S-nitrosylation of Inhibitor-κB kinase: Identifying Novel Targets of Curcumin

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Abstract In this study, we investigated the preventive effects of curcumin using LPS (Lipopolysaccharides)-induced Raw264.7 cells and the potential role of curcumin in regulation of anti-inflammation through *S*-nitrosylation. Western blot presented the protein expression of iNOS can be reduced by treated curcumin with 5, 10 and 15 μM separately for 12 and 24h. Consistently, pro-inflammatory cytokines, such as IL-1β, IL-6, TNFα and IFN-γ was also repressed. Moreover, 5, 10 and 15 μM curcumin reduced the amount of nitrite and nitrate in LPS-induced Raw264.7 cells maintained total *S*-nitrosylation level on proteins at 12h, the similar results was also observed at 24h that indicating curcumin inhibited NO oxidation. Furthermore, the protection of *S*-nitrosylation on IKKβ in LPS-induced Raw264.7 cells at 12h by curcumin caused the repression of IκB phosphorylation and NF-κB activation. In conclusion, this study verified that curcumin-mediated *S*-nitrosylation may be as an important regulator for anti-inflammation in LPS-induced Raw264.7 cells.

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1. Introduction

Inflammation is a complex immune response that can be induced by cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor (TNF-α) and interferon-γ (IFN-γ). Another crucial mediator of inflammation is inducible nitric oxide synthase (iNOS) that produces nitric oxide (NO) may cause direct cellular injury and pro-inflammatory stimulation [1]. However, NO also participates in the cellular signal transduction, which is considered the second-messenger in the organism by modified proteins via *S*-nitrosylation. For inflammation, *S*-nitrosylation occurs in the most proteins and causes it to lose functions, including inhibitor-κB kinase (IKK), iNOS, NF-κB, matrix metalloproteinases (MMPs) and cysteine-aspartic proteases (caspases) [2,3,4].

The results of numerous studies show that the NO suppresses IKK through *S*-nitrosylation, which prevents the transcription of NF-κB. NF-κB is a protein that has either homodimers or heterodimers, controlling the gene transcription through which changes in the extracellular environment elicit altered genes expression at the cellular level involving inflammatory and apoptotic processes [5]. Inactivated NF-κB exists in the cytoplasm and attaches the inhibitor-κB (I-κB) in the normal status. Once the cell is stimulated by pro-inflammatory factors such as lipopolysaccharides (LPS) and cytokines, I-κB is phosphorylated by IKK and consequently dissociation by E3 ubiquitin ligase. The degradation of I-κB exposes the nuclear-localization signals on NF-κB, which

subsequently translocate into the nucleus and activates the transcription of multiple target genes simultaneously. In the complex regulation of NF-κB, the inhibitory molecule I-κB is a key step [6].

Curcumin, a common food component of Asian that is a natural polyphenolic compound from the *curcuma longa* plant, have anti-inflammatory and anti-oxidative potential. It has three crucial benefits: anti-oxidation, anti-carcinogenesis, and anti-inflammation [7]. Although the ability of curcumin to suppress NF-κB pathways is studied for many years; however, these studies focused on the correlation of the inhibition of the serial upstream proteins phosphorylation which induces NF-κB transcription.

In the previous study, we provided the data that reveals curcumin-mediated *S*-nitrosylation may be as an important regulator for anti-inflammation in dextran sulfate sodium (DSS) induced colitis of mice [8]. However, the gastroenterological microenvironment is very complex, the mechanism by which DSS passes through mucosal epithelial cells remains unclear, but DSS-induced damage in mice form the infiltration of immune cells which can cause the serious inflammation [9]. In order to confirm that curcumin represses the activity of inhibitor-κB kinase protein *S*-nitrosylation more accurately, the LPS-induced macrophage was used to verify curcumin maintains the IKK *S*-nitrosylation here. In this report, we investigated whether curcumin affects *S*-nitrosylation of IKK and subsequent NF-κB pathway activation by using LPS-induced macrophages model.

2. Materials and Methods

2.1. Reagents

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and antibiotics were purchased from Invitrogen (Grand Island, NY, USA). Plastic culture plates were manufactured by Corning Inc. (Corning, NY, USA). The Total Nitric oxide (NO) and Nitrate/Nitrite Assay Kit, antigens, and goat anti-mouse IgM Cy5-conjugated antibody for the cytokine microarray chips were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The microarray chips were obtained from CEL & Associates, Inc. (Los Angeles, CA, USA). The antibodies, including NF-κB, I-κB, IKK, iNOS, and β-Actin, were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). The Bicinchoninic Acid (BCA) Protein Assay Kit, Bradford Protein Assay Kit, and PEO-iodoacetyl-biotin were obtained from Pierce (Rockford, IL, USA). Other chemicals were purchased from Sigma - Aldrich (St. Louis, MO, USA).

2.2. Cell Culture

Macrophage cells (Raw264.7) were obtained from the ATCC (Manassas, VA, USA) and grown in an uncoated plastic cell culture dish (150 × 25 mm) with 20 ml of DMEM containing 10% bovine fatal serum (FBS) and 1% antibiotics (Invitrogen, Grand Island, NY, USA). The medium was changed every 2 d. All cell cultures were conducted at 37 °C in a 5% CO_2 incubator. At confluence, cells were passaged using trypsinization.

2.3. Determination of Cytotoxicity with Curcumin on Cell Viability

To determine the effects of curcumin on Raw264.7 cells growth, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (MTT assay). Cells were seeded in 96-well cell culture plates with approximately 1×10^4 cells per well. After overnight culture, the cells were treated with various concentrations of curcumin that is 0, 1, 2, 4, 8, 15, 30, 60, and 120 μM (containing 0.4% of DMSO to resolve curcumin) for 24 and 48 h. The cells were subsequently incubated with 5 mg/ml of MTT working solution for 4 h at 37 °C, followed by treatment with 20 μl of DMSO to dissolve the crystals [10]. The number of independent experiments (n=3) for cytotoxicity of curcumin on cell viability detection was determined based on statistical power calculation [11].

