDNA was synthesized from total RNA using strand-specific primers, and step-down PCR was performed using PC708 (Astec, Fukuoka, Japan),

as previously described [25], with minor modifications. For iNOS (257 bp), TNF- $\alpha$  (275 bp), IL-6 (286 bp), IL-10 (245 bp), CINC-1 (231 bp), IL-1RI (327 bp), and elongation factor-1 $\alpha$  (EF; internal control) (335 bp) mRNA, an oligo(dT) primer was used for RT and the indicated primers sets were used for PCR (Table 1). For the antisense transcript of iNOS (211 bp), the sense primer (5'-TGCCCCTCCCCACATTCTCT-3') was used for RT and the indicated primer set was used for PCR (Table 1). These mRNAs and antisense transcript levels were measured with real-time PCR using the Rotor-Gene Q 2plex HRM (Qiagen, Tokyo, Japan). The Rotor-Gene SYBR Green PCR Kit (Qiagen) was included in the reaction mixture, and the following touchdown protocol was applied: 1 cycle at 95°C for 5 min and 45 cycles at 95°C for 5 s and 60°C for 10 s. The cDNAs for the rat iNOS mRNA and antisense transcript were deposited in the DNA Data Bank of Japan/European Bioinformatics Institute (DDBJ/EMBL)/GenBank under the accession numbers AB250951 and AB250952 respectively.

### ***Electrophoretic mobility shift assay (EMSA)***

Nuclear extracts were prepared and an electrophoretic mobility shift assay was performed as previously described [26]. Briefly, nuclear extracts from hepatocytes (4  $\mu$ g) were mixed with 1  $\mu$ g of poly(dI-dC) and a probe for 20 min at room temperature (total mixture, 15  $\mu$ l). To prepare the double-stranded DNA probe, annealed oligonucleotides harboring a  $\kappa$ B site (5'-AGTTGAGGGGACTTTCCCAGGC-3'; only the sense strand is shown) were labelled with [ $\gamma$ -<sup>32</sup>P]-adenosine-5'-triphosphate (ATP; DuPont-New England Nuclear Japan, Tokyo, Japan) and T4 polynucleotide kinase (Takara Bio Inc., Shiga, Japan). Samples were resolved on a 4.8% polyacrylamide gel, followed by drying and autoradiography. Protein concentrations were measured using the Bradford method [27] with a binding assay kit (Bio-Rad) using bovine serum albumin as a standard.

### ***Transfection and luciferase assay***

Transfection of cultured hepatocytes was performed as previously described [28]. Briefly, hepatocytes were cultured at  $4 \times 10^5$  cells/dish (35  $\times$  10 mm) in WE supplemented with serum, dexamethasone, and insulin for 7 h, before being subjected to magnet-assisted transfection (MATra). The reporter plasmid pRiNOS-Luc-SVpA or pRiNOS-Luc-3'UTR (1  $\mu$ g) and the CMV promoter-driven  $\beta$ -galactosidase plasmid pCMV-LacZ (1 ng) as an internal control were mixed with MATra-A reagent (1  $\mu$ l; IBA GmbH, Göttingen, Germany). After incubation for 15 min on a magnetic plate at room temperature, the medium was replaced with fresh WE containing serum. Cells were cultured overnight, and then treated with IL-1 $\beta$  in the presence or absence of metformin. Luciferase and  $\beta$ -galactosidase activities of cell extracts were measured using PicaGene (Wako Pure Chemicals) and Beta-Glo (Promega, Wisconsin, USA) kits respectively.

**Statistical analysis**

The results shown are representative of three to four independent experiments yielding similar findings. All data are expressed as the mean  $\pm$  SD. Differences were analyzed by the Bonferroni–Dunn test, and a value of  $P < 0.05$  was considered to indicate a significant difference.

