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Full Length Research Paper

Inhibition of nitric oxide production and mRNA expressions of proinflammatory mediators by xylarinic acid A in RAW 264.7 cells

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Mushrooms possess vast and yet unknown source of powerful new pharmaceutical products, which contain active biomolecules. Xylarinic acid A isolated from a mushroom, *Xylaria polymorpha,* has been reported to have free radicals scavenging property and anti-fungal activity. However, information of its anti-inflammatory activity is scarce. Therefore, the objective of this study was to examine the effect of the compound on the gene expression levels of inflammatory mediators and pro-inflammatory cytokines. MTT assay, NO assay, semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real time PCR (qPCR) were performed to determine the effect of xylarinic acid on cell viability, NO production and mRNA expression levels in LPS-treated RAW 264.7 mouse macrophage-like cells. As such, xylarinic acid A inhibited nitric oxide production and mRNA expressions of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β) and interleukin-6 (IL-6) in a concentration dependant pattern without cytotoxicity effect. Our preliminary result indicates that xylarinic acid A might have anti-inflammatory property.

Key words: Xylarinic acid A, macrophages, inflammation, inflammatory mediators.

INTRODUCTION

Inflammation is a complex process involving numerous mediators of cellular and plasma origin with interrelated biological effect (Vane et al., 1994). Macrophages produce a variety of inflammatory mediators during inflammation (Guha and Mackman, 2001; Laskin and Pendino, 1995). Indeed, a number of inflammatory stimuli including lipopolysaccharide (LPS) and pro-inflammatory cytokines activate immune cells and up-regulate inflammatory states in primary macrophages and RAW 264.7 cells. Therefore, they are important in exploring molecular mechanisms of action for the subsequent development of potential anti-inflammatory agents (Zamora et al., 2000). As a consequence, macrophages play a central role in managing many different immunopathological phenomena such as the overpro-duction of pro-inflammatory cytokines (that is, tumor

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Abbreviations: iNOS, inducible nitric oxide synthase; **COX-2,** cyclooxygenase-2; **IL-1β,** interleukin-1β; **IL-6,** interleukin-6; **RT-PCR,** reverse transcription-polymerase chain reaction; **LPS,** lipopolysaccharide; **TLR 4,** toll-like receptor 4; **NF-κB,** nuclear factor kappa B; **NO,** nitric oxide; **CAD,** coronary artery disease; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **qPCR,** quantitative real time PCR.

necrosis factor (TNF)-α. interleukin (IL)-1β and interleukin (IL)-6) and inflammatory mediators such as (that is, tumor necrosis factor (TNF)-α. interleukin (IL)-1β and interleukin (IL)-6).

LPS can bind to toll-like receptor 4 (TLR 4) and activate nuclear factor kappa B (NF-□B) for nitric oxide (NO) production and mRNA expressions of iNOS, COX2, IL-1β and IL6 in murine macrophage RAW 264.7 cells (Beg, 2002; Xiang et al., 2009). As such, NO, produced by iNOS, plays a central role in immunomodulatory and inflammatory processes such as cancer, rheumatoid arthritis, diabetes, septic shock and cardiovascular diseases (Carreras et al., 2004; Guzik et al., 2003).

The production of prostaglandin E (PGE) depends on the activity of cyclooxygenases (COX) and isoforms of the COX enzyme (COX-1 and COX-2), which have been identified (Smith et al., 2000, 1996), whereas, COX-1 is expressed constitutively in most types of cells and is involved in many physiological functions (Smith et al., 2000) and COX-2 is an inducible enzyme (Muraoka et al., 1999). Many inflammatory stimuli, including LPS are capable of inducing COX-2 expression in different cell types, particularly in macrophages (Barrios-Rodiles et al., 1999; Muraoka et al., 1999). Increased COX-2 synthesis by tissue macrophages is responsible for the accumulation of large amounts of PGE in local tissues (Smith et al., 2000). Secreted PGE, especially PGE2, promotes inflammation by increasing the vascular permeability and vasodilatation and by directing cell migration into the site of inflammation through the induction of inflammatory cytokines (Muraoka et al., 1999; Simon, 1999). Thus, controlling PGE synthesis is a critical element in regulating inflammatory reactions during bacterial infection, tissue injury and autoimmune responses.

