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Anti-inflammatory Effects of Novel Thiazolidinone Derivatives as Bioactive Heterocycles on RAW264.7 Cells

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ABSTRACT

The inhibition of the inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2) and nuclear factor-ϰB (NF-ϰB) production are research targets of attract in the field of anti-inflammatory drug development.

Therefore, this study was designed to investigate the anti-inflammatory effects of novel thiazolidinone derivatives using a cellular model of lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7. In the present study, five new derivatives (A to E) of thiazolidinone were synthesized and screened for anti-inflammatory activities.

Cell viability of LPS-stimulated RAW 264.7 macrophages clearly decreased in >55 μg/mL of synthesized A-E compounds especially in the presence of C; therefore, up to 50 μg/mL of compounds were selected for the subsequent analysis. A majority of these compounds showed significant inhibition on the production of NO in LPS-stimulated macrophages in a dose-dependent manner. Compounds B and D (10-50 μg/mL) significantly inhibited LPS-induced NF-ϰB (p65) production in a dose-dependent manner. The effects of B and D on iNOS and COX-2 mRNA and protein expression in LPS-stimulated RAW 264.7 cells were detected by real time-PCR and western blot. B derivative significantly suppressed the iNOS and COX-2 mRNA level and as well as protein expression.

Taken together, these results reveal that compound B as new thiazolidinone derivative decreased expression of the inflammatory-related signals (NO, iNOS and COX-2) through regulation of NF-ϰB; hence, this compound could be suggested as a novel therapeutic strategy for inflammation-associated disorders.

Keywords: Anti-inflammatory effects; COX-2; iNOS; NF-ϰB (p65); Thiazolidinone

INTRODUCTION

Experimental, clinical and epidemiological studies have revealed that chronic inflammation is involved in the development of approximately 15–20% of malignancies worldwide.[1-4] During inflammation, macrophages play a central role in managing many different immunopathological phenomena including the overproduction of pro-inflammatory cytokines and

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inflammatory mediators.[5,6] The well-known nuclear factor-κB (NF-κB) is an important transcription factor complex that regulates the expression of many genes involved in immune and inflammatory responses.[4,7,8] In unstimulated cells, NF-κB is constitutively localized in the cytosol as a heterodimer and bound to inhibitor κB (IκB).[8,9] NF-κB would be activated with many stimuli such as lipopolysaccharides (LPS), cytokines, and activators of protein kinase C, oxidants, and viruses via several signal transduction pathways which leads to IκB phosphorylation.[10] Following NF-κB activation, the heterodimer formed is rapidly translocated to the nucleus, where it activates the transcription of target genes, including the genes encoding the pro-inflammatory cytokines, adhesion molecules, chemokines such as interleukin-6, tumor necrosis factor (TNF)-α, and inducible enzymes such as COX-2 and iNOS.[11-14] COX-2 and iNOS expression has been indicated in human ovarian tumors and in tumor associated macrophages; therefore, these pro-inflammatory mediators are considered as essential anti-inflammatory and anti-tumor targets.[15,16] Nitric oxide (NO) is a free radical produced from L-arginine by constitutive nitric oxide synthase (cNOS) and iNOS in numerous mammalian cells and probably generated in excess level during the inflammatory conditions.[17,18] The overproduction of NO is mainly caused by iNOS, which is up-regulated in macrophages during the inflammatory conditions by cytokines and/or bacterial LPS.[17] Several clinical studies have established the role of COX-2 in colon tumorigenesis and have also shown the importance of COX-2 inhibition in the human cancer prevention,[19,20] The usage of COX-2 inhibitors in non-steroidal anti-inflammatory drugs (NSAIDs) led to reduction of diverse tumors risk (colorectal, esophagus, lung, stomach and ovary).[21,22] Despite the fact that there have been many efforts to develop anti-inflammatory agents, there is still a large challenge for developing effective agents.[23] The steroids and NSAIDs are commonly used widely for the treatment of chronic inflammatory diseases. However, long term use of these drugs can induce serious side effects such as gastrointestinal disorders, colorectal cancer, cardiovascular diseases and hypertension.[24,25] It is well-known that a number of heterocyclic compounds containing nitrogen and sulfur exhibit a wide range of pharmacological activities.[26] Thiazolidine and its derivatives as bioactive heterocycles containing nitrogen and sulfur play a key role in medicinal chemistry and they have been widely used as scaffolds for drug discovery. On the other hand, thiazolidine showed a wide range of biological activities.[27-29] Out of these biological activities, the anti-inflammatory potential of thiazolidinone-containing compounds is of particular interest recently.[30] For example, Sharma et al. reported synthesis and evaluation of a series of thiazolidinone derivatives and identification of novel thiazolidinone derivative as a potential anti-inflammatory.[31] Also, Ottana et al. identified a series of thiazolidin-4-one derivatives as novel inhibitors of COX-2.[32] Therefore, we investigated the effects of novel thiazolidinone derivatives on changes in inflammation-related signals (nitric oxide, NF-κB, COX-2, and iNOS) in RAW 264.7 macrophage cells, which can be stimulated by LPS to mimic the condition of infection and inflammation.

