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Establishment and Evaluation of Biotechnological Platform for Screening Health Food with Anti-inflammation Ability

Wang-Ju Hsieh[1,§], Shih-Ting Chiou[2,§], Min-Hsiung Pan[3], and Shu-Chen Hsieh[2,*]

[1] Department of Biochemical Science and Technology, National Taiwan University, Taipei, Taiwan
[2] Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan
[3] Department of Seafood Science, National Kaoshuiung Marine University, Kaoshiung, Taiwan
[§] Contributed equally

Abstract

Chronic inflammation leads to a progressive inflammation in certain types of cells. Recent studies report that the activation of nuclear factor kappa B (NF-κB) increases the expression of inflammation-related protein such as inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), which further enhance the chronic inflammation, thus conduct the development of disorders. The aim of the study is to develop an efficient method for screening food components with anti-inflammation function. Here we employed a reporter plasmid, which contains NF-κB response element followed by a minimal promoter for driving the down-stream luciferase reporter gene. After transfection of this plasmid to a mouse cell line RAW264.7, we obtained stable clones by using Hygromycin selection. Our results reveal that the luciferase activity of the cell based platform can be induced by the inflammation inducing reagent LPS and can be further suppressed by the administration of CAPE, an anti-inflammation chemical. The results estimated by our platform present good correlation to that analyzed by RT-Q-PCR. Additionally, the known anti-inflammation factors such as resveratrol, significantly counteracted the effect of LPS on our platform. Furthermore, the screening result of various mushroom extract showed that some fractions revealed NF-κB activating effects. Therefore, we conclude that the platform is effective in large scale screening for inflammatory regulating compounds.

Key words: Inflammation, NF-κB, Luciferase assay, Cell platform

Introduction

Chronic inflammation promotes progression of cardiovascular disease and the consequent heart attack and stroke (Hansson and Hermansson, 2011), and also destroys nerve cells in the brains of Alzheimer's victims and facilitates the development and progression of cancer (Terzić et al., 2007). Recently, NF-κB has been reported to act as a pro-inflammatory factor in the development of chronic inflammation (Baker et al., 2011).

The stimulators of NF-κB include cytokines such as interleukin 1 (IL-1), tumor necrosis factor alpha (TNF-α), and lipopolysaccharide (LPS) (Anisowicz et al., 1991; Jones et al., 2007). Activated NF-κB plays a pivotal role in driving the expression of other proinflammatory genes such as cytokines, chemokines, and can enhance several inflammatory genes, including adhesion molecules, induced nitric oxide synthase (iNOS) and cycloxygenase-2 (COX-2).

Correspondence to:
Dr. Shu-Chen Hsieh. Graduate Institute of Food Science and Technology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan, Tel: +886-2-3366-9871, Fax: +886-2-2362-0849, E-mail: scjhsieh@ntu.edu.tw

iNOS belongs to NOS family. As compared to the other two family members nNOS and eNOS, which exhibit constitutive expression (Huang, 2009; Kobayashi, 2010), the expression of iNOS is inducible upon many stimulus like cytokines, viruses, or LPS, leading to the production of large amounts of nitric oxide (NO). Subsequently, high concentration of NO causes cell proliferation, invasion (Ambs et al., 1997), and local or systemic inflammatory disorders (Bogdan et al., 2000).

COX-2, the other downstream target of NF-κB, catalyzes the production of Prostaglandin E2 (PGE2), and may function as a proinflammatory mediator (Dubois et al., 1998; Yang et al., 2011).

Because the relationship between chronic diseases and inflammation has been well established, to find out compounds with anti-inflammation effects becomes an important issue. Typically, Western blot and RT-Q-PCR are employed to analyze the anti-inflammation activity of components by detecting the expression of inflammation and proinflammation genes. The whole process is both time and money consuming. In order to develop a better method, we establish a cell based platform containing NF-κB response element fused reporter plasmid. We further proved that the platform can be used for high throughput screening for food components with anti-inflammation function.