COX-2 and iNOS have been found to be induced in a wide array of inflammatory models and disorders and in many cases, inhibition of NO release has been associated with profound suppression of prostaglandin production. In addition, co-induction or co-regulation of COX-2 and iNOS has been demonstrated in a number of cell culture studies and animal inflammatory models (Posadas et al., 2000; Salvemini et al., 1995; Vane et al., 1994). High amounts of PGE2 derived from COX-2 following induction by many proinflammatory mediators, including bacterial LPS, TNF-α and IL-1β have also been implicated in the pathogenesis of sepsis and inflammation (Beg, 2002; Xiang et al., 2009).

As mentioned earlier, co-expression of COX-2 and iNOS enzymes have been observed under diverse experimental conditions. Since both 5'-flanking promoter regions of COX-2 and iNOS genes have NF-κB binding sequences, the same pathophysiologic stimuli may turn on the expression of both genes simultaneously. Alternatively, NO produced through NF-κB-induced iNOS expression or via another pathway independently of NF-κB, may affect COX-2 expression/catalytic activity (Smith et al., 2000). Thus, anti-inflammatory agents that could target such pathways may have a potential to treat inflammation related diseases.

On the other hand, the search for natural compounds that could possess anti-inflammatory activity with limited side effects continues. In this regard, mushrooms possess vast and yet unknown source of powerful new pharmaceutical products, which contain active biomolecules including antitumor, anti-inflammatory and immunomodulatory properties. Xylarinic acid A isolated from a mushroom, *Xylaria polymorpha,* has been reported to have free radicals scavenging property and anti-fungal activity. However, information on the anti-inflammatory activity of this mushroom derived compound is limited. Therefore, we reported here a preliminary result of the compound's effect on the gene expression levels of inflammatory mediators and pro-inflammatory cytokines.

MATERIALS AND METHODS

Materials

RAW264.7 cells were obtained from Korean Cell Line Bank (Seoul, Korea), while RT and PCR premix were purchased from Bioneer Co. (Daejeon, Korea). DMEM and FBS were from WelGene Co. (Daegu, Korea) and LPS was from Sigma Co. (St Louis, MO, USA). However, all other reagents were of the first grade.

Preparation of xylarinic acid

The new polypropionate designated as xylarinic acid A was isolated from the fruiting body of *Xylaria polymorpha*. The structure of the compound used in this study was established as 4, 6, 8-trimethyl-2, 4-decadienoic acid on the basis of extensive spectroscopic analysis.

Cell culture

RAW 264.7 cells were maintained in DMEM supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin and 5% FBS. Cells were grown at 37℃ and 5% CO_2 in humidified air.

Measurement of nitrite

The nitrite in the culture medium was measured as an indicator of NO production based on Griess reaction (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2% phosphoric acid) (Cho et al., 2006). Briefly, RAW 264.7 cells ($1X10^6$cells/ml) were preincubated in 60 mm plates for 24 h. The cells were treated with xylarinic acid A (10, 25, 50 and 100 µM) for 30 min and then incubated with LPS (0.1 µg/ml) for 18 h. The cell supernatants (100 µl) were mixed with an equal volume of Griess reagent to determine the absorbance at 540 nm using Spectramax 250 microplate reader. The concentrations of nitrite were calculated by a regression analysis using serial dilutions of sodium nitrite as a standard.

Total RNA isolation and semi quantitative RT-PCR amplification

Total RNA was isolated using easy-BLUE™ reagent (iNtRON Biotechnology Co., Korea), according to the manufacturers'

protocol. The isolated RNA was stored at -70℃ until use. Semi-quantitative RT reactions were performed using RT premixes (Bioneer Co., Korea). Briefly, 2 µg of total RNA was incubated with oligo-dT_{18} at 70℃ for 5 min and cooled on ice for 3 min. The reaction mixture was incubated for 90 min at 42.5℃ after the addition of RT premix and then suspended at 95℃ for 5 min. The PCR reactions were continued using PCR premixes containing sense and antisense primers (Bioneer Co., Korea): GAPDH (sense: 5'-CAC TCA CGG CAA ATT CAA CGG C-3'; antisense: 5'-CCT TGG CAG CAC CAG TGG ATG CAG G-3'), iNOS (sense: 5'- CCC TTC CGA AGT TTC TGG CAG CAG C-3'; antisense: 5'- GGC TGT CAG AGC CTC GTG GCT TTG G-3'), COX-2 (sense: 5'- CAC TAC ATC CTG ACC CAC TT -3'; antisense: 5'- ATG CTC CTG CTT GAG TAT GT -3'), IL-1□ (sense: 5'- CAG GAT GAG GAC ATG AGC ACC -3'; antisense: 5'- CTC TGC AGA CTC AAA CTC CAC -3') and IL-6 (sense: 5'- GTA CTC CAG AAG ACC AGA GG -3'; antisense: 5'- TGC TGG TGA CAA CCA CGG CC -3'), under incubation conditions (a denaturation time of 45 s at 94℃, an annealing time of 45 s between 55 and 60℃, an extension time of 45 s at 72℃ and a final extension of 10 min at 72℃). The PCR products were separated on a 1% agarose using electrophoresis method of BioRad Co. The relative intensity levels were calculated using Eagle eyes image analysis software (Stratagene Co., La Jolla, USA). The resulting density levels of the iNOS, COX-2, IL-1β and IL-6 bands were expressed relative to the corresponding density amounts of the GAPDH bands, which were from the same RNA sample.