MATERIALS AND METHODS

All chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Merck Chemical (Germany) companies and were used without further purification. Antibodies against iNOS, COX-2 and β-actin were obtained from Santa Cruz Biotechnology (CA, USA). Dulbecco's modified essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA).

Synthesis of Thiazolidinone-containing Compounds (A-E)

All the investigated compounds in the present work were synthesized by treating the corresponding aryl diazonium salts with 5-(3-hydroxybenzylidene) thiazolidine-2,4-dione in alkaline media using diazotization-coupling reactions, as previously described.[33,34] The structures of prepared compounds were confirmed by analyzing their spectral characteristics. The chemical structure of synthesized compounds A-E is shown in Figure 1. All melting points were determined on an electrothermal melting point apparatus Infrared spectra were recorded on a Shimadzu 8400 FT-IR spectrophotometer (Japan). The proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a FT-NMR (400 MHz) Brucker apparatus spectrometer (Germany). The purity determination of the substrates and reaction monitoring were accompanied by thin layer chromatography TLC with silica gel SIL G/UV 254 plates.

Cell Culture and Cytotoxic Activity

The RAW264.7 murine macrophages were purchased from the Iranian Biological Resource Center (IBRC; Iran). The cells were maintained in complete Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotics (P/S) (100 U/mL of penicillin and 100 µg/mL of streptomycin) at 37 ◦C in 5% CO_2. In all experiments, RAW264.7 cells were incubated with various concentrations of compounds A–E (0 to 100 µg/mL) for 18 h and cell viability was determined by mitochondrial respiration dependent reduction of 3-(4, 5- dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide (MTT) to formazan method.[35] MTT (0.5% w/v) in phosphate buffer saline was added to each well and was allowed to incubate for 4 h at 37°C. After the incubation medium was removed and the formazan crystals resolved with 150 µL/well of dimethyl sulfoxide (DMSO) and the optical density was measured at 570 nm.

Measurement of Nitrite Concentration

The presence of nitrite was used as NO production indicator in the medium as previously described by Kim.[36]

