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Establishment of Reporter Platforms Capable of Detecting NF-κB Mediated Immuno-Modulatory Activity

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ABSTRACT: Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are transcriptional targets of nuclear factor kappa B (NF-κB) that are involved in inflammatory responses. The aim of this study is to develop a method for efficiently detecting inflammation modulatory activities. Here we established RAW264.7 macrophage cells stably expressing a luciferase reporter gene directed by iNOS or COX-2 promoter. Lipopolysaccharide (LPS) treatment stimulated the luciferase activity which paralleled with increased iNOS and COX-2 mRNA levels determined by RT-q-PCR. The LPS-stimulated luciferase activity was blocked by NF-κB inhibitor CAPE and by nobiletin, an anti-inflammatory natural product from citrus peels. We have applied the platforms to screen various mushroom species; analysis by scatter plot revealed a strong correlation to the results obtained by ELISA-based detection of TNF-α. Together we have established luciferase reporter systems sensitive to NF-κB-dependent iNOS and COX-2 activation, which provides an alternative screening method for identifying food components with immune-modulatory activities.

KEYWORDS: *iNOS, COX-2, luciferase assay, RAW264.7, cell platform*

INTRODUCTION

Inflammation is the body's response to exogenous invasion and insults, which triggers the activation of the immune system to remove the invasive agents and repair damages. Much evidence suggests that chronic inflammation is linked to diseases including cardiovascular disorder, Alzheimer, and cancer.[1,2] Recent studies have established a tight linkage between chronic inflammation and the activation of NF-κB induced by stimuli including interleukin 1 (IL-1), tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS).[3−5] As a transcription factor, NF-κB activates a number of pro-inflammatory genes including cytokines, chemokines, the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). The expression of iNOS results in the release of nitric oxide (NO) known to be involved in cell proliferation, invasion,[6] as well as local or systemic inflammatory disorders.[7−9] Cyclooxgenase (COX) has been shown to be responsible for production of the pro-inflammatory mediator prostaglandin E2 (PGE2). Two COX isoenzymes have been identified; COX-1 is a constitutive enzyme whereas expression of COX-2 is induced in activated macrophages at sites of inflammation.[10,11]

Because prolonged inflammation is tied to many chronic diseases, the search for molecules with anti-inflammation activities is critical. Analyses by immunoblotting and RT-q-PCR have been employed to detect the abundance of inflammation and pro-inflammation molecules. However, these methods are time-consuming, not cost-effective, and not suitable for high throughput screening. Other commonly used methods for detecting immune-modulatory activities include measuring the concentrations of TNF-α or NO in the medium of cultured macrophage cells. In the present study, we have established macrophage cell-based platforms containing either iNOS or COX-2 promoter-fused luciferase gene. By measuring the changes in luciferase activity, we have demonstrated that the efficacy of the platform is comparable to that of the TNF-α screening system and is potentially applicable as an alternative high throughput screening system for detecting compounds with immuno-modulatory activities.

MATERIALS AND METHODS

Plasmids, Transfection, and Stable Clone Selection. For construction of plasmids pGL4.32-iNOS and pGL4.32-COX-2, the pGL4.32 (Promega, WI, USA) were digested with *Bam*HI and *Kpn*I to yield a DNA fragment containing Hygromycin and Ampicillin resistant markers, respectively. The DNA fragment was then ligated downstream to the iNOS or COX-2 promoter driven luciferase gene generated from the *Bam*HI/*Kpn*I digestion of pGL3-iNOS or pGL3-COX-2 (kindly provided by Dr. Wu MJ[12]). For transfection, the RAW264.7 macrophage cells were plated in a 60 mm dish and grown to 90% confluence. Cells were replaced with serum free DMEM medium for 1 h and transfected with pGL4.32-iNOS or pGL4.32-COX-2 by the Lipofecatime 2000 (Invitrogen, NY, USA) according to the manufacturer's protocols. The cells were cultured in medium containing 400 μg/mL Hygromycin B for 9 more days, and 60 colonies were selected for testing the response to LPS by luciferase assays.