Quantitative real time PCR (qPCR)

The inflammatory mediators and cytokine mRNA expressions were quantified by real-time PCR in a 25 µl total reaction mixture using TaqMan probe (Applied Biosystems, UK). First-strand cDNA synthesis was carried out using oligo$(dT)_{18}$ and RT premix (Bioneer Co., Daejeon, Korea). DNA amplification and detection were carried out in Applied Biosystems 7500 real time PCR system (Perkin Elmer Applied Biosystems, Foster City, CA, USA) in MicroAmp optical 96-well reaction plates. The cycle threshold (C_T) values corresponding to the PCR cycle number at which fluorescence emission in real time reaches a threshold above the base-line emission were determined using ABI Prism- 7700 software. The real-time PCR conditions were as follows: initial denaturation at 95℃ for 10 min, amplification for 40 cycles of denaturation at 95℃ for 15 s and annealing at 60℃ for 1 min. However, the sequence of primers used was as follows:

Mouse-iNOS: (sense: 5'- GGCAGCCTGTGAGACCTTTG-3' and antisense: 5'- GCATTGGAAGTGAAGCGTTTC-3');
Mouse-COX-2: (sense: 5'- GGGTGTCCCTTCACTTCTTTCA-3' and antisense: 5'- TGGGAGGCACTTGCATTGA-3');
Mouse-IL-1β: (sense: 5'- CAACCAACAAGTGATATTCTCCATG-3' and antisense: 5'- GATCCACACTCTCCAGCTGCA-3');
Mouse-IL-6: (sense: 5'- TCCAGTTGCCTTCTTGGGAC-3' and anti-sense: 5'- GTGTAATTAAGCCTCCGACTTG-3');
Mouse GAPDH: Probe sequence: 5' TGCATCCTGCACCACCAACTGCTTAG 3' (VIC).

Statistical analysis

A one–way ANOVA was used to determine statistically, the significant differences between values of the experimental and control groups. The data represent the means ± SEM of three experiments, conducted in triplicate. P values of 0.05 or less were considered to be statistically significant.

RESULTS

The inhibitory effect of xylarinic acid A on NO production and iNOS expression

Since NO is produced by LPS-activated murine macrophage RAW264.7 cells and plays an important role in the development of innate and acquired immunity, we first determined the effect of xylarinic acid A on LPS-induced NO production. As shown in Figure 1B, LPS (0.1 µg/ml) markedly increased NO production, which was dose-dependently inhibited by xylarinic acid A. This inhibitory effect of xylarinic acid A on NO production was examined without affecting the viability of RAW264.7 cells at the concentrations used (10, 25, 50 and 100 µM) (Figure 1A). Thus, we investigated whether xylarinic acid A modulates mRNA expression of iNOS in LPS-activated RAW264.7 cells. LPS (0.1 µg/ml) noticeably increased the expression of iNOS mRNA, which was inhibited by xylarinic acid A in a concentration-dependent manner in both semi-quantitative RT-PCR (Figures 2A and B) and quantitative real time PCR (Figure 2C), suggesting that LPS-induced iNOS mRNA expression is attenuated by Xylarinic acid A pretreatment.

Effect of xylarinic acid A on a lipid-prostanoid inflammatory mediator

We next determined whether xylarinic acid A modulated the expression of COX-2 mRNA, a key regulator of another inflammatory mediator prostanoids, such as prostaglandin and thromboxane (Chun and Surh, 2004). As shown in Figures 3A, B and C, xylarinic acid A significantly and dose-dependently decreased the expression of COX-2 mRNA. The inhibitory effect of xylarinic acid A with the real time PCR (Figure 3C) is more prominent when compared with the semi-quantitative RT-PCR (Figures 3A and B). Hence, it is notable that xylarinic acid A decreased COX-2 mRNA level in LPS-stimulated RAW264.7 cells. This is of great value, because COX-2 plays an essential role in the biosynthesis of the inflammatory mediators such as PGE2 (Scher and Pillinger, 2009; Woodward et al., 2008).