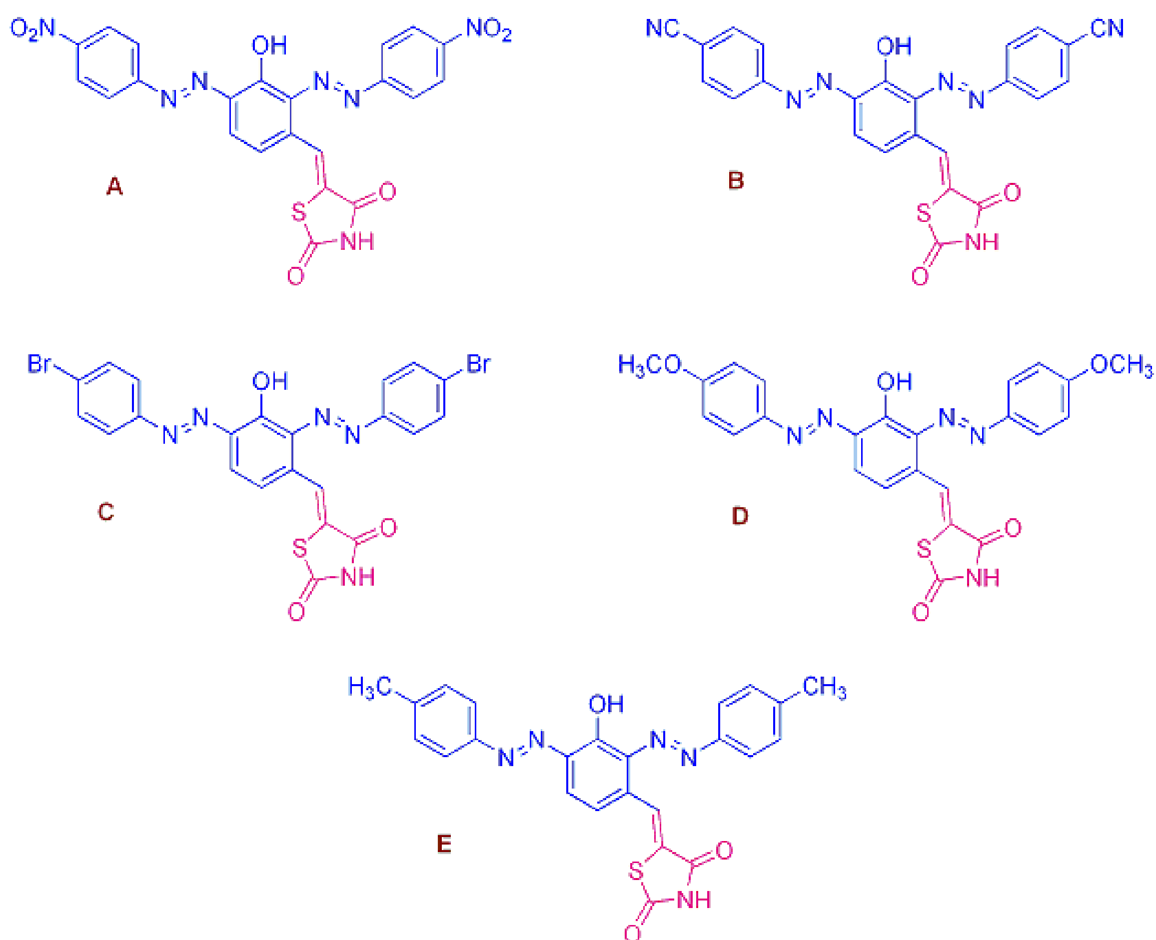

Figure 1. Chemical structure of synthesized compounds A-E. This chart describes the chemical structure of compounds A-E with different functional groups in diazo components, including NO2 (compound A) and CN (compound B) as electron-withdrawing groups, Br (compound C) as neutral functional group, and OCH3 (compound D) and CH3 (compound E) as electron-donating groups.

μg/mL) for 24 h. Then, 100 μL of cell culture supernatant was mixed with 100 μL of Griess reagent Briefly, RAW 264.7 cells were plated at a density of 1×10^5 cells/well on a 24-well plate and were incubated overnight. The cells were then stimulated with LPS (1 μg/mL), in the absence or presence of various concentrations of compounds A–E (10-50 (1% sulfonamide and 0.1% N-(1-naphtyl) ethylenediaminedihydrochloride in 2.5% H_3PO_4) and incubated for 10 min and absorbance at 540 nm was detected. The values were compared with a standard curve of $NaNO_2$ (0–100 μM) to enable calculation of the nitrite concentration produced during the assay.

ELISA Assay on NF-κBp65

The NF-κB transcription factor plays a key role in the induction of iNOS and COX-2 by LPS. The enzyme-linked immunosorbent assay (ELISA) was employed to detect expression of NF-κBp65.

Briefly, to investigate the effects of synthetic compounds A–E on NF-kB level from LPS-treated cells, RAW 264.7 cells (4×105 cells/mL) seeded into 24-well plates were pretreated with 5, 10, 20, 40 μg/mL of compounds for 1 h prior to 24-hourtreatment with 1 mg/L LPS in a 37 °C, 5% CO_2 incubator. In order to quantify the NF-κBp65 under different treatment schedules with synthetic compounds, we used ELISA kit according to the manufacturer's instructions (Invitrogen #KHO0371, CA, USA). The intensity of colored product was directly proportional to the concentration of NF-κBp65. The sensitivity of this assay was <50 pg/mL of NF-κBp65 and linearity of the assay was tested by applying standard NF-κBp65 solution from 0–2500 pg/mL.

Western Immunoblot Analysis

RAW264.7 cells were plated at a density of 1×10^6 cells per well in a 6-well cell culture plate with 2 mL of culture medium and incubated for 24 h. Following treatments with various concentrations of compounds B and D for 2 h and stimulated with LPS (1 μg/mL) for 18 h, cells were washed once with phosphate-buffered saline (PBS) and then whole cell extracts were prepared by lysing with lysis buffer [20 mMTris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, protease and phosphatase inhibitor cocktails] for 30 min on ice. Protein concentration was determined by Bradford assay.[37] A standard curve of absorbance against the amount of bovine serum albumin (BSA) was constructed to calculate equal total cellular protein amounts. Equal amounts of total protein (50 μg) were resolved on 8% SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. The immunoblot was incubated overnight with blocking solution (5% nonfat milk) at 4 °C, followed by incubating with specific antibodies against iNOS, COX-2 and β-actin (Santa Cruz Biotechnology, Santa Cruz, USA). The blots were washed twice with Tween 20/Tris-buffered saline (TTBS) and incubated for 1 h at 37 ºC with horseradish peroxidase-conjugated anti-mouse (1:2000 dilutions, Santa Cruz Biotechnology, Santa Cruz, USA). The intensity of the bands was then quantified by spot densitometry using MACHEREY-NAGEL software (version 1.03, GmbH & Co. KG, Germany