Material and methods

Cell culture and sample treatment

RAW264.7 were grown at 37 ℃ in DMEM medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin sulfate (100 U/ml) in a humidified atmosphere of 5% CO2. Cells were pretreated with different samples for 1 h, and followed by 1 μg/ml LPS (Sigma-Aldrich MO, USA) treatment for 6 h before luciferase assay.

Transfection and Stable clone selection

RAW264.7 cells were plated in 60 mm dish at a density of 90% confluence. Before transfection, the culture medium was replaced by serum-free DMEM medium for 1h, and then pGL4.32 [luc2P/NF-kB-RE/Hyrgo (with five copies of 5 NF-κB binding site (GGGAA/CTTTCC); Promega, WI, USA) was transfected into the cells by Lipofecatime 2000 (Invitrogen, NY, USA) according to user's guide. The cells were treated with 400 μg/ml Hygromycin B for 9 days, and 60 colonies were picked up separately for growing to larger amount. We further examined the response of each clone to LPS by luciferase assay. Only clones gave significant response to LPS were kept.

Assay of Luciferase activity

The cells were washed with 1X PBS buffer for three times, and the protein lysate was extracted by 1X lysis buffer according to the suggestion in the user's guide (Promega, WI, USA). The cell lysates were harvested and followed by determining the luciferase activities and the total protein concentration. luciferase activities were measured by the Luciferase Assay System (Promega, WI, USA) through the procedures suggested in user's guide. The total concentration of protein was measured by Bio-Rad Protein Assay (CA, USA), and the results served as an internal control to normalize the luminescence values.

RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using Tripure Isolation Reagent (Roche, IN, USA). For each sample, 2 μg of RNA was reverse-transcribed (RT) using SMART™ MMLV reverse transcriptase, 1 mM deoxyribonucleotide triphosphate (dNTP), 1X First-Strand Buffer, 10 mM DTT, and 2.5 μM random hexamer, adjusted by DEPC H2O to the final volume of 20 μl in a 0.2 ml PCR tube. The tubes were then incubated under 42℃, 60 min for reverse transcription. Finally, heat at 72℃ for 15 min to inactivate the reaction.

Quantitative polymerase chain reaction (Q-PCR)

Q-PCR analyses were performed on aliquots of the cDNA preparations to detect iNOS, COX-2 and 18S (as an internal standard) gene expresion using a thermal cycler (Applied Biosystems™, CA, USA). Reactions were carried out in a volume of 10 μl containing 5 μl of 2 x of KAPA SYBR® Master Mix, 0.4 μl of 10 μM forward and reverse primers, 2.6 μl PCR water, and 2 μl cDNA. After initial enzyme activation for 3 min at 95 ℃, 40 amplification cycles were performed for three genes (3 sec of 95 ℃ denaturation, 20 sec of 60 ℃ annealing and extension). The PCR primers used in this study are listed below and were purchased from Sigma-Aldrich (MO, USA). For gene detection of iNOS, sense strand: 5'-CCT GGT ACG GGC ATT GCT-3'-, antisense strand: 5'-GCT CAT GCG GCC TCC TTT-3'-. For gene detection of COX-2, sense strand: 5'-ACA ACA GAG TGT GCG AC-3'-, anti-sense strand: 5'-TGA GTT TGA AGT GGT AAC CG-3'-. For gene

detection of 18S sense strand: 5'-TAT TCC CAT GAC CCG CC-3'-, anti-sense strand: 5'-GTG AGG TTT CCC GTG TT-3'.