**Table 1.** Primers and nucleotide sequences

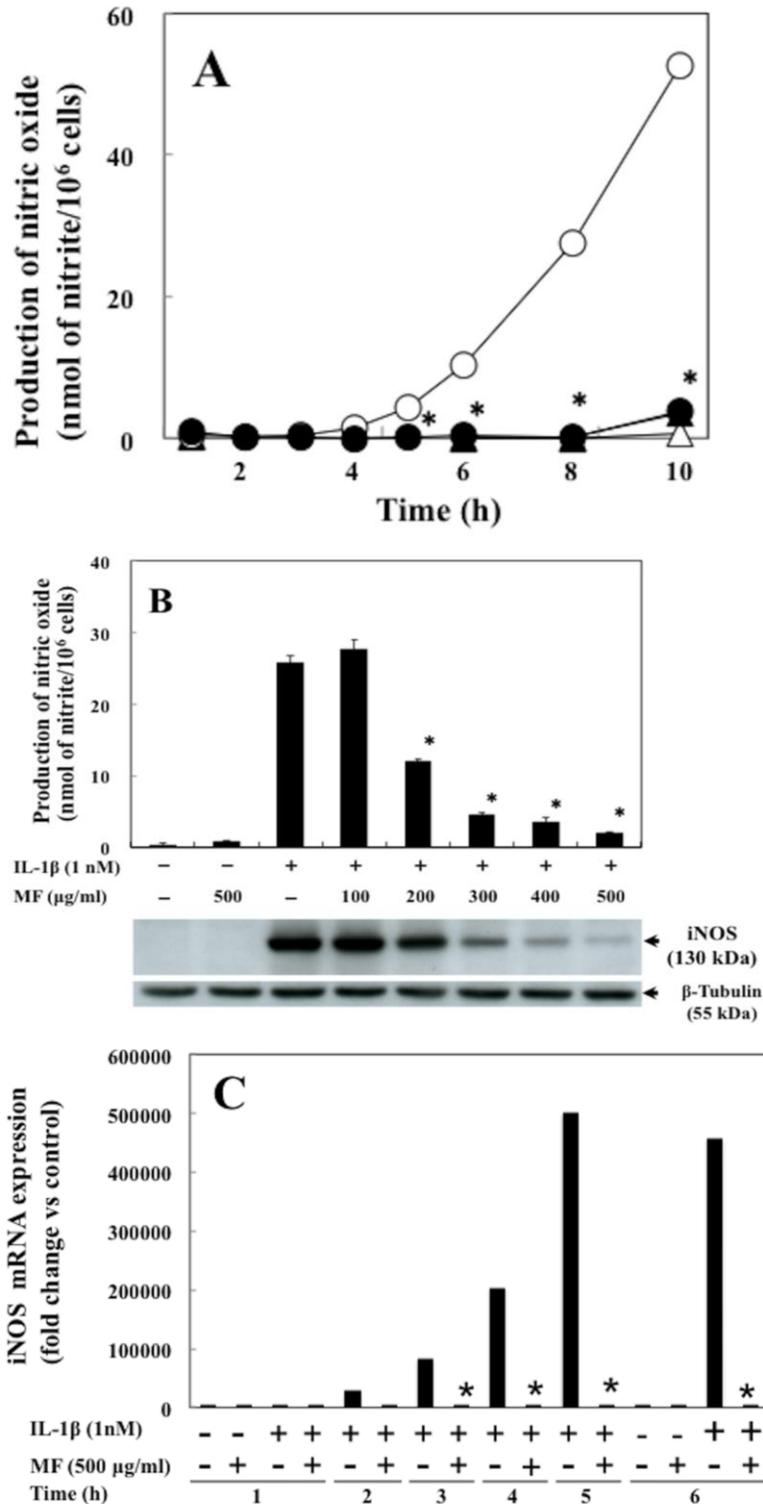
Primer name	Nucleotide sequence
iNOS F/R	5' CCAACCTGCAGGTCTTCGATG 3' GTCGATGCACAACCTGGGTGAAC 3'
as iNOS F/R	5' CCTTTGCCTCATACTTCCTCAGA 3' ATCTTCATCAAGGAATTATACACGG 3'
TNF- $\alpha$ F/R	5' TCCCAACAAGGAGGAGAAGTTCC 3' GGCAGCCTTGTCCTTGAAGAGA 3'
IL-6 F/R	5' GAGAAAAGAGTTGTGCAATGGCA 3' TGAGTCTTTTATCTCTTGTTTGAAG 3'
CINC-1 F/R	5' GCCAAGCCACAGGGGCGCCCGT 3' ACTTGGGGACACCCTTTAGCATC 3'
IL-1RI F/R	5'-CGAAGACTATCAGTTTTTGGAAAC-3'/5'- GTCTTTCCATCTGAAGCTTTTGG-3'
IL-10 F/R	5' GCAGGACTTTAAGGGTTACTTGG 3' CCTTTGTCTTGGAGCTTATATAA 3'
EF F/R	5' TCTGGTTGGAATGGTGACAACATGC 3' CCAGGAAGAGCTTCACTCAAAGCTT 3'

iNOS, inducible nitric oxide synthase; as, antisense transcript; TNF- $\alpha$ , tumor necrosis factor-alpha; IL-6, interleukin-6; CINC-1, cytokine-induced neutrophil chemoattractant-1; IL-1R1, type I IL-1 receptor; IL-10, interleukin-10; EF, elongation factor-1 $\alpha$ ; F/R, forward/reverse