Total RNA Isolation and Real Time PCR

To evaluate COX-2 and iNOS mRNA expression levels, total RNA was extracted from the treated RAW264.7 cells using the Qiagen RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. RAW264.7 cells were pretreated with the indicated concentrations of compounds B and D for 2 h and treated with LPS (1μg/mL) for 18 h. Quantitative real-time reverse-transcription PCR analysis for iNOS, COX-2 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA were performed using Applied Biosystems (Warrington, UK) instrument. The working stock solution of SYBR Green was 1:100. The sequences of the real time PCR primers and their features are listed in Table 1. The copy number of each transcript was calculated as the relative copy number normalized by GAPDH copy number.

Statistical Analysis

All experiments were done in triplicates (n=3) and data were subjected to analysis of variance performed using SPSS 19 (ANOVA) test. Significant differences between means were determined by Duncan's multiple range tests. p values less than 0.05 were considered statistically significant.

Table 1. Primer sequences used in real time PCR for analysis of iNOS, COX-2 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH)

Gene		Primer (5→3)
iNOS	F:	GGAGAGACTATCAAGATAGTGATC
	R:	ATGGTCAGTAGACTTTTACAGCTC′
COX-2	F:	CCCTTCCGAAGTTTCTGGCAGCAGC
	R:	GGCTGTCAGAGAGCCTCGTGGCTTTGG
G3PDH	F:	TGAAGGTCGGTGTGAACGGATTTGGC
	R:	CATGTAGGCCATGAGGTCCACCAC

RESULTS

Effect of Compounds A-E on the Viability of RAW 264.7 Cells

Initially, synthesized compounds A-E were screened for their cytoxicity effects on LPS-stimulated (1 µg/mL) RAW 264.7 cells. RAW 264.7 cells were incubated with compounds A-E in amounts ranging from 0 to 100 µg/mL and cell viability was measured by MTT assay 18 h later. Since, cell viability of LPS-stimulated RAW 264.7 macrophages clearly decreased in >55 µg/mL of synthesized compounds especially in B (Figure 2), therefore up to 50 µg/mL of compounds were selected for subsequent analysis. These results confirmed that the effects of synthesized compounds A-E on RAW 264.7 cells were not due to a reduction in cell viability at the various concentrations used.

Inhibitory Effects of Compounds A-E on Nitrite Production in LPS-induced RAW 264.7 Cells

Increased amount of NO as a signaling molecule is observed in pathophysiological conditions such as inflammation. This analysis indicated that placing non-stimulated RAW 264.7 cells in culture medium for 24 h produced a basal amount of nitrite, but when cells were stimulated with LPS, the NO in the supernatant increased significantly compared to non-stimulated cells. The results showed that all five compounds had inhibitory effects on the production of NO in LPS-induced RAW 264.7 cells, in a concentration-dependent manner. Three compounds (B, D and E) were found to exhibit strong NO inhibitory activity, giving IC_{50} values of 40.33, 28.24 and 41.51 µg/mL, respectively. However, two other compounds (A and C) displayed moderate inhibitory capacity with the IC_{50} values of 47.54 and 51.16 µg/mL (Figure 3). This means that compounds A-E can inhibit NO production but do not have cytotoxicity effect on LPS-stimulated RAW 264.7 cells at the various concentrations used.

Inhibitory Effects of Synthesized Compounds A-E on NF-κB (NF-κBp65) in LPS-induced RAW 264.7 Cells

NF-κB transcription factor has been evidenced to play an important role in LPS-induced expression of pro-inflammatory proteins including iNOS and COX-2. The samples were assayed in triplicate by a NF-κBp65 detection kit and a standard curve using different NF-κBp65 concentrations (0–2500 pg/mL) was generated for quantification in each experiment. The results of ELISA showed NF-κBp65 concentration was significantly decreased by B and D, especially B. The compound B significantly inhibited LPS-induced NF-κB expression in a concentration-dependent manner, corresponding to 7.65% at 10 µM, 21.95% at 20 µM, 32.63% at 30 µM, 41.1% at 40 µM and 48.17% at 50 µM. Also compound D at the same concentrations inhibited NF-κB expression by 9.98%, 15.61%, 23.62%, 26.47% and 36.06% in LPS-stimulated RAW 264.7 cells, respectively. On the other hand the data showed compound E has moderate inhibitory effect, also compounds A and C have slight effect on NF-κBp65 concentration in LPS-stimulated RAW 264.7 cells at the same concentrations (Figure 4). Our findings indicate that among the active compounds above, two compounds, B and D, demonstrated the highest anti-inflammatory activities and low cytotoxicity in LPS-stimulated RAW 264.7 cells. Thus, they were chosen for further investigation of their concentration-dependent inhibitory effects against LPS-induced expression of pro-inflammatory proteins including iNOS and COX-2.