Luciferase Assay. Cells were washed three times with PBS and lysed in lysis buffer according to the manufacturer's protocols (Promega, WI, USA). Luciferase activity was measured using the

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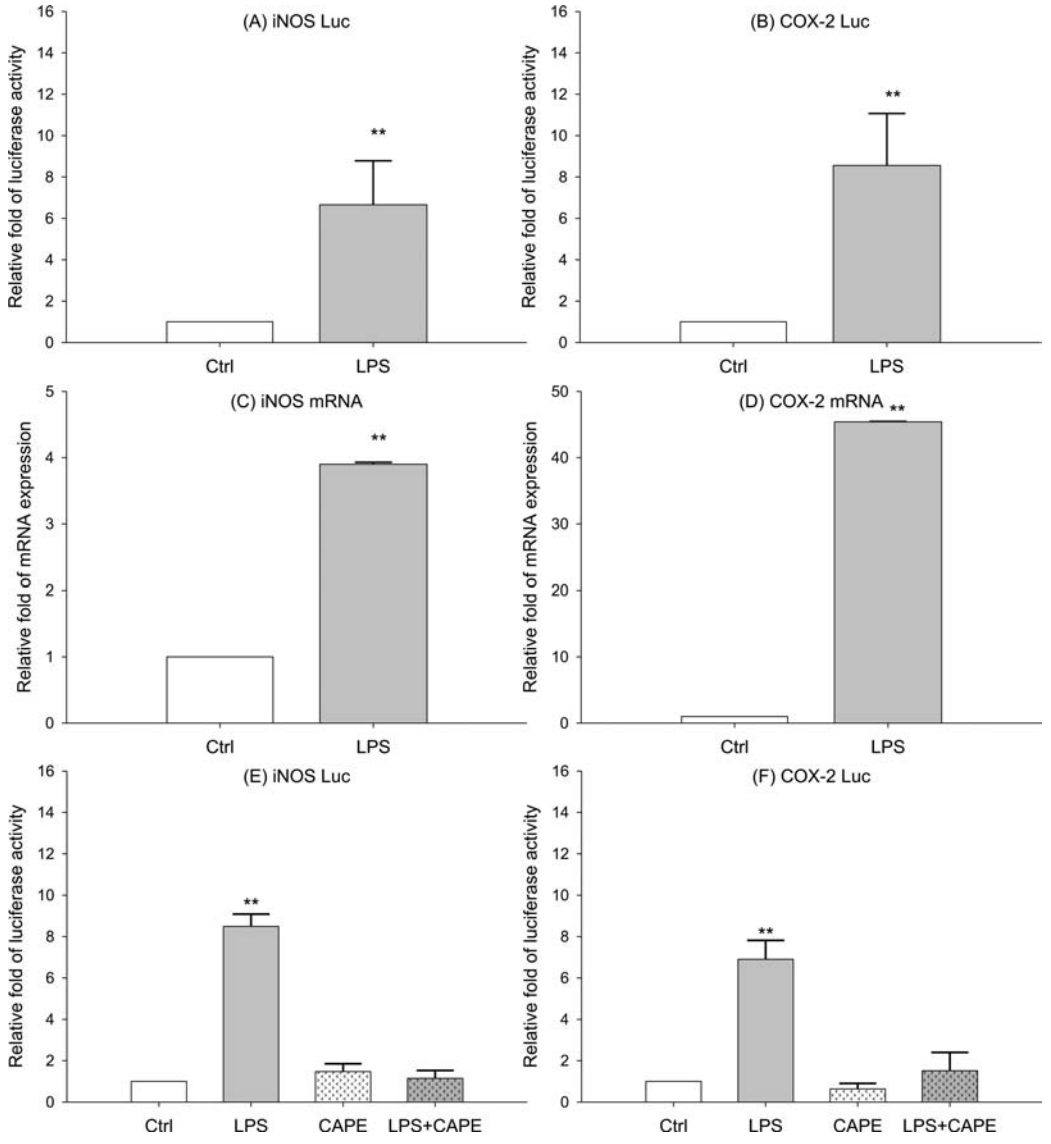