**RESULTS*****Metformin inhibits NO production and iNOS induction.***

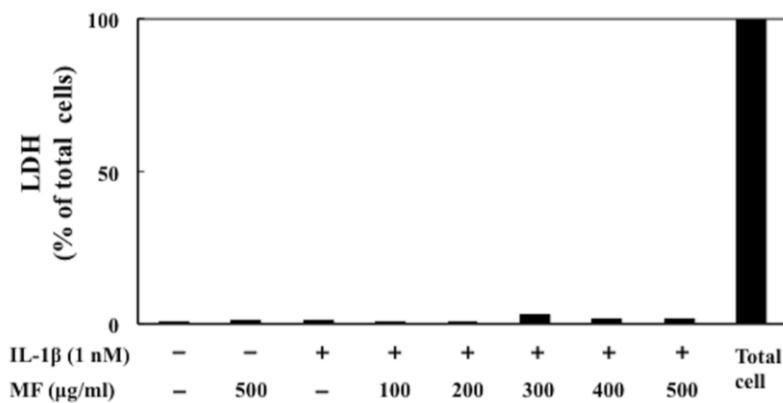
The proinflammatory cytokine IL-1 $\beta$  stimulated iNOS induction, which was followed by production of NO in primary cultured rat hepatocytes. Simultaneous addition of metformin and IL-1 $\beta$  reduced the levels of nitrite (a stable metabolite of NO) in a time and dose-dependent manner in the culture medium (Fig. 1A and 1B). Metformin demonstrated more than 90% inhibition at 500  $\mu$ g/ml. Metformin had no cellular cytotoxicity at the various concentrations, as evaluated by the release of LDH into the culture medium (Fig. 2) and Trypan blue exclusion in hepatocytes (data not shown). Western blotting analysis revealed that metformin dose-dependently reduced iNOS

protein expression, showing its IC50 at 200  $\mu\text{g}/\text{ml}$  (Fig. 1B). RT-PCR analysis revealed that metformin decreased iNOS mRNA expression in a time-dependent manner (Fig. 1C). These results suggested that metformin inhibited the induction of iNOS gene expression at the transcriptional and/or post-transcriptional level



**Figure 1.** Effects of metformin on induction of NO production and iNOS in IL-1 $\beta$ -stimulated hepatocytes. Cultured hepatocytes

were treated with interleukin (IL)-1 $\beta$  (1 nM) in the presence or absence of metformin (100–500  $\mu$ g/ml). (A) Effect of metformin (500  $\mu$ g/ml) treatment for the indicated times on nitric oxide (NO) production (IL-1 $\beta$ , open circles; IL-1 $\beta$  + metformin, filled circles; metformin, filled triangles; controls [without IL-1 $\beta$  and metformin], open triangles). (B) Effects of treatment with various doses of metformin (100–500  $\mu$ g/ml) for 8 h on NO production (upper panel) and inducible nitric oxide synthase (iNOS) protein levels (medium). Nitrite levels were measured in the culture medium. Data are presented as the mean  $\pm$  SD for  $n = 3$  dishes/point; \* $P < 0.05$  versus IL-1 $\beta$  alone. In the western blot panels, cell lysates (20  $\mu$ g of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 7.5% gel, and immunoblotted with an anti-iNOS or anti- $\beta$ -tubulin antibody. (C) Effects of metformin (500  $\mu$ g/ml) treatment for the indicated times on iNOS mRNA expression. Total RNA was analyzed by strand-specific RT-PCR to detect iNOS mRNA, using EF mRNA as an internal control.



**Figure 2.** Effects of metformin on cellular cytotoxicity. Cells were treated with IL-1 $\beta$  (1 nM) in the presence or absence of metformin (100–500  $\mu$ g/ml) for 8 h. Lactate dehydrogenase (LDH) activity was measured in the culture medium (data are presented as the mean  $\pm$  SD,  $n = 3$  dishes/point).