Effects of B and D on Expression Levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 Cells

To evaluate whether the inhibitory effects of B and D on NO production were via inhibition of corresponding gene expression, the protein and mRNA expression levels of iNOS were determined by western blot analysis and real time PCR, respectively. Additionally, the COX-2 protein and mRNA expression levels were also investigated in this study.

In LPS-free RAW 264.7 cells, COX-2 and iNOS mRNA levels were undetectable, but LPS (1µg/mL) stimulation significantly increased iNOS and COX-2 expression. It was also found that treating RAW 264.7 macrophages with compounds B and D (0, 20 and 40 µg/mL) significantly attenuated LPS-induced expression of iNOS and COX-2.

Accordingly, iNOS and COX-2 mRNA levels were significantly inhibited by B compared to LPS (1 µg/mL) alone, corresponding to 32.3% at 20 µM and 51% at 40 µM for iNOS (Figure 5a) and 49.3% at 20 µM and 56.2% at 40 µM for COX-2 (Figure 5b). However unlike B, compound D did not show valuable inhibitory activity in LPS-stimulated RAW 264.7 cells.

On the other hand, the COX-2 and iNOS proteins, when the cells were treated with B in presence of LPS, the intensities of bands for COX-2 and iNOS decreased, corresponding to 45.23% at 20 µM and 51.2% at 40 µM for COX-2 and 34.3 % at 20 µM and 58.2% at 40 µM for iNOS, compared to LPS (1µg/mL) alone. However, when D at the same concentrations was added into the culture medium of LPS-stimulated RAW 264.7 cells, the concentration of iNOS and COX-2 were slightly reduced, compared to the control, but it was not a significant difference (Figure 5c).

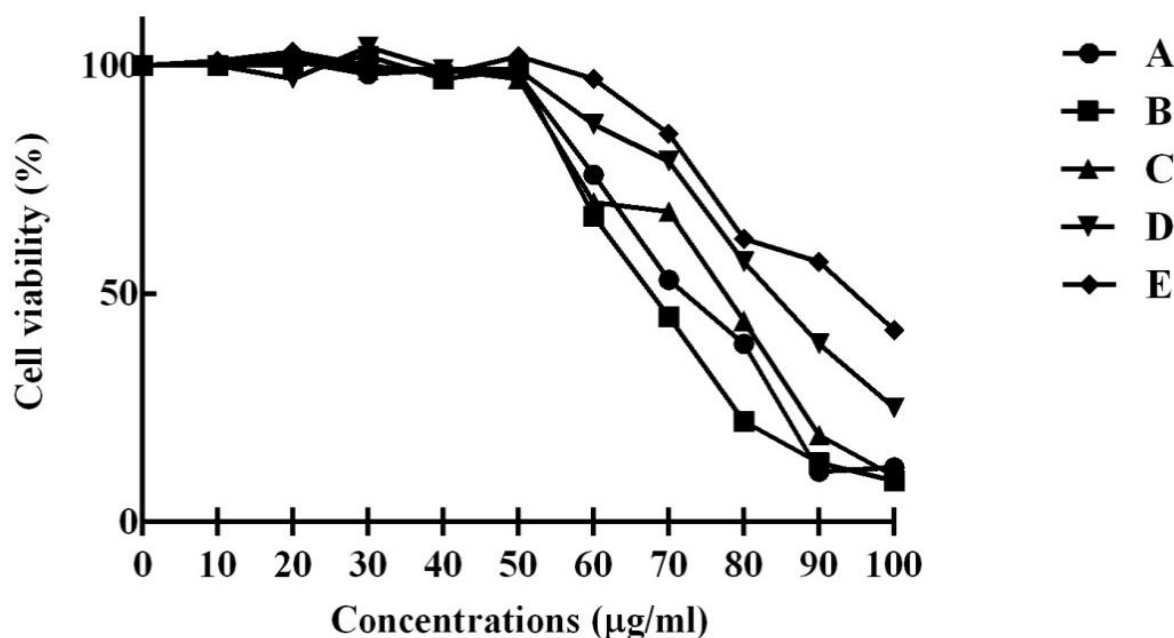

Figure 2. Cytotoxicity of compounds A-E in RAW 264.7 macrophages. RAW 264.7 cells were incubated with compounds A-E in amounts ranging from 0 to 100 µg/mL and cell viability was measured by 3-(4, 5- dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide (MTT) assay 18 h later. Cell viability of RAW 264.7 cells clearly decreased in >55 µg/mL of synthesized compounds. The values are expressed as a relative percentage to the control value. Values represent the means±SDs of three independent experiments.