Figure 1. Establishment of stable macrophage cells reporting the NF-κB-mediated, iNOS or COX2 promoter-directed luciferase expression. (A and B) Generation of macrophage stable clones expressing luciferase sensitive to inflammation stimulation. The RAW264.7 macrophage cells containing (A) iNOS or (B) COX-2 promoter-directed luciferase gene were seeded in 12-well plates and grown to 70% confluence. Cells were treated with LPS (1 μg/mL) for 6 h, and lysates were prepared and assayed for luciferase activity. (C and D) Analysis of iNOS and COX-2 mRNA expression by RT-q-PCR. Cells were treated as above, total RNA was extracted and analyzed by RT-q-PCR for iNOS (C) and COX-2 (D) mRNA expression. (E and F) The iNOS-luciferase (E) or COX-2-luciferase (F) cells were cultured as stated in panels A and B. Cells were treated with 25 μg/mL CAPE in the presence or absence of LPS and assayed for luciferase activity. Shown is luciferase activity relative to the nontreated control (Ctrl) set as 1, and values are mean $\pm$ SD from at least three independent experiments. ** represents a significant difference compared to the Ctrl (Student's t test, $p < 0.01$).

Luciferase Assay System (Promega, WI, USA) according to the protocols provided by the manufacture. For normalizing the luciferase value of each reaction, the value of luminescence measurement was divided by the concentration of protein for every individual sample. Data was shown as the relative fold to control, whereas control was set as 1.

Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-q-PCR). Q-PCR analyses were performed on aliquots of cDNA for mRNA levels of iNOS, COX-2 and 18S (as an internal standard) using a thermal cycler (Applied Biosystems, CA, USA). Reactions were carried out in a volume of 10 μL containing KAPA SYBR Master Mix, forward and reverse primers, and cDNA. After initial enzyme activation at 95 °C for 3 min, the reactions were proceeded by 40 amplification cycles (denaturation at 95 °C for 30 s followed by annealing and extension at 60 °C for 20 s). The PCR primers used in this study were as follows: iNOS-forward, 5′-CCT GGT ACG GGC ATT GCT-3′ and reverse, 5′-GCT CAT GCG GCC TCC TTT-3′; COX-2-forward, 5′-ACA ACA GAG TGT GCG AC-3′, and reverse, 5′-TGA GTT TGA AGT GGT AAC CG-3′; and 18S-forward, 5′-TAT TCC CAT GAC CCG CC-3′ and reverse, 5′-GTG AGG TTTCCC GTG TT-3′

Preparation of Chinese Olive Extract. Fresh Chinese olives were crushed and mixed with water and extracted for 4 h at room temperature. The paste was centrifuged to separate the aqueous part and dried, solubilize with methanol at ratio of 20 v/w (mL/g) for three times. The extract was evaporated and resuspended with water at a ratio of 10 v/w (mL/g) and then partitioned with the same volume of hexane, ethyl acetate, and butanol at room temperature, respectively, to obtain fractions with different polarity.

Preparation of the Nondigestible Water-Soluble Polysaccharides (ND-WSP) from Mushrooms. Eighteen different mushrooms were collected from local grocery stores, including *Agaricus blazei*, *Agaricus bioporus*, *Hypsizygus marmoreus* (two species), *Lentinula edodes* (dried and fresh), *Pleurotus cystidiosus*, *Pleurotus*