Although the protein expression of inflammatory mediators and the molecular mechanism by which xylarinic acid A inhibition was not shown in this study, the downregulation of iNOS and COX-2 mRNA expression might indicate that the agent could posses a sound anti-inflammatory property.

The inhibitory effect of xylarinic acid A on pro-inflammatory cytokine expression

We next examined whether xylarinic acid A modulated the mRNA expression of pro-inflammatory cytokines such as

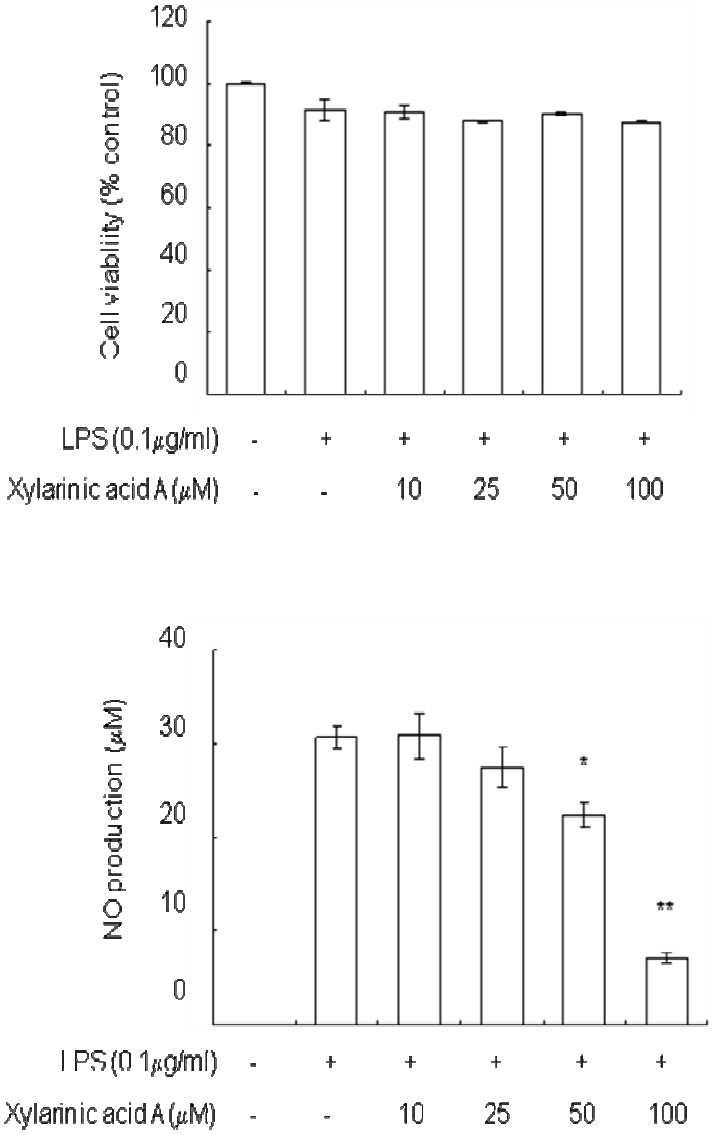
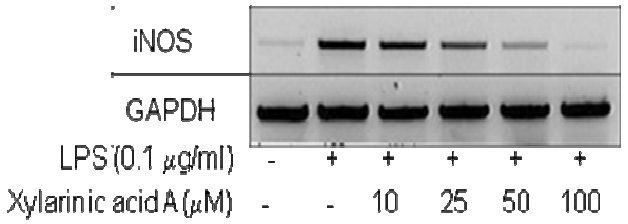
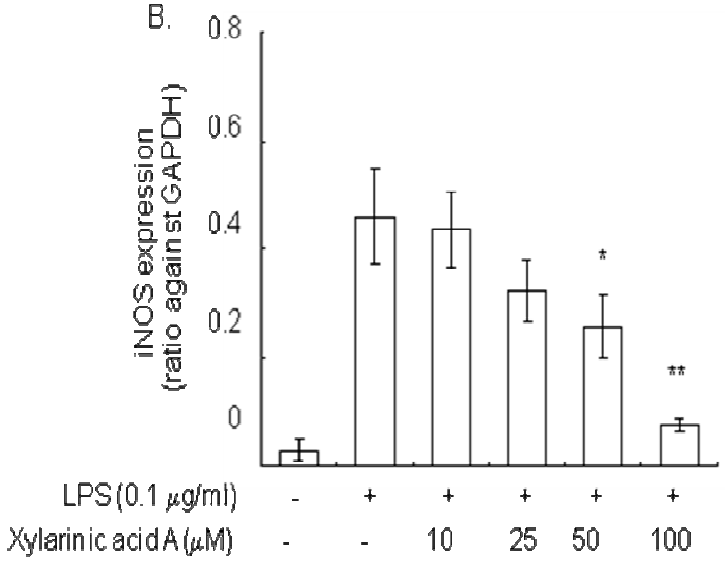