2.4. Determination of Total *S*-nitrosylated Proteins

To detect the variation of total *S*-nitrosylated protein expression with different concentration of curcumin on the Raw264.7 cells that was treated with LPS and curcumin. The 1×10^6 Raw264.7 cells were seeded overnight in a 150 × 25 mm culture dish with 20 ml of DMEM medium. After the cells were attached to the dish completely, discarded the cultured medium and washed gently with 1X phosphate buffered saline (PBS) for 3 times. The cells were cultured with a fresh medium that contained LPS with 0.1μg/ml. The curcumin (Sigma-Aldrich, St. Louis, MO, USA) that was diluted from the stock was subsequently added and the final concentrations of the

medium were determined as 0, 5, 10 and 15 μM. At the end of 12 and 24h culture periods, the culture medium was collected and centrifuged at 12 000 rpm for 5 min for the preparation of cytokine microarray, nitrite and nitrate production assay. The cells were scraped and collected after washing 3 times with 4 °C 1X PBS. The total lysates from the Raw264.7 cells were prepared as follows: the cells were washed with 4 °C 1X PBS and disrupted in the lysis buffer containing the HEN buffer (250 mM HEPES at pH 7.7, 1 mM EDTA, and 0.1 mM neocuproine), 1% NP-40, 150 mM NaCl, 1 mM PMSF, and 1/100 (v/v) protease inhibitor cocktail set III. Cellular debris was removed by centrifugation at 13 000 rpm at 4 °C for 15 min. The protein concentration of the remaining supernatant, designated as the total cell lysates, was determined using the BCA Protein Assay Kit.

S-nitrosylated proteins were labeled with PEO-iodoacetyl-biotin using the biotin switch method (BSM) with several modifications [12]. First, *S*-alkylation on free cysteine thiols in the total cell lysates at the concentration of 4 mg/ml were obtained by adding 3 volumes of a blocking buffer (270 mM iodoacetamide (IAM) in HEN buffer plus 5% (w/v) SDS) to the protein mixture and incubating at 37°C for 2 h in the dark, and removed excess IAM by acetone precipitation. The mixture was centrifuged at 13 000 rpm for 20 min, and the pellet of proteins was washed with 95% ice-cold ethanol. After the proteins were resuspended in the HEN buffer with 1% SDS, the concentration was adjusted to 2 mg/ml. Subsequently, the *S*-nitrothiols of the protein mixture were labeled with PEO-iodoacetyl-biotin (2 mM) at 37 °C for 2 h in the dark after reduction by sodium ascorbate (5 mM) with vortex 10 sec. Acetone precipitation was conducted for the purified *S*-nitrosylated proteins. After identified the concentration of *S*-nitrosylated proteins, 2 μg of biotinylated protein was separated by 10% SDS PAGE and transferred to a polyvinylidene fluoride membrane. After blocked and treated with the anti-biotin monoclonal antibody (1:5000) for 1h at room temperature. The immunoreactive bands were visualized using an enhanced chemiluminescence detection system. All procedures of the detection on *S*-nitrosylated proteins were performed in accordance with Chen et al [13].

2.5. Analysis of Expression of *S*-nitrosylated IKK Using Immunoprecipitation

To determine the variation of *S*-nitrosylated IKK treated with 0, 5, 10, and 15 μM of curcumin on LPS-indeced macrophages, the cells were treated with or without curcumin for 12 and 24 h. A total of 250 μg of *S*-nitrosylated proteins were obtained from Step 2.4., and resuspended with an avidin loading buffer (2X PBS, 40 mM NaH_2PO_4 at pH 7.2 and 300 mM NaCl). Protein concentration was adjusted to 2 mg/ml. These *S*-nitrosylated proteins were purified with streptavidin agarose (Sigma-Aldrich) for incubation for 1h. After washing with an avidin wash buffer I (1X PBS at pH7.2), wash buffer II (50mM ABC at pH8.3 and 20% methanol) and 1 ml Milli Q water were used. All samples were eluted by an avidin eluting buffer (30% ACN, 0.4% TFA). The purified sample was separated by 10% SDS PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was blocked and incubated with an IKK

primary antibody. The condition was set according to the instructions provided by the manufacturer. The protein bands were visualized using an enhanced chemiluminescence detection system.

2.6. Detection of the Effect of Curcumin on NF-κB Pathway Using Western Blotting.

To profile the relationship of *S*-nitrosylated IKK and curcumin on LPS-induced macrophage cells, the proteins of the NF-κB pathway, which included iNOS, IKK, NF-κB, I-κB, and phosphorylated inhibitor-κB (p-IκB), were detected using western blotting. The total lysates were prepared in Step 2.4.; however, this step was not used for BSM. Equal amounts of protein from each lysate were separated by 10% SDS PAGE and transferred to a nitrocellulose membrane, blocked, and subsequently incubated with the relevant blotting antibodies.

2.7. Determination of Inflammatory Cytokines Expression

The cytokine microarray including interleukin-1β (IL-1), interleukin-6 (IL-6), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were manufactured by the robotic Cartesian/Microsys arrayer (Cartesian technologies, Irvine, CA, USA). The procedure of the microarray chips was performed in accordance with Quintana et al [14]. The chips were stored at 4 °C overnight, and blocked for 1 h at 37 °C with 1% BSA. After blocking, each chip was washed with 1X PBS and 1X PBST (1X PBS contained 0.025% tween-20) for 3 min and incubated for 1 h at room temperature with 10 μl of the test cultured medium. The arrays were subsequently washed and incubated for 1 h with a 1:500 dilution of a goat anti-mouse IgM Cy5-conjugated antibody. After washed and dried, Arrays were

scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). The rules were recorded as TIFF files.