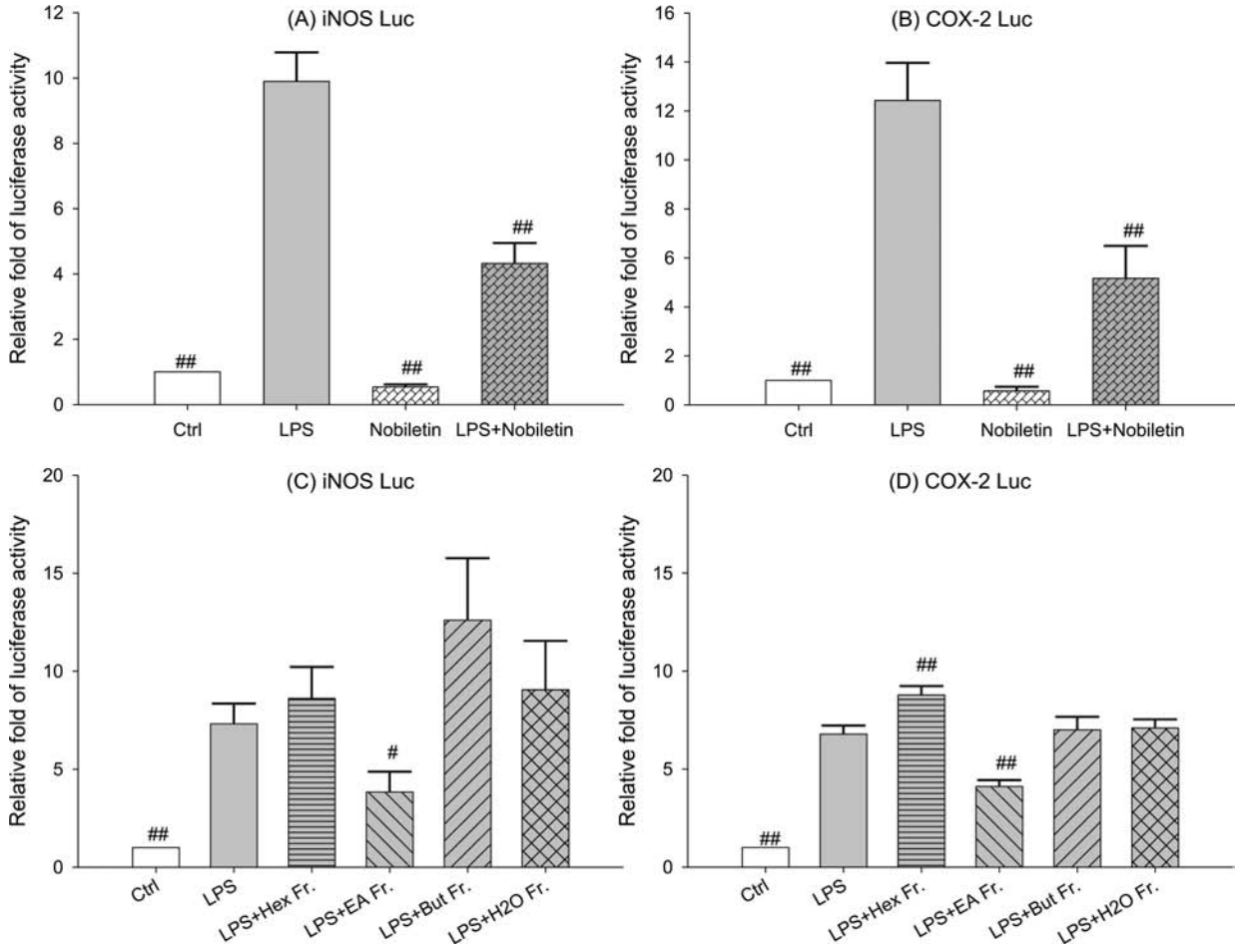

Figure 2. iNOS and COX-2-directed luciferase systems which are sensitive to anti-inflammatory activities from natural products. The iNOS (A) and COX-2 (B) luciferase cells were plated in 12-well plates and cultured to 70% confluence. The cells were preincubated with nobiletin (100 μM) for 1 h and treated with LPS (1 μg/mL) for 6 h before assaying for luciferase activity. Alternatively, the iNOS (C) and COX-2 (D) luciferase cells were treated with different organic solvent extractions of Chinese olives for 1 h, followed by LPS treatment for 6 h before assaying for luciferase activity. Shown is luciferase activity relative to the nontreated control (Ctrl) set as 1, and values are mean ± SD from at least three independent experiments. ## represents a significant difference compared to the LPS group (Student's t test, $p < 0.01$, whereas # indicates $p < 0.05$).