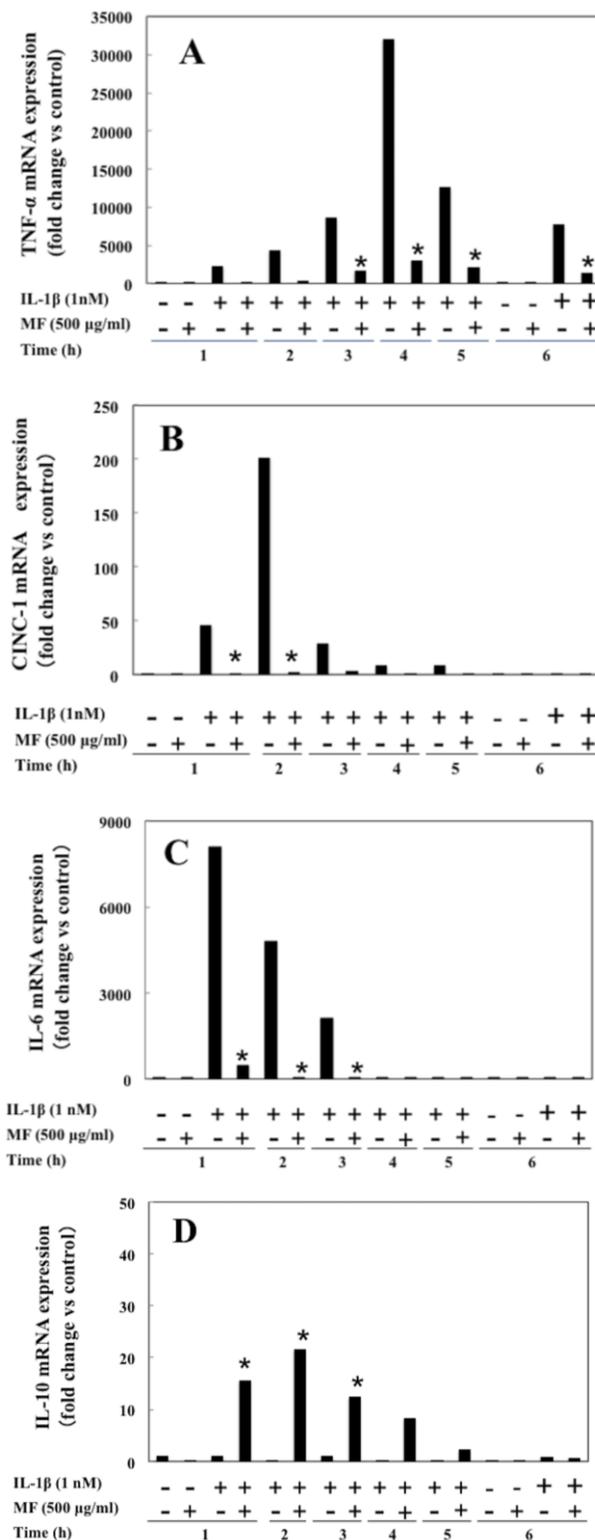
### Metformin affects mRNA expression of proinflammatory cytokines.

The expression of other mRNAs was examined. IL-1 $\beta$  increased the levels of proinflammatory cytokine expression, such as TNF- $\alpha$ , CINC-1, and IL-6, and metformin decreased TNF- $\alpha$ , CINC-1, and IL-6 mRNA levels (Fig. 3A, 3B, and 3C). However, metformin increased the mRNA levels of the anti-inflammatory cytokine IL-10, while IL-1 $\beta$  had no effects on IL-10 mRNA levels (Fig. 3D).

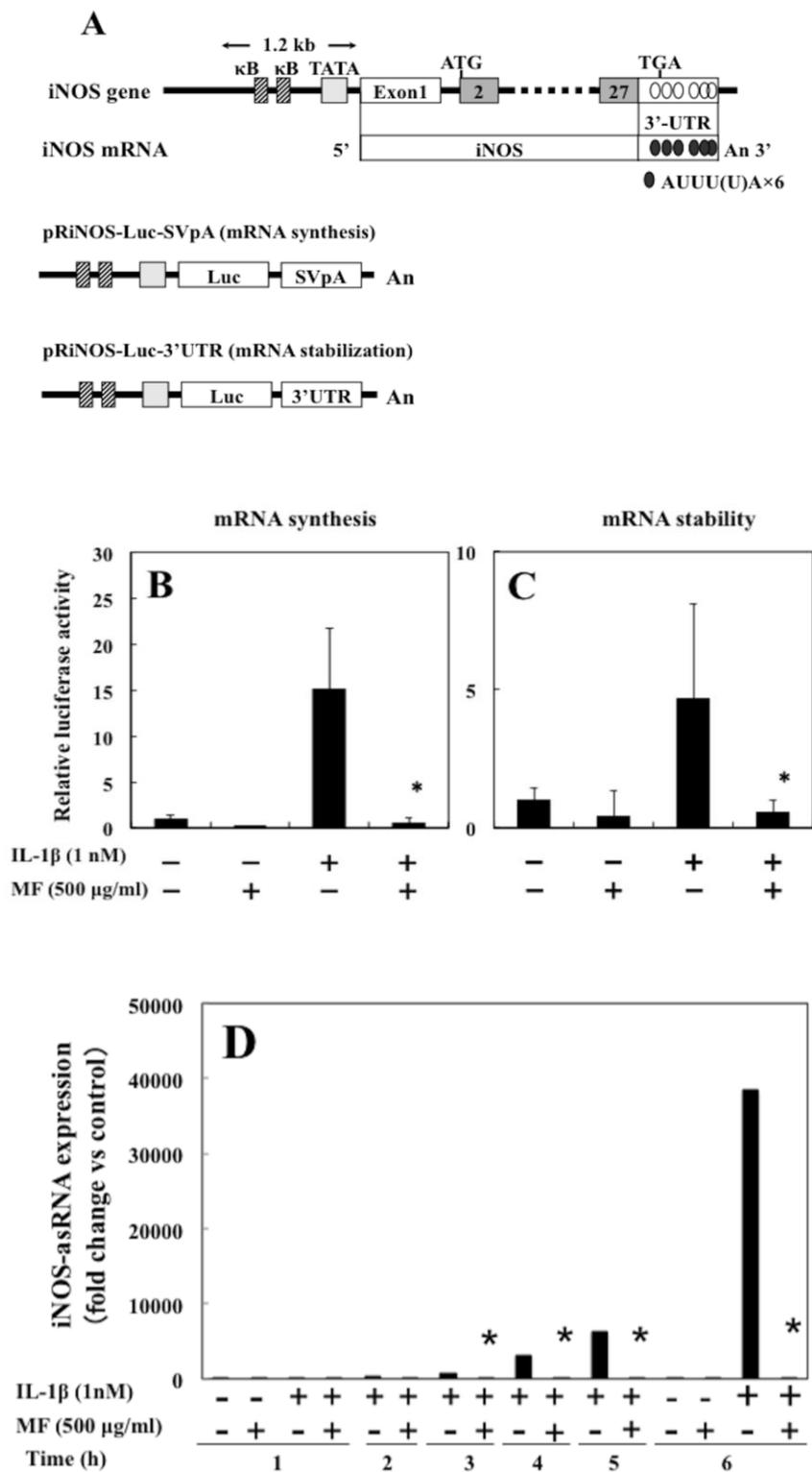
### Metformin decreases iNOS mRNA synthesis and stabilization.