Results

Single clone selection and confirmation of the efficacy of the cell based platform

We transfected pGL4.32-NF-κB, a reporter plasmid containing NF-κB response element fused with the luciferase reporter gene, into mouse macrophage cell line RAW 264.7. The cells stably expressing the reporter plasmid survived after hygromycin selection. For the stable lines derived from each single colony we selected, we employed the luciferase assay to test whether they could respond to inflammation signaling. As shown in Figure 1A, the representative stable line we obtained after antibiotics selection dramatically responded to the inflammation signaling induced by LPS. In consistent with the luciferase result, the inflammation signal which triggered the activity of NF-κB measured in our cell based platform, could turn on the mRNA expression of iNOS and COX-2, the downstream targets of NF-κB (Figure 1B, 1C), suggesting that our platform can successfully detect the inflammation signaling in culture environment.

Inflammation signal blocked by NF-κB inhibitor can be detecting by the cell based platform

After confirming the efficacy of the platform on sensing the inflammation signaling, we next examined whether the anti-inflammation signaling could be detected by our platform. We thus used CAPE, a NF-κB inhibitor, to block the signaling of inflammation. As expected, the results revealed that the platform we established was sensitive to the addition of CAPE, which suppressed the LPS induced luciferase activity. Additionally, the endogenous NF-κB activity could be suppressed by CAPE (Figure 2). The results together indicate that the platform is capable of detecting both inflammation and anti-inflammation signaling.

The application of the platform for screening components with immune regulation activity

We further estimated the capacity of our platform by using a well-studied compound, resveratrol, which has the effects on decreasing the protein expression of iNOS, and COX-2 (Donnelly et al., 2004). As can be observed in Figure 3, the luciferase activity induced by LPS could be reduced by resveratrol in a dose dependent manner. We next tried to screen food components with