Figure 1. Effects of xylarinic acid A on cell viability (A) and NO production (B) in LPS-activated RAW 264.7 cells. RAW 264.7 cells ($1X10^6$ cells/ml) were incubated with xylarinic acid A (10, 25, 50 and 100 μM) either in the presence or absence of LPS (0.1 μg/ml) for 18 h. The cell viability test was performed using MTT assay. For NO production, the supernatant was removed and the nitrite was determined using Griess reagent. Each value is the means ± SEM of the three to five independent experiments performed in triplicate. ** $p < 0.01$ and * $P<0.05$ versus vehicle control.

IL-1β and IL-6 in LPS (0.1 μg/ml)-activated RAW264.7 cells. IL-1β and IL-6 mRNA levels in RAW264.7 cells were evaluated by semi-quantitative RT-PCR and quantitative real time PCR. As shown in Figures 4A and B, xylarinic acid A marginally decreased the expression of IL-1β mRNA using semiquantitative RT-PCR. However, using quantitative real time PCR and xylarinic acid A notably reduced LPS-stimulated IL-1β gene expression (Figure 4C). During treatment of maximal dose (100 μM) of xylarinic acid A, LPS-induced IL-1β up- regulation was completely reversed in comparison to the vehicle control expression level. In line with this, xylarinic acid A dose-dependently inhibited the expression of IL-6 mRNA, which was also induced by LPS (0.1 μg/ml). The inhibitory effect of xylarinic acid A on IL-6 mRNA expression was shown more clearly with real time PCR





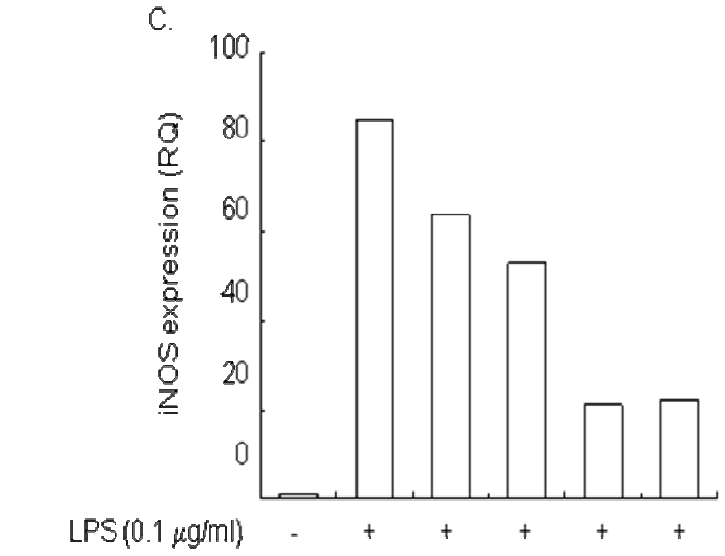


Figure 2. Effects of xylarinic acid A on iNOS mRNA expression in LPS-activated RAW264.7 cells. The cells were pretreated with xylarinic acid A (10-100 μM) for 30 min, and then incubated with LPS (0.1 μg/ml) for 18 h. Total RNA was isolated using easy-Blue™ reagent under the manufacturer's instruction. The semi-quantitative RT-PCR (A and B) and quantitative real time PCR (C) were performed and each value is the means ± SEM of the three experiments. ** $p < 0.01$ and * $P< 0.05$ versus vehicle control.