2.8. Nitrite and Nitrate Production Measurement

Nitrite and nitrate production in treated LPS macrophage cells was determined using a commercial nitrite/nitrate colorimetric assay kit (Cayman chemical company, Ann Arbor, MI). All procedures were conducted according to the instructions of the manufacturer. The cell culture medium was mixed with a Griess reagent and incubated for 10 min at 37 °C. The absorbance was measured at 540 nm with a 96-well plate reader. The nitrite and the nitrate concentration were calculated using sodium nitrite/nitrate as a standard.

2.9. Statistical Analysis

The data were reported as mean ± SEM, using one-way analysis of variance and Student's Newman-Keuls test for post hoc comparisons to determine differences between the control and experimental groups. A *p* value of less than 0.05 was considered significant.

3. Results

3.1. Cytotoxicity of Various Levels of Curcumin on Raw264.7 Cells Viability

The death of Raw264.7 cells was induced with curcumin in a dose and time-dependent manner (Figure 1). Concentrations of 5, 10, and 15 μM were selected to present the effects of low, medium, and high levels of curcumin, respectively, on LPS-treated Raw264.7 cells.
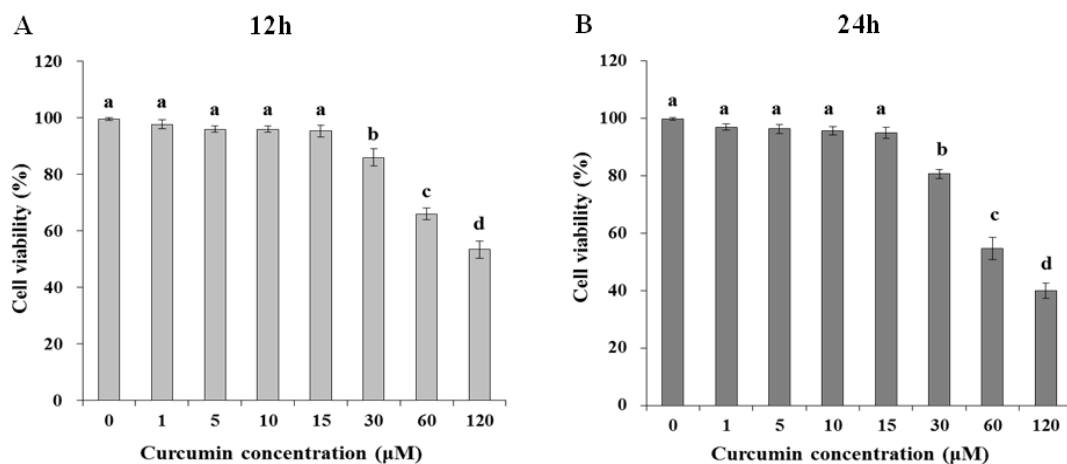

Figure 1. The cytotoxicity of curcumin on Raw264.7 cells. Cells were incubated with samples for (A) 24 h or (B) 48 h and viability was assessed using an MTT assay. Experiments were repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different (*p*< 0.05) at corresponding concentrations between different treatments

3.2 Curcumin Inhibits LPS-induced Inflammatory Factors and NO-derived Nitrite Production in Raw264.7 Cells

To investigate the manner in which curcumin regulated iNOS, we observed the relation of curcumin on LPS-induced iNOS, nitrite and nitrate expression. Raw264.7 cells were treated with LPS (0.1 μg/ml), and 5, 10, and 15 μM of curcumin were subsequently added for 12 and 24 h,

respectively. The cell lysates was subjected to Western blot assay using an iNOS antibody. Results showed curcumin prevents LPS-induced iNOS expression in a dose- and time-dependent manner (Figure 2). To verify these results, nitrite (NO_2) and nitrate (NO_3), the NO oxide production was examined from cell cultured medium. The cultured medium was collected from LPS-induced Raw264.7 cells and incubated with 5, 10, and 15 μM curcumin simultaneously for the same period. NO_2

production was reduced by curcumin not only in a dose-dependent manner, but also in a time-dependent manner (Figure 3A-B). Similar results were obtained for nitrate (NO_3) (Figure 3C-D). The data reveals curcumin significantly diminished NO oxide production.
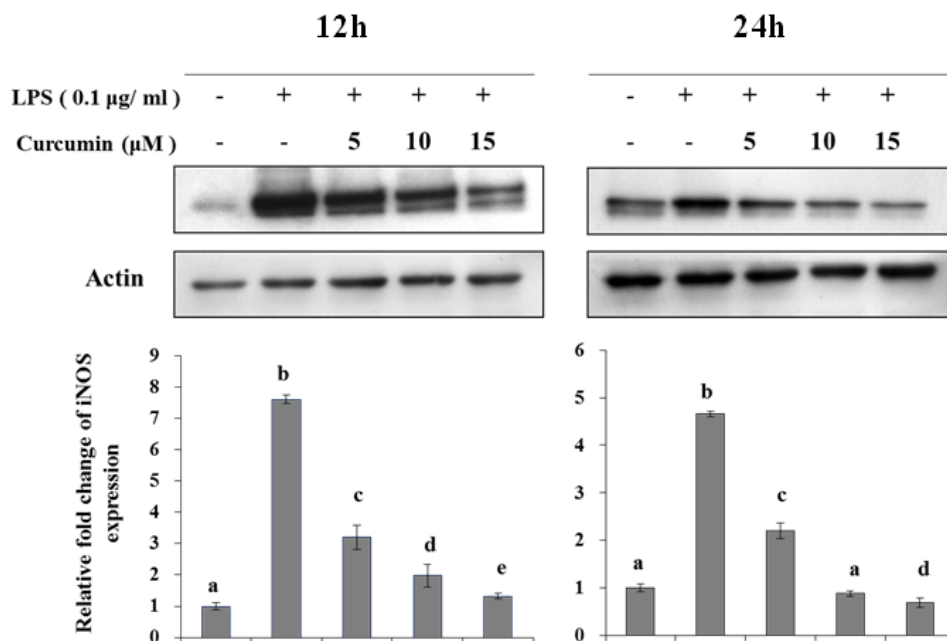