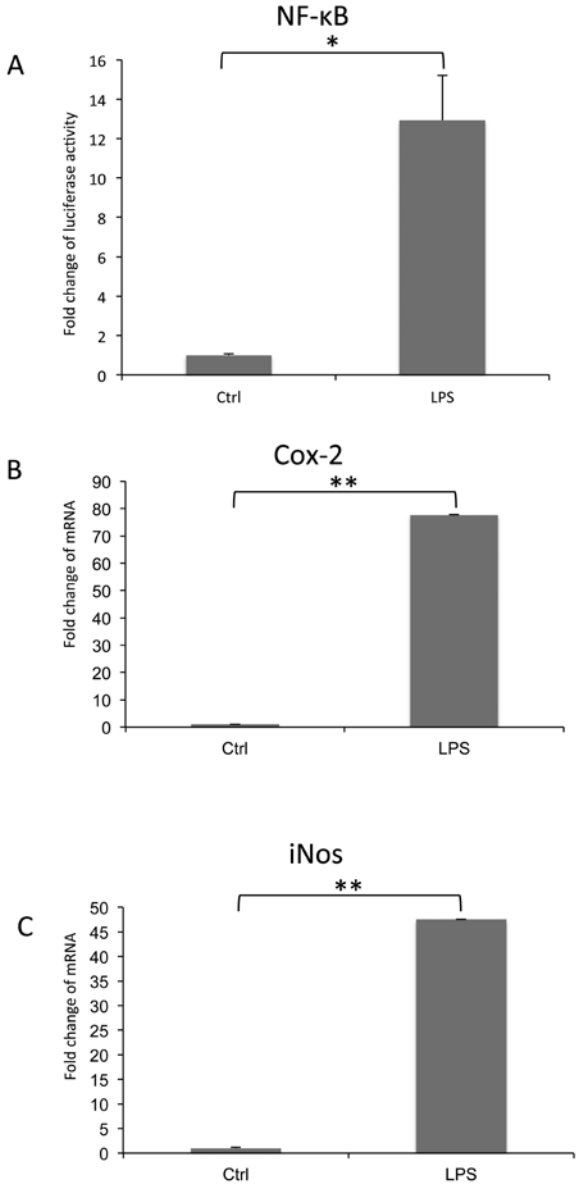

Figure 1. The cell based platform significantly responds to the inflammation inducing reagent LPS.
(A) The Raw264.7 cells stably expressing pGL4.32-NF-κB plasmid. were seeded in 12 well plate and grew up to the density of 70% (the details for constructing the cell based platform was described in materials and methods). After being treated with 1 μg/ml LPS for 6 h, the total protein lysate were collected and luciferase activity was measured by Luminoskan Ascent. Under the same treatment as described in (A), the total RNA was extracted, and 2 μg RNA was used to be converted into cDNA, followed by Q-PCR, the mRNA expression of (B) iNOS and (C) COX-2 were estimated. The results were shown as means ±SD and analyzed by Student's T-test to show the statistical significance. ** $p<0.01$ and * $p<0.05$ indicate significant difference when compared to control group (Ctrl).

inflammation regulating activity by using our platform. Mushrooms are known for the ability in regulating immune response; we therefore screened various mushroom fermentation extracts to estimate their immune modulating ability. As revealed in Figure 4, among the screening samples, the fermentation extract

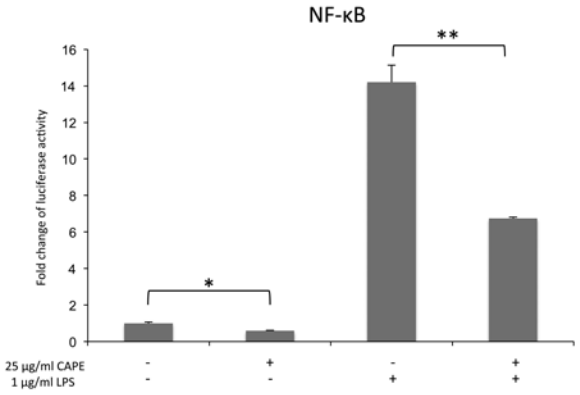

Figure 2. The platform can monitor the anti-inflammation signal in the culture environment.
The Raw264.7 cells stably expressing pGL4.32-NF-κB plasmid were seeded in 12 well plate and grew up to the density of 70%, then either pretreated with 25 μg/ml CAPE or left non-treated for 1 h and followed by the presence or absence of 1 μg/ml LPS treatment for 6 h. Total protein lysates were collected and the luciferase assay was then taken by Luminoskan Ascent. The relative folds were shown as means ±SD and analyzed. ** $p<0.01$ and * $p<0.05$ reveals statistical significance between the samples compared using Student's T-test.

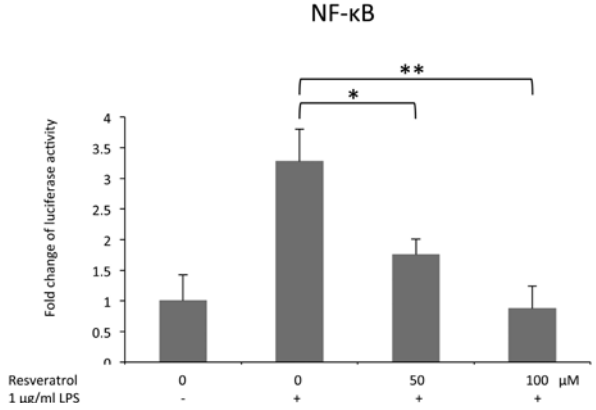

Figure 3. The anti-inflammation effect of resveratrol can be monitored by the cell based platform.
The Raw264.7 cells stably expressing pGL4.32-NF-κB plasmid were seeded in 12 well plate and grew up to the density of 70%, then the cells were pretreated with 50 or 100 μM Resveratrol for 1 h and then incubated with 1 μg/ml LPS for 6 h. Total protein lysates were collected and the luciferase activity was estimated by Luminoskan Ascent and results were shown as a relative fold to control with means ±SD and analyzed by Student's T-test to show the statistical result. ** $p<0.01$ and * $p<0.05$ indicates the significant differences between samples compared.