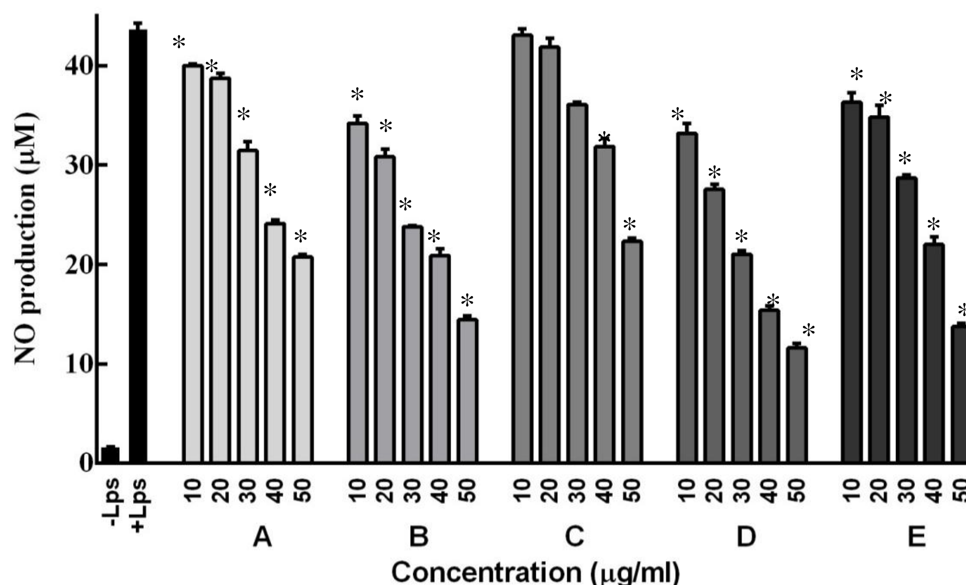

Figure 3. Effects of compounds A-E on LPS-induced nitric oxide production in a dose-dependent manner in RAW 264.7 macrophages. Cells were treated with lipopolysaccharides (1μg/mL), in the absence or presence of various concentrations of compounds A–E (10-50 μg/mL) for 24 h. NO levels in culture media were determined using Griess assays. The results showed that all five compounds had inhibitory effects on the production of nitric oxide in RAW 264.7 cells, in a concentration-dependent manner. Among these compounds, B, D and E showed strong inhibitory abilities in NO production. Values represent the means ± SDs of three independent experiments. **$p<0.05$; vs culture treated with only LPS.**