Figure 2. The repression of iNOS in LPS-induced Raw264.7 cells by curcumin. Total lysates was extracted from LPS-induced Raw264.7 macrophage cells of each experimental group at 12 and 24h. The expression of iNOS was detected by Western blot, and β-actin was as the loading control. Experiments were repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different ($p< 0.05$) at corresponding concentrations between different treatments
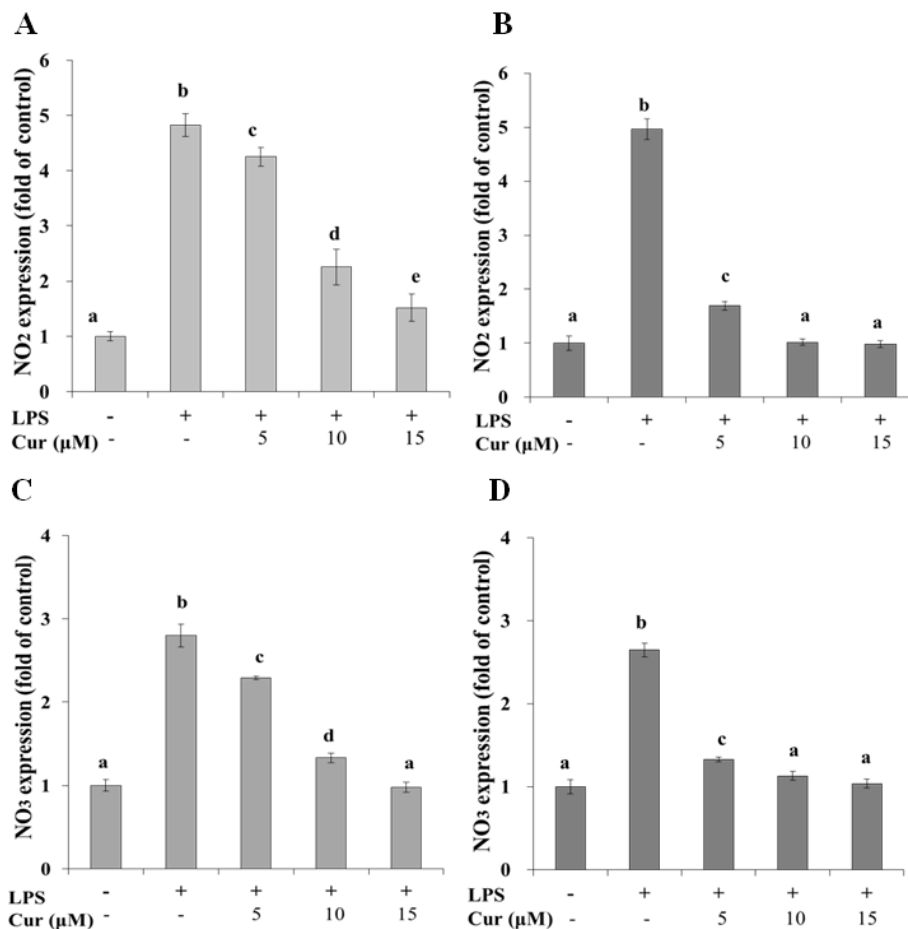

Figure 3. Inhibition of nitrite and nitrate in LPS-induced Raw 264.7 cells by curcumin. Nitrite and nitrate were detected from Raw264.7 cells cultured medium of each experimental group at 12(A, C) and 24h (B, D) by used Griess reagent. The data was repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different ($p< 0.05$) at corresponding concentrations between different treatments

Numerous studies have addressed abnormal cytokine expression, especially the inflammation. In previous decades, curcumin is thought to have multiple benefits to modulate inflammation [15,16]. To confirm the ability of curcumin to inhibit inflammation, we investigated the correlation between curcumin and cytokine expression. The cultured medium was collected from LPS- induced macrophages, and 0, 5, 10 and 15 μM curcumin were added simultaneously at the end of 12 and 24 h culture periods. After the supernatant was centrifuged at 15 000

