Establishment and Evaluation of the Reporter Platforms for Screening Health Food with Anti-inflammation Ability

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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 inflammation modulation activity. Here we employed reporter plasmids, which contain either the promoter of iNOS or COX-2 to drive the down-stream luciferase reporter gene. After transfection of these plasmids to a mouse macrophage cell line RAW264.7, we obtained stable clones derived from each single cell colony 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 nobiletin significantly counteracted the effect of LPS on our platform. Furthermore, the screening result of various mushroom extract showed that some extracts revealed iNOS and COX-2 activating effects. Therefore, we conclude that the platform is effective in large scale screening for inflammatory regulating compounds.

Keywords: Inflammation, Luciferase assay, iNOS, COX-2, Platform.

1. Introduction

Chronic inflammation promotes progression of many chronic diseases, including cardiovascular disease, Alzheimer's disease, and cancers[1, 2]. Recently, NF-κB has been reported to act as a pro-inflammatory factor in the development of chronic inflammation [3]. The stimulators of NF-κB include cytokines such as interleukin 1 (IL-1), tumor necrosis factor a (TNF-α), and lipopolysaccharide (LPS) [4, 5]. Activated NF-κB plays a pivotal role in driving the expression of other pro-inflammatory genes such as cytokines, chemokines, and can enhance several inflammatory genes, including adhesion molecules, induced nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).

iNOS belongs to NOS family. As compared to the other two family members nNOS and eNOS, which exhibit constitutive expression [6, 7], the expression of iNOS is inducible upon many stimuli 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 [8], and local or systemic inflammatory disorders [9]. The other downstream target of NF-κB, COX-2, catalyzes the production of Prostaglandin E2 (PGE2), and may function as a pro-inflammatory mediator [10, 11].

Because the relationship between chronic diseases and inflammation has been well established, to find out compounds with inflammation regulating 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 pro-inflammation genes. The whole process is both time and money...
consuming. In order to develop a better method, we established the cell based platforms containing either iNOS or COX-2 promoter fused reporter plasmid. We further proved that the platforms can be used for high throughput screening for food components with inflammation modulation ability.

2. Materials and Methods

2.1. Transfection and Stable Clone Selection

RAW264.7 cells were plated in 60 mm dish at 90% confluence. Before transfection, the culture medium was replaced by serum-free DMEM medium for 1h, and then pGL4.32-iNOSand pGL4.32-COX-2 (we have constructed the plasmids by inserting the promoter of iNOS or COX-2 into the upstream of luciferase gene of pGL4 plasmid (promega, WI, USA) respectively) were transfected into the cells by Lipofectamine 2000 (Invitrogen, NY, USA) respectively 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 upon LPS treatment by luciferase assay. Only clones exhibiting significant response to LPS were kept.

2.2. 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 user’s guide (Promega, WI, USA). The cell lysates were harvested and used for 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 the user’s guide. The total concentration of protein was measured by Bio-Rad Protein Assay (CA, USA), and the total protein amounts served as an internal control to normalize the luminescence value.

2.3. RNA Preparation and Reverse Transcriptase Quantitative polymerase Chain Reaction (RT-PCR)

The whole procedure was described in our previous study [12].

3. Results and Discussion

3.1. Single Clone Selection and Confirmation of the Efficacy of the Cell Based Platform

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

3.2. 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 signalling, we next examined whether the anti-inflammation signalling could be detected by our platform. We thus used CAPE, a NF-κB inhibitor, to block the signalling of inflammation. As expected, the results revealed that the platforms we established were sensitive to the addition of CAPE, which suppressed the LPS induced luciferase activity. Additionally, the gene expression of iNOS and COX-2 measured by luciferase assay could be suppressed by CAPE (Figure 2). The results together indicate that the platform is capable of detecting both inflammation and anti-inflammation signalling.

3.3. The Application of the Platform for Screening Components with Immune Regulation Activity
We further estimated the capacity of our platforms by using a well-studied compound, nobiletin, which has the effects on decreasing the protein expression of iNOS, and COX-2 [13]. As revealed in Fig. 3, the luciferase activity induced by LPS could be reduced by nobiletin, indicating the efficacy of the platforms. We next tried to screen food components with inflammation regulating activity by using our platform. Mushrooms are known for the ability in modulating immune response; we therefore screened various mushroom extracts to estimate their immune regulating ability. As revealed in Fig. 4, among the screening samples, the extracts of *Agrocybe cylindracea* and *Auricularia auricula-juda* showed ability in activating both iNOS and COX-2, consistent with the fact that compounds within mushrooms can induce moderate inflammation signal to enhance body defense when encountering pathogens[14].

In this study, we have successfully established the cell based screening platforms 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 can be applied in a 96 well plate, and the measurement of 96 samples takes only few minutes. Furthermore, our cell based platforms can stably express the reporter plasmid, implying that we won’t have to go through transfections before performing each luciferase assay. 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. We believe that the success of establishing the cell based platforms demonstrates an alternative way to establish a time and cost saving screening platform for identifying components with immune regulating ability.

![Fig. 1](image)

**Fig. 1:** The cell based platform significantly responds to the inflammation inducing reagent LPS.

The Raw264.7 cells stably expressing pGL4.32-iNOS (A) or pGL4.32-COX-2(C) plasmid were seeded in a 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 6-h treatment of LPS (1 μg/ml), the total protein lysate was collected and luciferase activity was measured by Luminoskan Ascent. Under the same treatment as described in (A, C), 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 (D) COX-2 were estimated. The results were shown as means ±SD.

The Raw264.7 cells stably expressing pGL4.32-iNOS (A) or pGL4.32-COX-2(B) plasmid were seeded in a 12 well plate and grew up to the density of 70%, then either pre-treated 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.
The Raw264.7 cells stably expressing pGL4.32-iNOS or pGL4.32-COX-2 plasmid were seeded in 12 a well plates and grew up to the density of 70%, then the cells were pre-treated with 100 μM Nobiletin for 1 h and followed by incubation 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.

The Raw264.7 cells stably expressing pGL4.32-iNOS or pGL4.32-COX-2 plasmid was seeded in a 12 well plate and grew up to the density of 70%, then the cells were pre-treated with each of the extracts from different mushrooms at the concentration of 100 μg/ml for 6 h. 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.

4. Acknowledgements

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5. References


Fig. 3: The anti-inflammation effect of nobiletin can be monitored by the cell based platform.

Fig. 4: The screening results of the platform.