eryngii, *Pleurotus ferulae*, *Pleurotus nerbrodensis*, *Pleurotus ostreatus*, *Pleurotus salmoneostramineus*, *Auricularia auricula-judae*, *Coprinus comatus*, *Flammulina velutipes*, *Hericium erinaceus*, *Pholiota nameko*, and *Tremella fuciformis*. The ND-WSP was prepared according to the dietary fiber assay protocols of Sigma TDF-100A kit (Sigma, MO, USA). Briefly, lyophilized powders of mushrooms were gelatinized at 95 °C with heat stable α-amylase and then digested with protease and amyloglucosidase to remove digestible proteins and starch. The mixture was filtered, and the ND-WSP was precipitated by adding ethanol to the filtrate.

Determination of TNF-α by ELISA. Cells were seeded into the 24-well culture plates at a density of 3×10^5/well in DMEM for 24 h. The medium was removed and cells were incubated in medium containing ND-WSP prepared from various mushroom species. Amounts of TNF-α in the cultured medium were determined using the Mouse TNF-α ELISA Ready-SET-Go kit from eBioscience (CA, USA) according to the manufacturer's instructions.

Determination of Nitric Oxide (NO). Cells were cultured in DMEM for 24 h and treated with extractions from various mushroom species for 24 h. Concentrations of NO released into the medium was determined by mixing 50 μL of the medium with 50 μL of the Griess reagent (Sigma, MO, USA) for 15 min. The absorption of the mixture was measured at 540 nm with an ELISA reader. A serial dilution of sodium nitrite ($NaNO_2$) was used as the standard.

Statistical Analysis. Values are expressed as the mean ± SD from at least three experiments. Data were analyzed by Student's t test for two-group comparisons. The correlations between analysis results from luciferase-based platform (iNOS, COX2) and cytokine production (NO, TNF-α) were performed using the computer software-Statistical Package for the Social Sciences (SPSS). The correlation coefficient was determined by Spearman analysis.

RESULTS

Establishment of Stable Macrophage Cells Reporting NF-κB-Mediated iNOS and COX-2 Gene Activation. To report the NF-κB-directed inflammation signaling pathways, we selected iNOS and COX-2 promoters, instead of consensus NF-κB binding sequence, which has been adopted in our previous study,[13] for modulating luciferase reporter activities. The reporter plasmids, pGL4.32-iNOS and pGL4.32-COX-2 which contain iNOS and COX-2 promoter-driven luciferase genes, respectively, were separately transfected into mouse RAW264.7 macrophage cells. Cells resistant to hygromycin were selected, and single foci-derived clonal cells were further examined for their ability to express luciferase in response to LPS treatment. A total of eleven highly sensitive clones containing iNOS promoter and four COX-2 containing clones were obtained. All of these clones were subjected to cycles of freeze−thaw treatment and shown to maintain their responsiveness to LPS stimulation. As shown by representative experi-