rpm for 1 min at 4 °C, it was incubated with the microarray chip for 1 h at room temperature. Cytokine expression was detected by the scanner after combining with the IgM Cy5-conjucated antibody. The results showed that curcumin attenuated the inflammation by decreasing pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and IFN-γ at 12 and 24 h (Figure 4A-B). The results as well as previous reports that indicates curcumin can decrease pro-inflammatory cytokine [17,18].
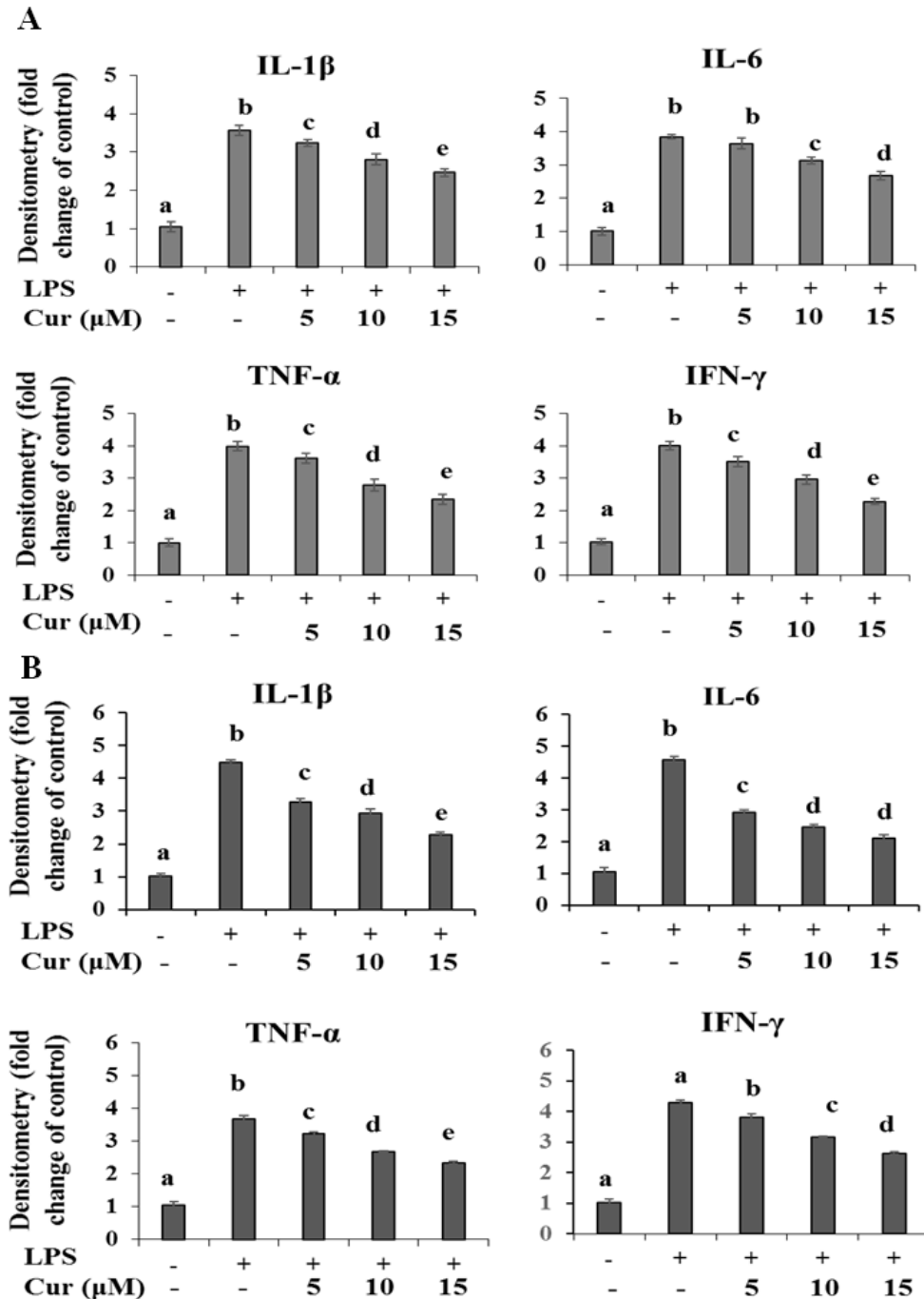

Figure 4. Cytokine expression in LPS-induced Raw 264.7 cells by curcumin. IL-1β, IL-6,TNF-α and IFN-γwere detected from Raw264.7 cells cultured medium of each experimental group at 12(A) and 24h (B) by used microarray.The data was repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different (*p*< 0.05) at corresponding concentrations between different treatments

3.3. Curcumin Involves Protein *S*-Nitrosylation

S-nitrosylation, which is the covalent attachment of a nitrogen monoxide group to the thiol side chain of

cysteine, is a critical mechanism for dynamic post-translational regulation of most or all main classes of protein; moreover, it conveys a large part of the ubiquitous influence of NO on cellular signal transduction, and

provides a mechanism for redox-based physiological regulation [19,20]. In order to elucidate the relationship between curcumin and *S*-nitrosylation and determine the effects of iNOS expression and NO release on LPS- induced Raw264.7 cells by treatment with curcumin. *S*-nitrosylated proteins were sequentially tested. The results showed that curcumin reduced *S*-nitrosylation of

total proteins at 12 and 24h (Figure 5A-B). The decrease of protein *S*-nitrosylation by treatment with curcumin in a dose-dependent manner was substantial at 24 h. However, the decrease tendency of protein *S*-nitrsylation after treated curcumin that was not consistent with the iNOS and NO oxide expression at 12 h.
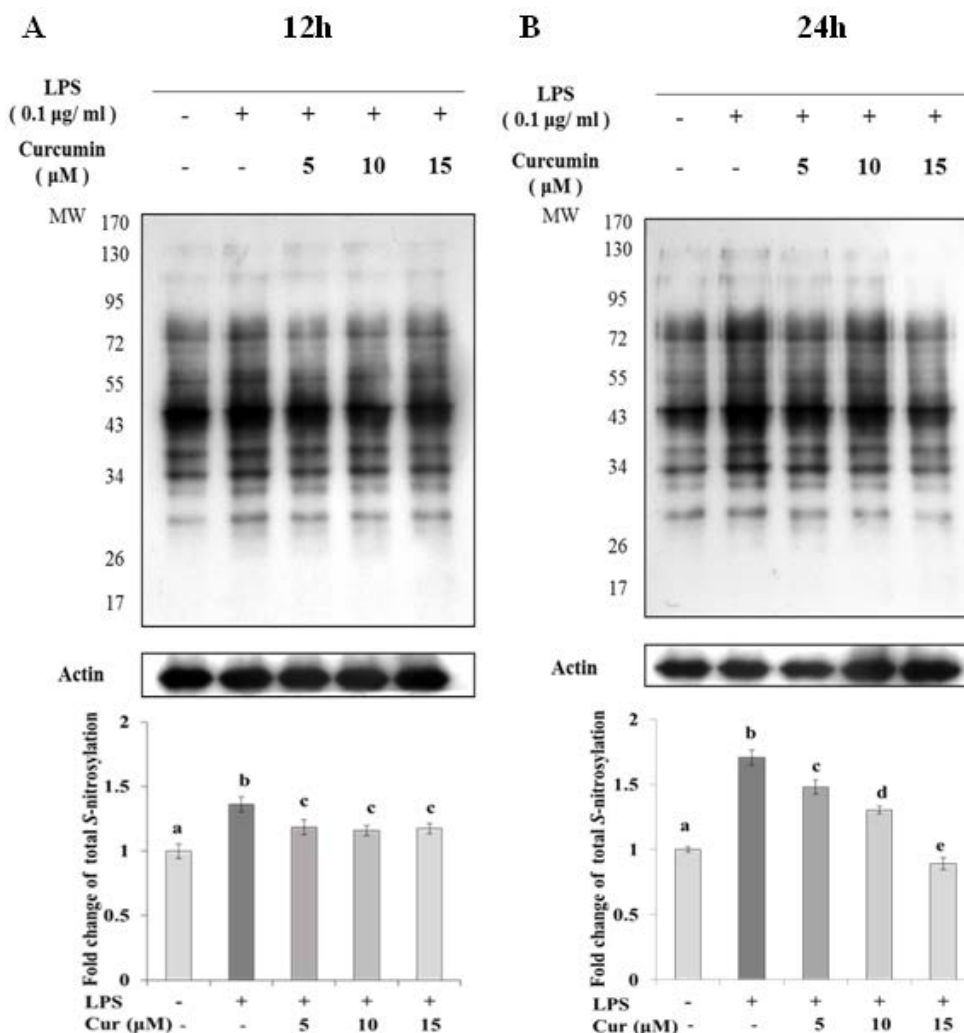