We examined the mechanisms that are involved in inhibiting iNOS induction. iNOS mRNA expression is regulated by iNOS promoter transactivation with transcription factors, such as NF- $\kappa$ B, and by post-transcriptional modifications, such as mRNA stabilization [29]. Therefore, we performed transfection experiments using constructs containing firefly luciferase controlled by the iNOS promoter (pRiNOS-Luc-SVpA and pRiNOS-Luc-3'UTR; Fig. 4A), which detects iNOS promoter transactivation (mRNA synthesis) and mRNA stabilization. IL-1 $\beta$  increased the luciferase activity of these constructs, and these effects were significantly inhibited by metformin (Fig. 4B and 4C). Additionally, iNOS antisense transcript (asRNA) analysis using real-time RT-

PCR revealed that IL-1 $\beta$  increased iNOS asRNA expression in a time-dependent manner and that metformin markedly inhibited this effect (Fig. 4D).



**Figure 3.** Effects of metformin on TNF- $\alpha$ , CINC-1, IL-6, and IL-10 mRNA expression. Cells were treated with IL-1 $\beta$  (1 nM) in the presence or absence of metformin (500  $\mu$ g/ml) for the indicated times. Total RNA was analyzed using strand-specific RT-PCR to detect (A) TNF- $\alpha$ , (B) CINC-1, (C) IL-6, and (D) IL-10 using EF mRNA as an internal control.



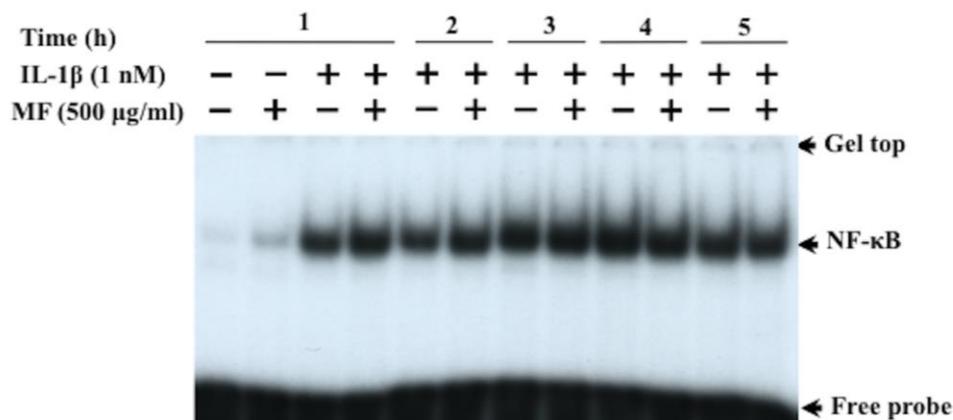
**Figure 4.** Effects of metformin on iNOS promoter transactivation and iNOS gene antisense transcript expression. (A) Schematic representation of the iNOS gene promoter region. Two reporter constructs are shown beneath the iNOS gene and mRNA. The constructs consist of the rat iNOS promoter (1.2 kb), a luciferase gene, and the SV40 poly(A) region (pRiNOS-Luc-SVpA) or iNOS 3'-UTR (pRiNOS-Luc-3'UTR). "An" indicates the

presence of a poly(A) tail. The iNOS 3'-UTR contains AREs (AUUU(U)A  $\times$  6), which contribute to mRNA stabilization. (B, C) Each construct was introduced into hepatocytes, and the cells were treated with IL-1 $\beta$  (1 nM) in the presence or absence of metformin (500  $\mu$ g/ml) for 8 h for pRiNOS-Luc-SVpA (B) and 5 h for pRiNOS-Luc-3'UTR (C). Luciferase activity was normalized by  $\beta$ -galactosidase activity. Fold activation was calculated by dividing luciferase activity by control activity (without IL-1 $\beta$  and metformin). Data are presented as the mean  $\pm$  SD for n = 4 dishes. \*P < 0.05 versus IL-1 $\beta$  alone. (D) The cells were treated with IL-1 $\beta$  (1 nM) in the presence or absence of metformin (500  $\mu$ g/ml) for the indicated times. Total RNA was analyzed by strand-specific RT-PCR to detect the iNOS gene antisense transcript (asRNA).