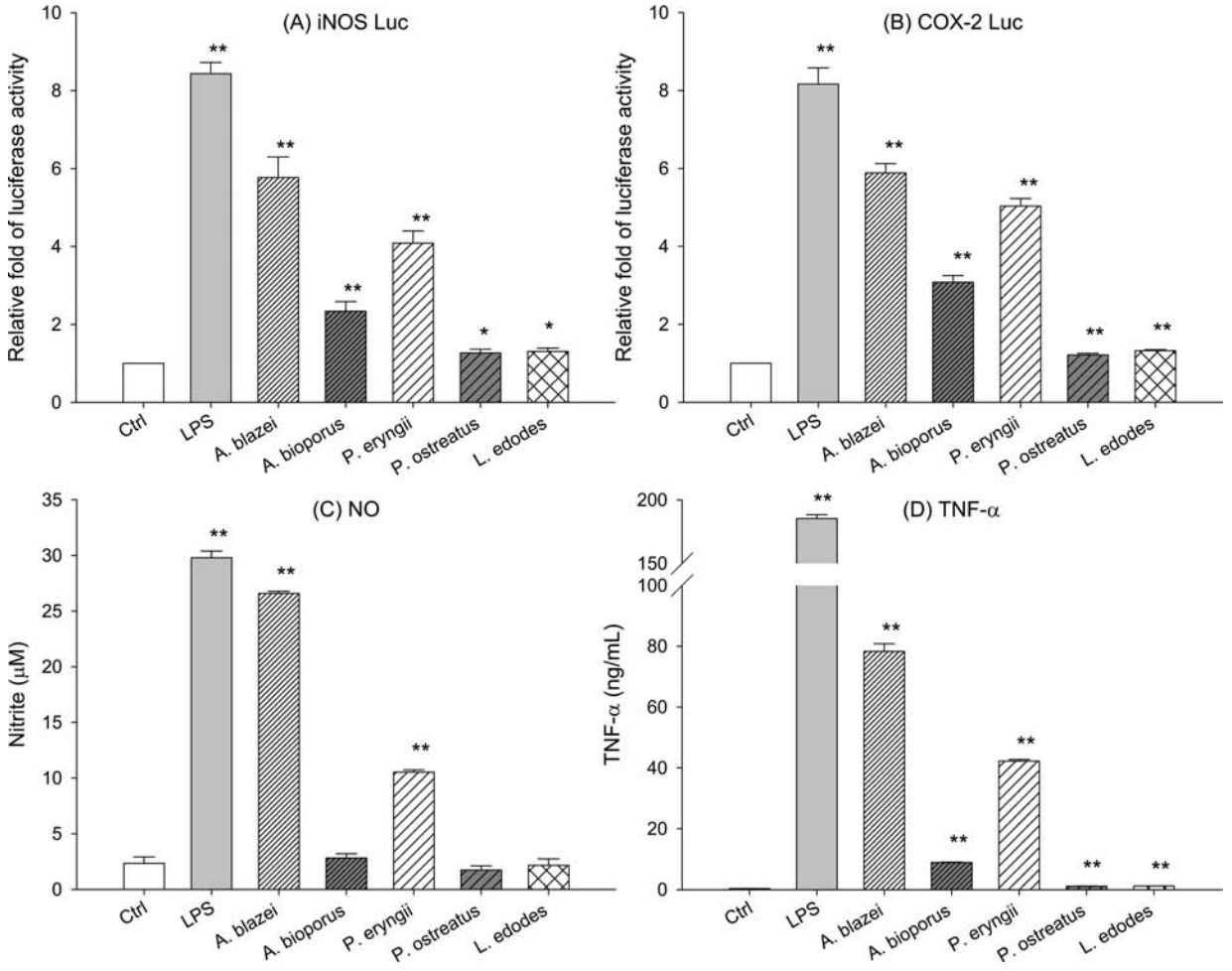

Figure 3. Comparisons of the luciferase-based assay systems to other immune-modulatory-activity detection methods. The iNOS (A) and COX-2 (B) luciferase cells were culture in 24-well plates and grown to 70% confluence. The cells were treated with 100 μg/mL of the nondigestible water-soluble polysaccharides (ND-WSP) prepared from the indicated mushroom species for 6 h. Cell lysates were prepared for luciferase assays. Alternatively, cells were treated as above, and concentrations of NO (C) and TNF-α (D) in the culture medium after 24 h of treatment were determined by chemical-based and ELISA-based methods, respectively, as described in the Materials and Methods. The results shown are mean ± SD from three independent experiments, and ** represents a significant difference compared to the Ctrl (Student's t test, $p < 0.01$, whereas * indicates $p < 0.05$).

ments in Figure 1, panels A and B, luciferase activities increased upon treatment with LPS, suggesting increased iNOS and COS-2 promoter activities. Analysis by RT-q-PCR demonstrated mRNA levels of iNOS and COX-2 increased accordingly (Figure 1, panels C and D), suggesting that the promoter-luciferase assays accurately reflect the abundance of iNOS and COX-2 mRNA levels upon inflammation stimulation.