Figure 5. Total *S*-nitrosylated protein expression in LPS-induced Raw264.7 cells by treated with curcumin. The total *S*-nitrosylated protein extracted from cell lysates of each experimental group at12(A) and 24h (B) that was prepared by biotin switch method and detected by Western blot using anti-biotin. The bar-chart presented the relative quantification of total S-nitrosylation from Western blot. The data was repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different ($p < 0.05$) at corresponding concentrations between different treatments

3.4. Curcumin Attenuated NF-κB Translocation by *S*-nitrosylayed IKK

Results of IKK subjected to NO in *S*-nitrosylation has previously been reported [21,22]. In consideration of the connection among curcumin, *S*-nitrosylation, and IKK activation was not studied in former researches. The study made an attempt on resolving the contradictory of curcumin on iNOS, NO oxide and protein *S*-nitrosylation expression by NF-κB pathway further. The key factor among procedures to active NF-κB is IKK, which is the stimulus-triggered phosphorylation of the I-κB-kinase complex. This kinase complex is responsible for the phosphorylation of I-κB, which is committed to proteasomal degradation by this signal [15,21]. Figure 6 indicated *S*-nitrosylated IKK was obtained from an LPS-induced macrophage by treated curcumin at 12 and 24h.

Compared to the data of 24h, *S*-nitrosylated IKK did not has significantly decrease with 5, 10 and 15 μM curcumin at 12 h, although it decreased iNOS and NO oxide expression in a dose-dependent on our previous experiments. Consistent with these findings, phosphorylation of IκB, the downstream substrate of IKKβ, also maintained a similar degree in LPS-induced Raw264.7 cells by treated curcumin with 5, 10 and 15 μM at 12h (Figure 7). The localization of NF-κB was also observed in nucleus by LPS activation and repressed in cytosol by curcumin. Taken together, the result explored that curcumin might repress the IKKβ activity through *S*-nitrosylation and sequentially inhibit the phosphorylation of IκB, as well as attenuate the activation of NF-κB. On the other hand, we also observed that IKK *S*-nitrosylation was decreased with 15 μM curcumin at 24h. Compared the data with iNOS and NO oxide

expression at 24h that might due to high concentration curcumin affected S-nitrosylation by prevented NO oxidation and promoted LPS-induced inflammation tends to the equilibrium [22].
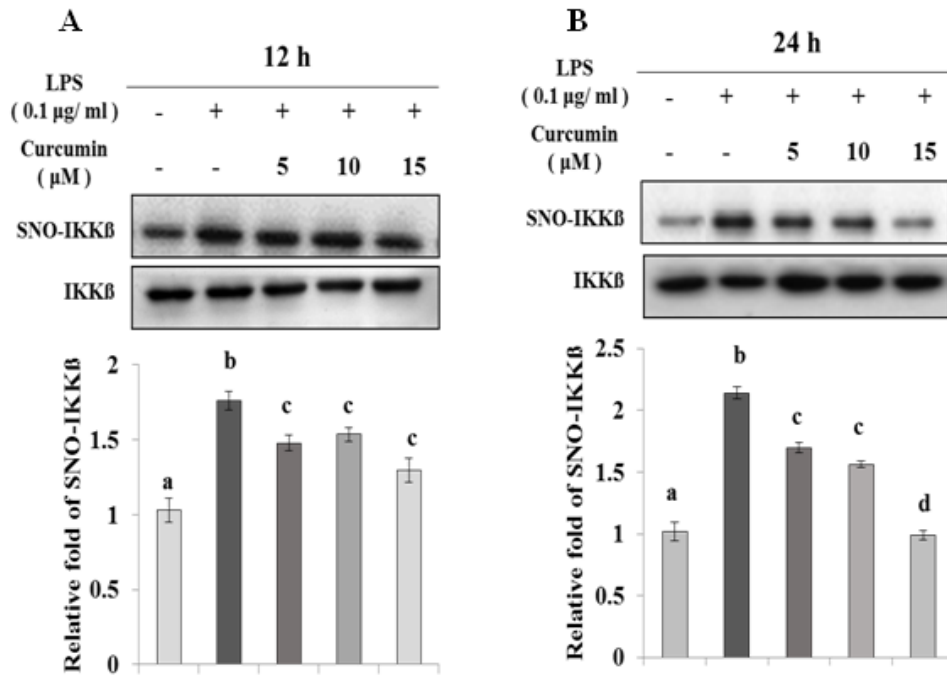