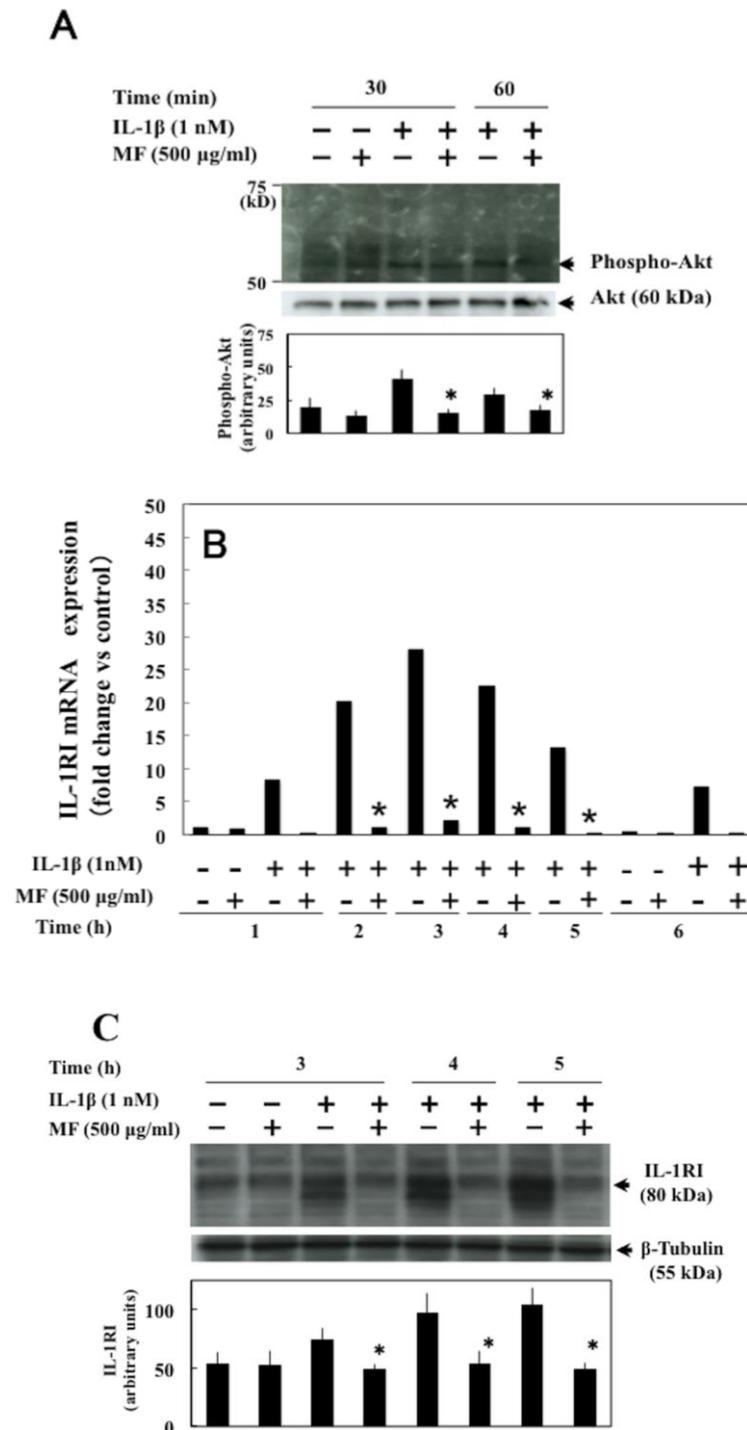
### Metformin has no effect on NF- $\kappa$ B activation but it inhibits IL-1RI upregulation.

There are two essential signaling pathways for induction of iNOS, called the I $\kappa$ B kinase and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. In the former pathway, IL-1 $\beta$  stimulates degradation of I $\kappa$ B proteins after phosphorylation by I $\kappa$ B kinase, which is followed by activation of NF- $\kappa$ B (i.e., translocation from the cytoplasm to the nucleus and DNA binding). An electrophoretic mobility shift assay with nuclear extracts demonstrated that metformin did not inhibit NF- $\kappa$ B activation at 1–5 h, which indicated no blockade of NF- $\kappa$ B nuclear translocation (Fig. 5). Metformin also had no effect on I $\kappa$ B degradation (data not shown).