Both iNOS and COX-2 have been shown to be NF-κB target genes, we next verified whether the LPS-stimulated luciferase activation is indeed mediated by NF-κB in the established cell systems. The RAW264.7 cells expressing iNOS and COX-2-directed luciferase genes were treated with LPS in the presence or absence of the NF-κB inhibitor CAPE. As shown in Figure 1, panels E and F, addition of CAPE blocked the LPS-stimulated luciferase activity in both macrophage clones. These results together demonstrated that we have established luciferase-containing macrophage cell systems which express luciferase activity in response to inflammatory stimulation in a NF-κB/iNOS or NF-κB/COX2 dependent manner.

iNOS and COX-2-Directed Luciferase Systems Can Detect Immune-Suppressive Activities in Natural Products. We examined the effectiveness of the established cell systems to detect the anti-inflammatory activities of natural products. The cells were treated with LPS in the presence or absence of nobiletin, an anti-inflammatory compound abundant in citrus peels shown previously to suppress the expression of iNOS and COX-2.[14] As shown in Figure 2 (panels A and B), the LPS-induced luciferase activities were significantly suppressed by the addition of nobiletin in both the iNOS- and COX-2-luciferase cells. We next tested the sensitivity of the luciferase cell systems to various organic solvent extractions of Chinese olives. The water extraction of Chinese olives was dried and solubilized by methanol and sequentially partitioned with hexane (Hex), ethyl acetate (EA), and butanol (But), the remaining water-soluble fraction was designated as H_2O. As shown, among the organic solvent extractions, the anti-inflammatory activity of the olives was exclusively contained in the ethylacetate fraction both the iNOS and the COX2 cell systems (Figure 2, panels C and D).

Comparisons of the Luciferase-Based Assay Systems to Other Immuno-Modulatory-Activities Detection Methods. We next asked whether the method developed in this study is comparable to other high throughput screening

methods. Because mushrooms are known to contain immunomodulatory activities, we prepared the water-soluble polysaccharides (ND-WSP, see the Materials and Methods) from various mushroom species and assessed their inflammation activities by the iNOS and COX2-modulated luciferase systems, the ELISA-based TNF-α release and the medium NO concentration determination. As revealed in Figure 3, panels A and B, analogous results were obtained using the iNOS or COX2 promoter-mediated luciferase assays. The ND-WSP prepared from *Agaricus blazei*, *Agaricus bioporus*, and *Pleurotus eryngii* significantly stimulated the iNOS and COX-2 promoter activities when compared with the nontreated control cells. Similar patterns of immuno-stimulatory activities were obtained when assayed for the concentrations of NO (Figure 3C) or TNF-α (Figure 3D) in the medium. One exception was *Agaricus bioporus*; in contrast to other detection methods, no significant stimulation on NO release could be observed (Figure 3C). To assess the correlation among these detecting methods, we further assayed the immuno-stimulatory activities of 18 mushroom extracts. Analysis by the scatterplot revealed positive correlations (correlation coefficient >0.7) when the data sets from separate methods were compared (Figure 4).