Figure 6. *S*-nitrosylated IKK-β Expression in LPS-induced Raw264.7 cells by treated with curcumin. *S*-nitrosylated IKKβ as obtained from cell lysates of each experimental group at12 (A) and 24h (B) by biotin switch method and detected by Western blot using anti-biotin. The expression level of IKKβ was as the control and relative fold change of *S*-nitrosylated IKKβ was normalized by IKKβ protein expression. The data was repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different (*p*< 0.05) at corresponding concentrations between different treatments
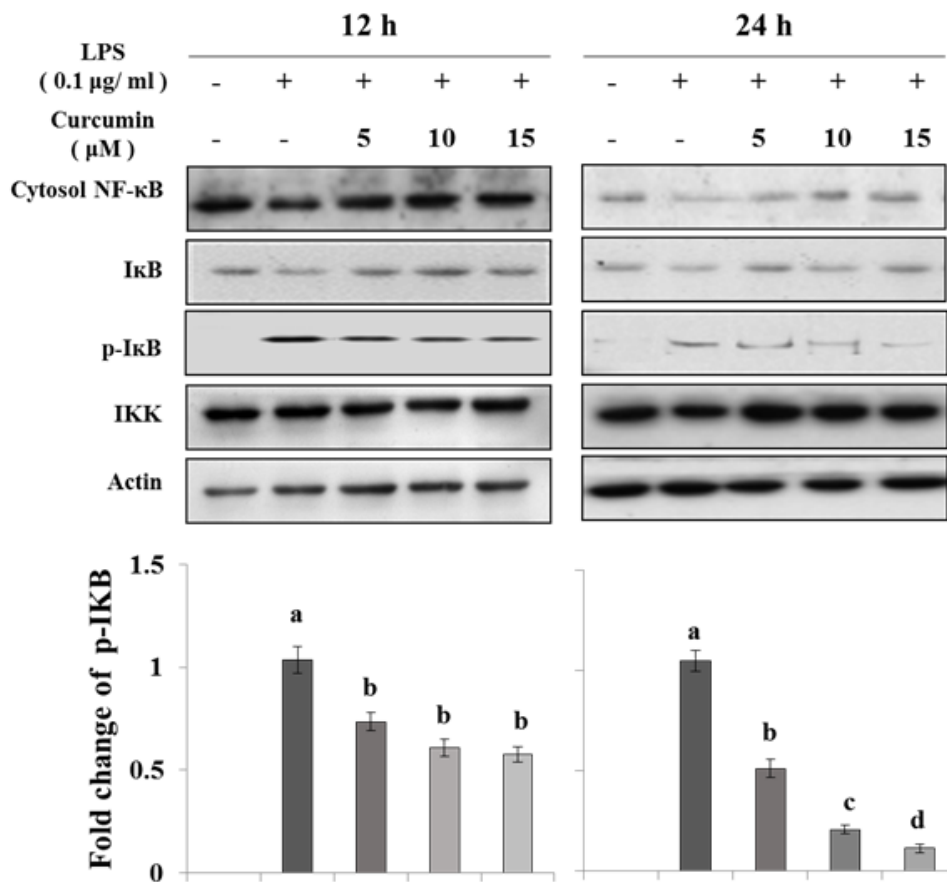

Figure 7. IκBphosphorylation and NF-κB activation in LPS-induced Raw264.7 cells by treated with curcumin. The expression level of phosphorylated IκB (pIκB), IκB, and cytosolic/nuclear NF-κB proteins were detected by Western blot. Act in expression was as the loading control. The bar chart presented the relative quantification of p-IκB expression normalized by IκB protein expression The data was repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different (*p*< 0.05) at corresponding concentrations between different treatments

4. Discussion

Curcumin contributes to the resistance of inflammation, oxidative damage, and carcinogenesis [23]. These studies showed that curcumin intervenes in the inflammation, decreases iNOS expression, and is involved in inflammation effectors, such as cytokines. Under inflammatory conditions, iNOS, IL-1β, IL-6, TNF-α and IFN-γ caused the rapid burst of NO and induced protein *S*-nitrosylation for cellular signal transduction. It is crucial to determine how to prevent excess NO production to process the correct signal transduction. Prior studies focused on the manner in which curcumin decreases inflammation on the activated immune system; however, the effect of curcumin on *S*-nitrosylation under inflammation was rarely addressed. This study presents the relationship between curcumin and post-translational modification in regulating inflammation. The data explored that curcumin might suppress LPS-induced NF-κB activation via protein *S*-nitrosylation. Nitrite and nitrate reduction was attributed to the direct sequestration of NO by curcumin [24,25,26], which may be the reason for curcumin decreased the NO oxide, yet had no effect on NO to *S*-nitrosylation. These results are consistent with the previous study that curcumin represses the activity of inhibitor-κB kinase in dextran sulfate sodium-induced colitis by *S*-nitrosylation [8]. In the normal state,

S-nitrosylation is responsible for most proteins, which mediate the activity of enzyme downregulation. It maintains the equilibrium of physiological variance in the organism for the redox-based regulation [27].