In the PI3K/Akt pathway, IL-1 $\beta$  stimulates upregulation of IL-1RI by activation of PI3K/Akt [30, 31]. Immunoprecipitation–western blotting analysis demonstrated that metformin inhibited phosphorylation (activation) of Akt, a downstream kinase of PI3K (Fig. 6A). Real-time RT-PCR and western blotting analyses revealed that metformin reduced IL-1RI mRNA and protein expression levels (Fig. 6B and 6C).



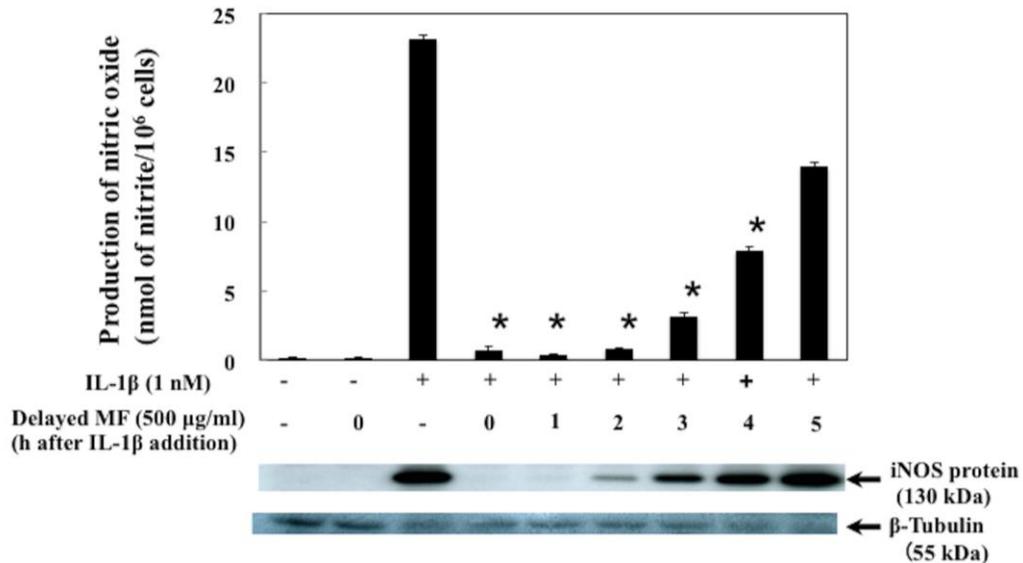
**Figure 5.** Effects of metformin on activation of NF- $\kappa$ B. Cells were treated with IL-1 $\beta$  (1 nM) in the presence or absence of metformin (500  $\mu$ g/ml) for the indicated times. Activation of NF- $\kappa$ B. Nuclear extracts (4  $\mu$ g of protein) were analyzed using an electrophoretic mobility shift assay. Representative results of three independent experiments are shown.



**Figure 6.** Effects of metformin on upregulation of type I IL-1 receptor. Cells were treated with IL-1 $\beta$  (1 nM) in the presence or absence of metformin (500  $\mu$ g/ml) for the indicated times. (A) Phosphorylation of Akt. Total cell lysates were immunoprecipitated with an anti-Akt antibody, followed by immunoblotting (SDS-PAGE in a gel with a gradient of 6–9%) with an anti-phospho-Akt or anti-Akt antibody. (B) Total RNA was analyzed using strand-specific RT-PCR to detect type I IL-1 receptor (IL-1RI) mRNA, using EF mRNA as an internal control. (C) Cell lysates (50  $\mu$ g of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-IL-1RI or anti- $\beta$ -tubulin antibody. The bands corresponding to phospho-Akt or IL-1RI were quantitated by densitometry (lower; mean  $\pm$  SD; n= 3 experiments). \*P<0.05 versus IL-1 $\beta$  alone

**Delayed administration of metformin inhibits iNOS induction.**

We examined whether delayed administration of metformin affects induction of iNOS. Metformin was added to the medium 0–5 h after the addition of IL-1 $\beta$ . Delayed administration of metformin up to 2 h after IL-1 $\beta$  addition still markedly inhibited NO production, although the magnitude of inhibition decreased in a time-dependent manner (Fig. 7).



**Figure 7.** Effects of delayed metformin administration on induction of iNOS in hepatocytes. Cells were treated with metformin (500  $\mu$ g/ml) at 0–4 h after addition of IL-1 $\beta$  (1 nM). The effects of metformin on NO production (upper panel) and iNOS protein (lower panel) were analyzed at 8 h after addition of IL-1 $\beta$ . Nitrite levels were measured in the culture medium. Data are presented as the mean  $\pm$  SD, n = 3 dishes/point; \*P < 0.05 versus IL-1 $\beta$  alone. In the western blotting panels, cell lysates (20  $\mu$ g of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-iNOS or anti- $\beta$ -tubulin antibody.