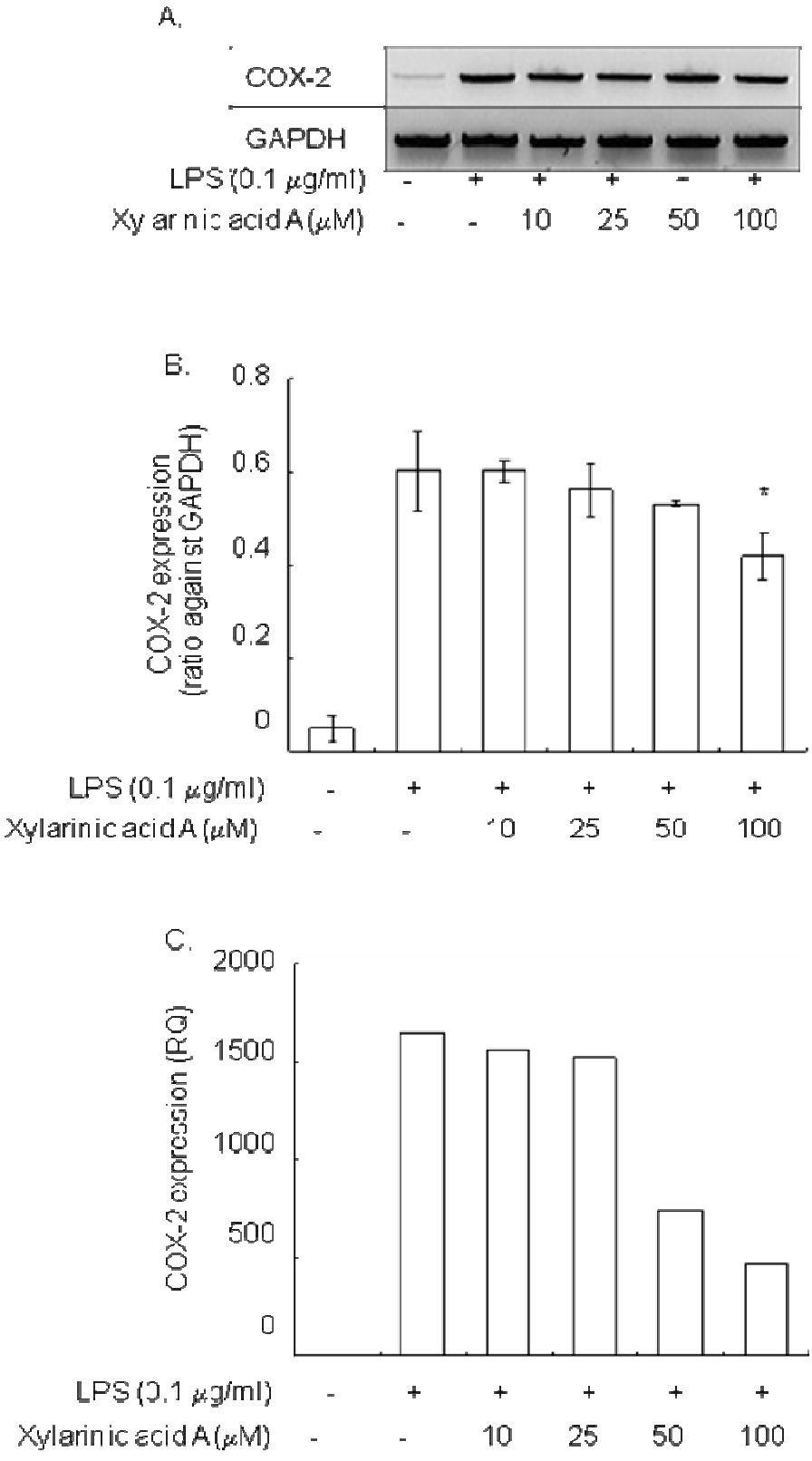


Figure 3. Effect of xylarinic acid A on COX-2 mRNA expression in LPS-activated RAW264.7 cells. COX-2 mRNA levels were determined by both semi-quantitative RT-PCR (A and B) and quantitative real time PCR (C). The indicated figures were from representative results of three separate experiments with similar results (A and C). * $p < 0.05$ versus vehicle control.