S-nitrosylated IKK lost the ability to induce I-κB from phosphorylation [28]. Curcumin maintained *S*-nitrosylated IKK with 5, 10 and 15 μM at 12h in our study, which showed curcumin might remain *S*-nitrosylation and caused the IKK to lose the ability for NF-κB activation. On the other hand, react to peroxynitrite (ONOO-), which is a more reactive and potential pathogenic molecule [29], curcumin played a predominant role in reducing the nitrite and nitrate from NO oxidation. In this study, the data shows the lowest level of SNO-IKK was from the LPS induced cell with curcumin that might due to curcumin probably has been decreased proinflammatory effector such as iNOS and cytokines. The cellular response is a flow process and the immune response involves multiple effectors, the phosphorylated IκB might be regulated for several signal transductions. Curcumin consists of multiple functional groups, including the β-diketone group, carbon-carbon double bonds, and phenyl rings containing varying amounts of hydroxyl and methoxy substituents. These functional groups enable curcumin to have multiple capabilities, especially for attenuating methylglyoxal-induced ROS formation [30,31,32].
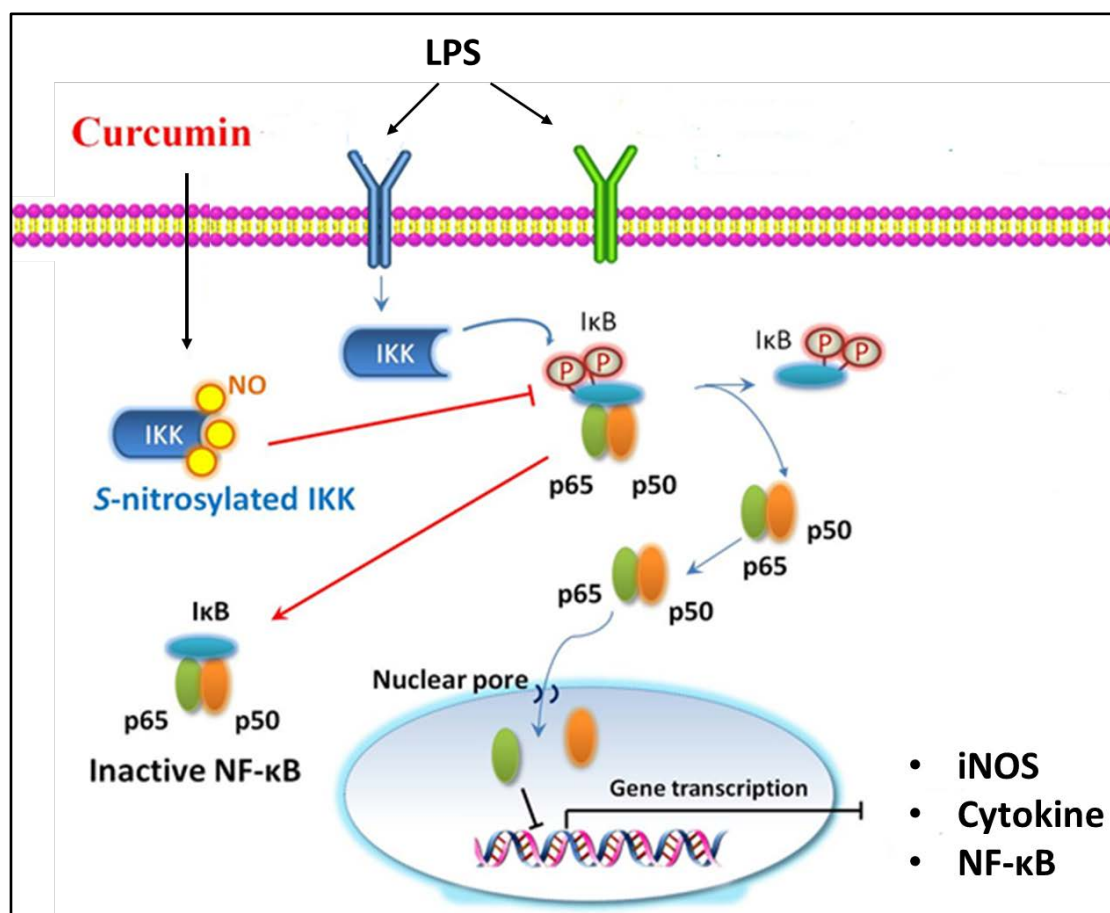

Figure 8. Proposed model of the mechanism responsible for the protective effects of curcumin in LPS-induced Raw264.7 cells

Our data showed that curcumin reduced pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IFN-γ from LPS-induced macrophage cells. Furthermore, the cytokines caused iNOS production, and subsequent NF-κB activation

was reduced. Consistent with previous studies, the pro-inflammatory cytokines were blocked by curcumin [33,34,35]. Therefore, our findings are crucial in the treatment of inflammatory conditions with curcumin,

because curcumin reduced I-κB phosphorylation by maintained *S*-nitrosylated IKK, and also reduced pro-inflammatory cytokines that cause iNOS expression, and subsequently eliminated the peroxide of NO to decrease the inflammation. In summary, curcumin decreases iNOS expression to prevent overproduction of the peroxide of NO, which is caused by the downregulation of pro-inflammatory cytokines. Conversely, curcumin promotes *S*-nitrosylation of IKK in LPS-induced macrophage cells, and *S*-nitrosylated IKK loses the ability to inhibit I-κB phosphorylation and subsequent NF-κB activation. Figure 8 shows the proposed model of the mechanism responsible for the protective effects of curcumin in LPS-induced Raw264.7 cells. The anti-inflammatory effect of curcumin may involve several independent or interrelated mechanisms [36,37,38]. However, the relationship of curcumin and *S*-nitrosylation provides insights into the mechanism of inhibition of NF-κB by phytochemicals, which have anti-inflammatory effects. Our observations may provide a novel aspect: the involvement of curcumin and *S*-nitrosylation in the NF-κB pathway. This study is the first attempt to examine the role of inhibitor-κB kinase and nitric oxide on the anti-inflammation of curcumin via target protein *S*-nitrosylation in LPS-induced macrophage. The results would be helpful to future investigation into the application of anti-inflammatory bioactivities or nutritional ingredients. These basic findings will have much potential in both nutrition and biochemistry research.

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