**DISCUSSION AND CONCLUSION**

In the current study, we used primary cultured rat hepatocytes stimulated by the proinflammatory cytokine IL-1 $\beta$  (*in vitro* liver injury model) [10] to investigate the effects and mechanisms of metformin in the injured liver. We found that metformin inhibited the induction of iNOS and its mRNA and protein expression, thereby preventing NO production [Fig. 1A, 1B and 1C]. Additionally, in transfection experiments with iNOS promoter constructs (containing two NF- $\kappa$ B binding sites, Fig. 4A), metformin decreased iNOS mRNA expression by inhibiting its mRNA synthesis and stabilization steps (Fig. 4B and 4C). For iNOS mRNA stabilization, metformin decreased iNOS asRNA expression (Fig. 4D). We recently reported that asRNAs are often

transcribed from many inducible genes, such as iNOS and TNF- $\alpha$  [25]. The iNOS asRNA interacted with and stabilized iNOS mRNA [32,33].

Proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, play an important role in injury to multiple organs in addition to the liver. Metformin also inhibited mRNA expression of TNF- $\alpha$ , CINC-1, and IL-6 (Fig. 3A, 3B, and 3C) and conversely enhanced mRNA expression of the anti-inflammatory cytokine IL-10 (Fig. 3D). Results suggested that metformin markedly inhibited the inflammatory pathway.

Metformin had no effect on NF- $\kappa$ B activation in the EMSA experiment (Fig. 5). Conversely, we found that metformin inhibited IL-1RI upregulation (Fig. 6B and 6C) by reducing Akt phosphorylation (Fig. 6A). Isoda et al. reported that metformin demonstrated anti-inflammatory effects by sustaining NF- $\kappa$ B activity through the PI3K/AKT pathway in human vascular wall cells [34]. Because transfection experiments with iNOS promoter luc-constructs containing two NF- $\kappa$ B binding sites (Fig. 4 B and 4C) demonstrated that metformin inhibited the activities of both constructs, we cannot rule out the possibility that metformin inhibits the promoter transactivation through NF- $\kappa$ B. It is known that a variety of inflammatory genes including TNF- $\alpha$  and CINC-1 is regulated by transcription factor NF- $\kappa$ B. NF- $\kappa$ B might be involved in mechanisms of the decreased mRNA expression of these genes.

Delayed metformin treatment after IL-1 $\beta$  addition also caused a marked reduction in iNOS induction and NO production (Fig. 7). This observation may be of clinical importance because initiation of therapeutic metformin treatment is usually delayed from the onset of disease. Liver steatosis induced by a high fat diet is associated with impaired insulin-induced liver vasodilation. These findings were documented after only 3 days of high fat diet administration, which is enough to induce liver steatosis and to impair insulin signaling, fibrosis, or other features of advanced NAFLD [35]. Appropriate regulation of inflammatory reactions is essential in preventing the onset of organ damage and early recovery.

Many studies, ranging from basic to clinical studies, have been conducted on metformin and its clinical applications in treating various diseases [37-41]. Recent reports suggest that metformin may have the therapeutic potential to treat liver injury [42, 43], in addition to its efficacy in treating type 2 diabetes [44]. Our results demonstrate that metformin may have anti-inflammatory effects in the liver. To link our results with previous work on metformin in the diabetic context, we speculate that metformin may block the inflammatory pathway associated with a wide variety of liver injury. Based on the range of metformin's effects, metformin itself may be useful in treating and preventing some diseases, but fully establishing its potential will require further studies and clinical applications.

Metformin can prevent IL-1 $\beta$ -stimulated liver injury in cultured hepatocytes by inhibiting induction of inflammatory mediators such as iNOS and TNF- $\alpha$ . Metformin may have a therapeutic potential to treat liver injury. However, it is reported that metformin should be used with caution

for patients with liver injury clinically. Consequently, metformin may have a therapeutic potential to treat chronic liver injury in NASH rather than acute liver injury.

**List of Abbreviations:** MF, metformin; iNOS, inducible nitric oxide synthase; TNF- $\alpha$ , tumor necrosis factor-alpha; NO, nitric oxide ; IL, interleukin; NF- $\kappa$ B, nuclear factor-kappa B; CINC-1, cytokine-induced neutrophil chemoattractant-1; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IL-1RI, the type I interleukin-1 receptor; RT-PCR, reverse transcriptase-polymerase chain reaction.

**Competing Interests:** The authors declare that there are no conflicts of interest.

**Authors' Contributions:** RN participated in the design of the study, data collection, statistical analysis, and drafting of the manuscript. TO participated in supervision and provided oversight when the manuscript was being drafted. IH, IM, KM, YN, and MK assisted in the design of the study. MN provided advice regarding development of the protocol for the study and assisted in the design of the study.

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