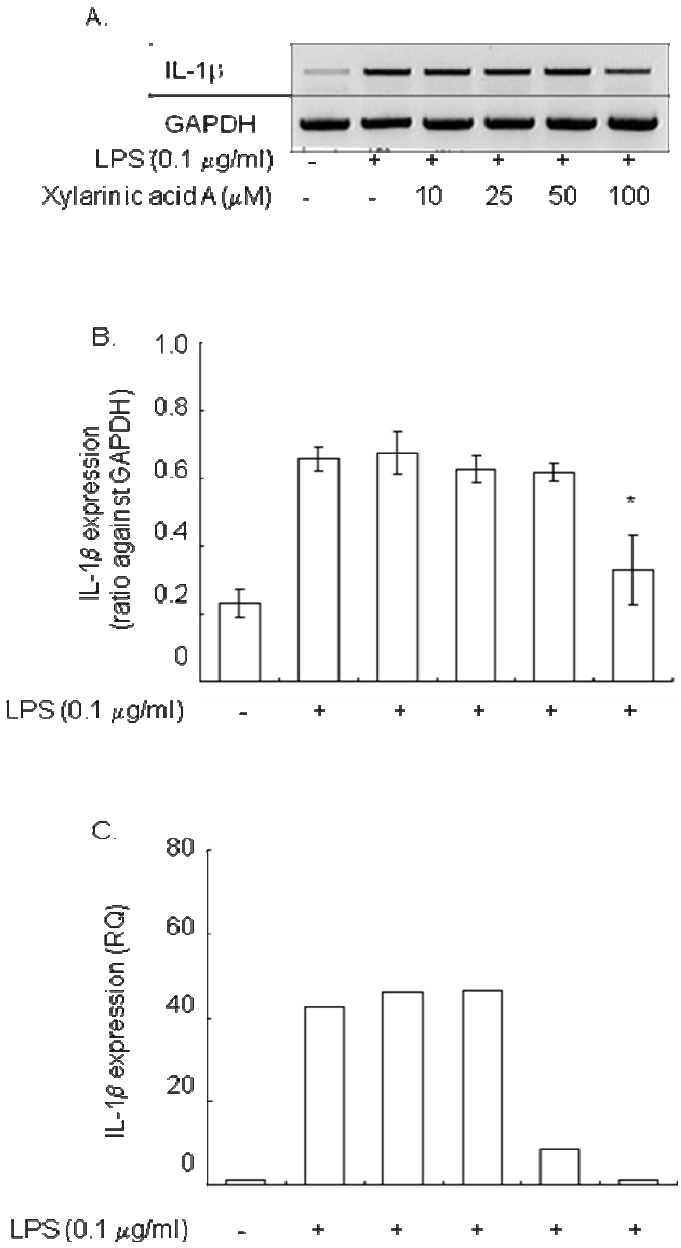


Figure 4. Effects of xylarinic acid A on the mRNA expression of pro-inflammatory cytokine IL-1β in LPS-activated RAW 264.7 cells. The mRNA levels of IL-1β gene were determined by semi-quantitative RT-PCR (A and B) and quantitative real time PCR (C). The results were representative of three similar independent experiments (A and C). * $p < 0.05$ versus vehicle control.

(Figure 5C) than with RT-PCR.

DISCUSSION

The use of natural products as anti-oxidant and anti-inflammatory agents has a long history that began with folk medicine. Focusing attention on plants and mushrooms that are medicinally used by the indigenous people is the preferable way to identify bioactive compounds. In this regard, the search for natural compounds that may have the potential to exhibit anti-inflammatory activity with limited side effects continues. Mushrooms possess vast and yet unknown source of powerful new pharmaceutical products, which contain active biomolecules including antitumor, anti-inflammatory