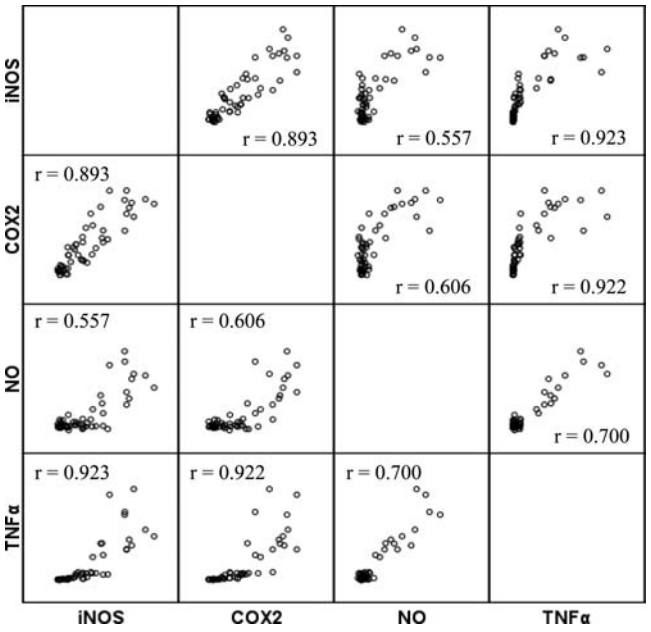

Figure 4. Analysis of the relationships between detection methods by Scatterplots. Immuno-modulatory activities of ND-WSP from 18 mushroom species were analyzed by the iNOS and COX-2-based luciferase assays, as well as by the release of NO and TNF-α into the culture medium. The relationships between sets of data were evaluated by displaying two each of the data set on Scatterplot. The correlation coefficients of greater than 0.7 were regarded as strong correlations. Data were from three independent experiments.

The correlation coefficient between iNOS and COX-2 monitoring platforms was 0.893. Moreover, the association between the luciferase platforms and TNF-α assessment exhibited strong correlation with a correlation coefficient of 0.92. These results demonstrated that the immuno-modulatory activity detecting system established in the present study is comparable to that of the high throughput TNF-α screening system for immunomodulatory materials.

DISCUSSION

In this study, we have established stable macrophage cell clones expressing luciferase reporter gene directed by promoters of the NF-κB transcriptional targets iNOS and COX-2. An array of NF-κB regulated genes has been identified and shown to be involved in cellular activities including proliferation, survival and invasion, in addition to inflammation. Because iNOS and COX-2 are inflammation-related targets, instead of the consensus NF-κB response element which is of broad specificity, promoters of iNOS and COX-2 were placed upstream of the luciferase gene in construction of the inflammation-sensing reporter. The established cell clones maintained stable responsiveness to immuno-stimulant or immuno-suppressant by altering luciferase activities even after cycles of freeze/thawing. Moreover, the LPS-stimulated luciferase activation was inhibited by the addition of CAPE, a specific NF-κB inhibitor. Using the established stable macrophage cells, we found that several mushroom-derived ND-WSP fractions possessed inflammation inducing activities. This result is in congruous with that obtained from analyzing the medium NO and TNF-α concentrations, and is consistent with reports that many polysaccharides from mushrooms can trigger immune response in macrophage cells.[15] These results demonstrated that the established luciferase assay system is capable of sensing inflammation signals in a NF-κB-dependent manner.

We have analyzed 18 mushroom extracts using different inflammation assessment methods and showed strong correlations between iNOS or COX-2-directed luciferase system and the ELISA-based assay for medium TNF-α. Despite the close relationships among these assay systems, the correlation coefficient was lower, but nevertheless significant, when comparing NO secretion and the luciferase-based assays. It is intriguing that the correlation between NO determination and iNOS-based luciferase assay did not exhibit tight association since NO is a reaction product of iNOS. One possibility is that the NO secretion into the medium was detected at a much later time point (24 h after stimulation) relative to the determination of luciferase activities (6 h), and also we cannot exclude the suggestion that only a small proportion of NO was released into the medium.

The inflammation signal-sensing system developed in the present study offers several advantages to be used as a high throughput screening assay. Relative to the ELISA-based antibody/antigen reactions, the luciferase reaction is rapid. When carried out in 96-well plates, the luminescent products can be detected by a luminometer in seconds. Moreover, using the established stable macrophage clones, the tedious plasmid transfection and the variation it caused between experiments can be avoided; reproducible results are thereby attainable. We have demonstrated that the promoter activities of iNOS and COX-2, as revealed by luciferase activities, paralleled with the mRNA expression of iNOS and COX-2, as estimated by RT-q-PCR assays. In this sense, the iNOS and COX2-dependent luciferase assays supersede the time- and cost-consuming RT-q-PCR in assaying immune-modulatory activities. In conclusion, we have established a luciferase-assay based system sensitive to external stimulation in an NF-kB/iNOS or COX-2-dependent manner. The platform is applicable as a high throughput screening for the detection of anti-inflammation compounds.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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