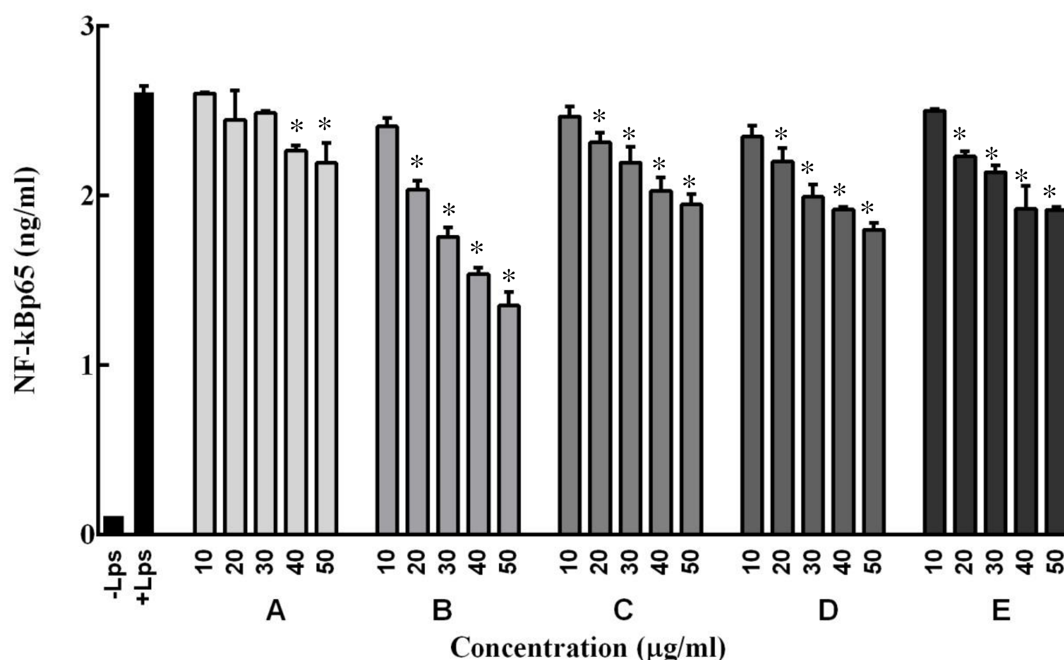

Figure 4. Effects of synthesized compounds A-E on lipopolysaccharide -induced NF-κBp65 in a dose-dependent manner in RAW 264.7 macrophages. The ELISA was employed to detect expression of NF-κBp65. RAW 264.7 Cells were pretreated with 5, 10, 20, 40 μg/mL of compounds for 1 h prior to 24 h treatment with 1 μg/mL LPS. The compound B significantly inhibited LPS-induced NF-κB expression in a concentration-dependent manner, corresponding to 48.17% at 50 μM. The values are expressed as a relative percentage to the control value. Values represent the means±SDs of three independent experiments. The linearity of the assay was tested by applying standard NF-κBp65 solution from 0–2500 pg/mL. **$p<0.05$; vs culture treated with only lipopolysaccharide.**

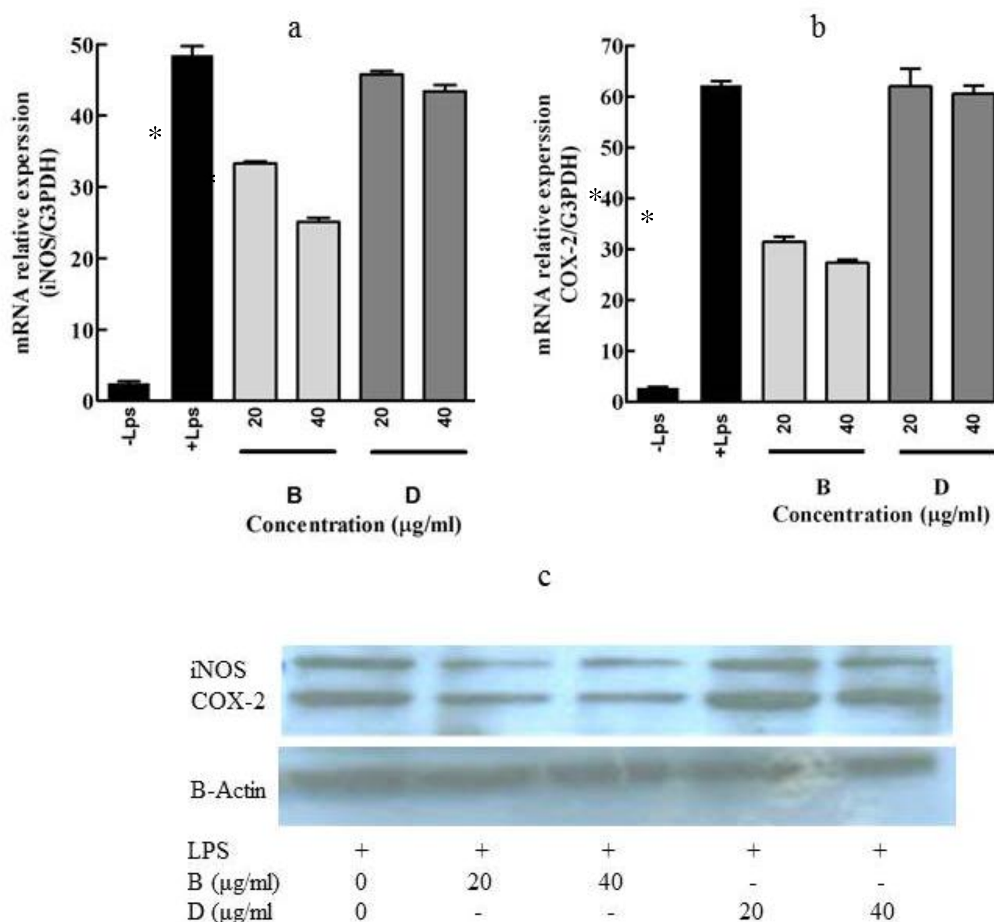

Figure 5. Effects of compounds B and D on inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) mRNA, and protein expressions in RAW264.7 macrophages. RAW264.7 cells were pretreated with the indicated concentrations of compounds B and D for 2 h and treated with LPS (1 μg/mL) for 18 h. Quantitative real-time reverse-transcription PCR analysis for iNOS, COX-2 and Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA were performed using Applied Biosystems (Warrington, UK) instrument. The values are expressed as a relative percentage to the control value, where the iNOS (a) and the COX-2 (b) were normalized to the G3PDH. Equal amounts of total protein were resolved on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Following treatments with various concentrations of compounds B and D for 2 h and stimulated with lipopolysaccharide (1 μg/mL) for 18 h (c). iNOS and COX-2 mRNA levels were significantly inhibited by B compared to control, corresponding to 56.2% at 40 μM for COX-2 and 51% at 40 μM for iNOS. *p<0.05; vs culture treated with only LPS.