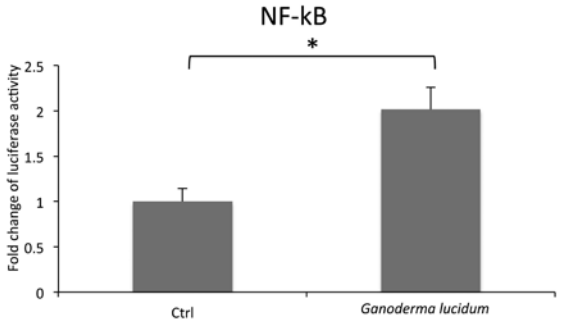

Figure 4. The screening results of the platform.
The Raw264.7 cells stably expressing pGL4.32-NF-κB plasmid were seeded in 12 well plate and grew up to the density of 70%, then the cells were pretreated with 100 μg/ml of the fermentation extract of *Ganoderma lucidum* 6 h respectively. Total protein lysates were collected and the luciferase activity was estimated by Luminoskan Ascent. The results show the relative fold to control. The results were shown as means ±SD and analyzed by Student's T-test to show the statistical significance. * $p<0.05$ means the result of treatment is significant different from that of control group (Ctrl).

of *Ganoderma lucidum* showed ability in activating NF-κB, consistent with the fact that compounds within *Ganoderma lucidum* can induce moderate inflammation signal to enhance body defense when encountering pathogens (Kuo et al., 2006)

Discussion

In this study, we have successfully established a cell based screening platform for searching food components with inflammation modulating activity. Compared with the traditional analyses such as Western blot and RT-PCR, our platform can be used for high throughput screening because the assay system adopted in our platform is luciferase reporter assay.

Reporter assay was first used in determining the gene expression in 1990. (Alam and Cook, 1990). In our project, we used pGL4.32-NF-κB to monitor the activity of NF-κB by measuring the luciferase activity. The principle is that the transcription activity of NF-κB correlates to the expression level of reporter gene driven, hence the higher luciferase activity, which leads to higher amount of luminescent products, means higher NF-κB activity. Because NF-κB activity is a good marker reflecting the extent of inflammation, we can detect the inflammation signaling by reading the value of luciferase activity.

Luciferase assay can be applied in a 96 well plate, and the measurement of 96 samples only takes few minutes, showing the efficacy for high throughput screening (Liu et al., 2009; Wehner et al., 2011). Furthermore, our cell based platform can stably express the reporter plasmid, implying that we don't have to go through transfections before performing each luciferase assay. In general, before doing the reporter assay, the plasmid must be transfected into cells, which costs time and money. Especially, the transfection efficiency of some cell lines is low and leads to the consequent low sensitivity and poor assay results. For example, the transfection efficiency of RAW264.7 is less than 0.5% in our preliminary test. Low level of transfection efficiency signifies that only few percentages of cells own the ability to drive reporter gene upon stimulus.

In the case of RAW264.7, it could hardly respond to the inflammation signaling or the anti-inflammation signaling using transient transfection. On the contrary, our platform can respond to LPS and CAPE efficiently, indicating the application value of our platform in screening the food components with inflammation modulating ability.

Our screening results of resveratrol and mushroom extract reveal that our platform can not only detect the anti-inflammation ability of well-studied pure compound identified from food resources, but also explore the inflammation regulating ability of extracts with unknown function. The results are consistent with the known function of resveratrol, which largely exists in several plants when under attack by pathogens, showing significant ability against inflammation (Donnelly et al., 2004). Our results are also in agreement with the fact that many types of mushrooms reveal the ability to modulate the immune response by moderately elevating the inflammation level. The success of establishing a cell based platform demonstrates an alternative way to establish a time and cost saving screening platform for identifying components with immune regulating ability.

In conclusion, we have established a cell based platform for screening anti-inflammation components from various food resources. The platform has been improved to have efficacy for high throughput screening and displays the potential in pharmatheutical application.

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