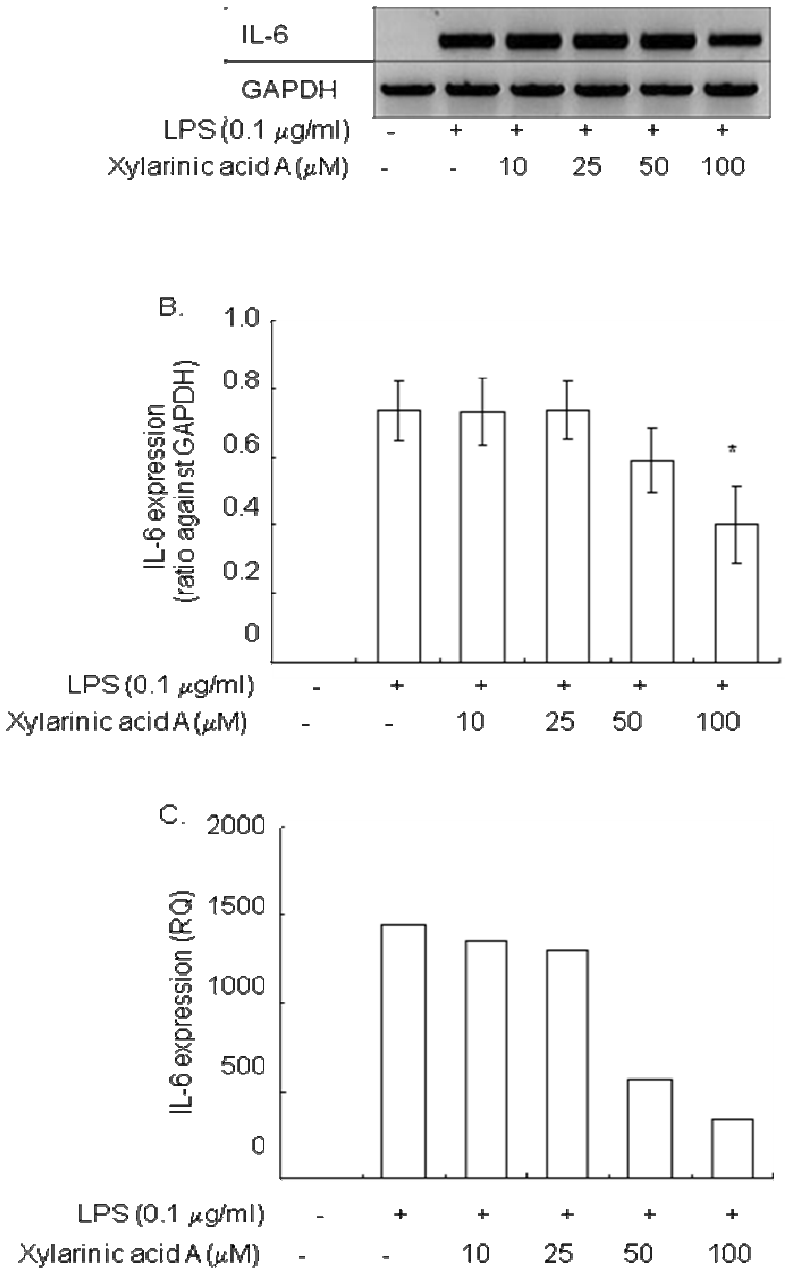


Figure 5. Effect of xylarinic acid A on mRNA expression of IL-6 gene in LPS-activated RAW 264.7 cells. The mRNA levels of IL-6 gene were determined by semi-quantitative RT-PCR (A and B) and quantitative real time PCR (C). The figures were representative of three separate experiments with similar results (A and C). * $p < 0.05$ versus vehicle control.

and immunomodulatory properties. Evidences indicated that xylarinic acid A isolated from a mushroom, *Xylaria polymorpha,* has been reported to have free radicals scavenging property and anti-fungal activity. However, to our knowledge, information on the anti-inflammatory activity of this mushroom derived compound is not reported. Here, from our preliminary data, it is reported that xylarinic acid A exhibited an inhibitory effect on LPS-induced NO production and iNOS mRNA expression. In addition, it attenuated COX-2 gene expression activated by LPS without affecting the cell viability. The indicated anti-inflammatory activity of xylarinic acid in the foregoing was further supported by the significant inhibition of IL-1β and IL-6 gene expression levels in the pretreated compound and LPS-activated cells. Evidences supported our preliminary report, in that iNOS and COX-2 have been found to be induced in a wide array of inflammatory models and disorders and in many cases, inhibition of

NO release has been associated with profound suppression of prostaglandin production. In addition, co-induction or co-regulation of COX-2 and iNOS has been demonstrated in a number of cell culture studies including RAW 264.7 cells and animal inflammatory models (Posadas et al., 2000; Salvemini et al., 1995; Vane et al., 1994). Therefore, the inhibitatory effect of xylarinic acid A on iNOS and COX-2 mRNA levels could probably result in the repression of iNOS and COX2 enzymes which are responsible for the production of NO and prostaglandin, respectively.

IL-1β and IL-6 are known to be pro-inflammatory cytokine that possess a variety of biological activities in acute and chronic inflammatory diseases such as septic shock, carcinogenesis, rheumatoid arthritis and autoimmune diseases (Abramson et al., 2001; Dinarello, 2002; Lv et al., 2009). In addition, the pro-inflammatory cytokine IL-1β has been reported as a potent stimulator for iNOS gene expression (Finder et al., 2001; Guan et al., 1999; Kim et al., 2006). Thus, the inhibitory activity of xylarinic acid A on LPS-induced IL-1β and IL-6 gene expressions might be due to the anti-inflammatory activity of the compound with the direct or indirect effect on the indicated cytokines.

In conclusion, we found that xylarinic aicd A inhibited LPS-induced NO production, iNOS and COX-2 gene expression without cytotoxic effect even at higher doses. In addition, xylarinic acid A significantly down-regulated the mRNA expression of pro-inflammatory cytokines, IL-1β and IL-6, in LPS-stimulated RAW 264.7 cells. Thus, xylarinic acid A might exhibit anti-inflammatory potential in LPS-activated RAW 264.7 cells. Further study need to be conducted to verify the anti-inflammatory activity of the compound at the protein expression level of the inflammatory mediators and the possible mechanism of action that may follow.

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