DISCUSSION

The pro-inflammatory factors such as NO and prostaglandins produced by stimulated macrophages play key roles in inflammatory diseases.[38] Hence, the inhibition of COX-2, iNOS and NF-κB expressions in inflammatory cells, offers us a novel therapeutic strategy for controlling inflammatory diseases.[39,40].[41] Thiazolidinones are derived from the thiazolidine structure containing sulfur and nitrogen in a five-member ring and the anti-inflammatory activity of thiazolidinone-containing compounds is of particular interest recently.[27,33,34] In recent years, a number of studies have suggested that some novel thiazolidine derivatives, suppress inflammation-related signals in LPS-stimulated RAW 264.7 cells by controlling NF-κB pathway.[30,42] In the present study, it was found that some novel thiazolidine derivatives inhibit

inflammation-related factors expression in LPS-stimulated macrophage cells.. A majority of tested compounds exhibited potent inhibitory effects on NO production in LPS-induced RAW 264.7 cells. Among these compounds, B, D and E showed strong inhibitory abilities in NO production, while pre-treatment of A and C exhibited slight effects with approximate IC_{50} values of 50 µg/mL. All compounds showed no cytotoxicity at their IC_{50} values for NO inhibitory activity. This result confirmed that the effects of synthesized compounds on RAW 264.7 cells were not due to a reduction in cell viability at the various concentrations used. Activation of NF-κB is critical for LPS induction of iNOS gene expression in macrophages.[43,44] Compounds A-E were also evaluated for their inhibitory activities in LPS-induced NF-κB expression in a concentration-dependent manner. We demonstrated that NF-κB expression decreased at treatment with some new thiazolidinone derivatives. Our findings indicate that among the active compounds above, two compounds, Band D, demonstrated the highest anti-inflammatory activities in LPS-stimulated RAW 264.7 cells, as their inhibitory rates reached 60% and 53%, respectively at 50µg/mL, compared to the LPS control. While A and C exhibited slight or no effect in LPS-induced NF-κB expression, respectively. Thus, B and D were chosen for further investigation against LPS-induced expression of pro-inflammatory factors including iNOS and COX-2. Accordingly, our findings suggested that the iNOS and COX-2 mRNA levels were extremely inhibited by B compared to the stimulation by LPS alone, As after treating RAW 264.7 macrophages with B (40 µg/mL) and LPS (1 µg/mL), iNOS and COX-2 mRNA levels were decreased about three and two fold, respectively. Furthermore, B significantly suppressed the gene expressions of iNOS and COX-2. These results indicate that the inhibition of iNOS expression by B occurs in parallel with inhibition of the productions of NO. This data suggests that B can down-regulate LPS-induced iNOS and COX-2 expression at the transcription level. Therefore, the synthesized compound with -CN substituent as bioactive group is effective against LPS-induced inflammatory mediators in RAW 264.7 cells. Expression of iNOS and COX-2 requires the activation of NF-κB, which is a key mechanism for the overproduction of the inflammatory mediators in macrophages in response to LPS. In summary, we found that B suppressed NF-κB signaling, which can also contribute to decreases in COX-2 and iNOS production. Previous studies have demonstrated that some of thiazolidinone derivatives have potent anti-inflammatory activity. For example, Sharma et al. revealed a series of thiazolidinone derivatives and found 2-hydroxyphenyl thiazolidinone as a potential anti-inflammatory agent.[31] In addition, some of 2-imino-4 thiazolidinones derivatives were recently synthesized and evaluated for COX-2 inhibition and anti-inflammatory activity.[32]

Summarizing, we synthesized a series of thiazolidinone derivatives A-E from thiazolidinone to enhance inhibitory effects on LPS-induced NO production, COX-2, iNOS and NF-κB expression in RAW 264.7 cells. Among the synthesized compounds, B having –CN moiety was found to significantly inhibit the productions of NO by LPS. Consistent with these results, B inhibited the LPS-stimulated expressions of iNOS, COX-2, iNOS and NF-κB in LPS-induced RAW 264.7 cells. Furthermore, our molecular data suggest that B inhibits these inflammation-related factors expression by suppressing NF-κB. Accordingly, our findings provide a partial description of the mechanism underlying the anti-inflammatory effects of B.

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