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ANTI-INFLAMMATORY ACTIVITY OF PECTIC ENZYME-TREATED PECTIN ON LIPOPOLYSACCHARIDE-INDUCED RAW 264.7 CELLS

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ABSTRACT

Even inflammation is a body defense response, but excessive inflammation causes chronic inflammatory conditions. The purpose of this study was to investigate the ability and pathway of the pectic enzyme-treated (PET) pectin to inhibit the inflammation of macrophage RAW 264.7 induced by lipopolysaccharide. The PET-pectin produced by commercial pectinase enzyme hydrolysis for 24, 48 and 72 h. Results showed that PET-pectin produced from 48 h reaction time had the highest antioxidative activity, thus these parameters were used to produce PET-pectin used in this study. PET-pectin showed no cell cytotoxicity to normal macrophage RAW 264.7 and reduce the nitrite secretion from LPS-induced RAW 264.7 by 20%. Finally, the expression of cytokines, including NO synthase (iNOS), nitric oxide (NO), cyclooxygenase-2 (COX-2), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and tumor necrosis factor (TNF-α) were analyzed by western blot. In the western blot method, it was found that iNOS, COX-2, NF-κB, TNF-α and other proteins that activated NO production had a downtrend. It was found that PET-pectin possess promising activity to mitigate the inflammatory response. Further study on experimental animals is needed to conclude its activity against inflammation.

Keywords: *Pectic enzyme-treated pectin; inflammation; lipopolysaccharide; RAW 264.7.*

ABSTRAK

Meski inflamasi merupakan respon pertahanan tubuh, inflamasi yang berlebihan dapat menyebabkan kondisi inflamasi kronis. Tujuan dari penelitian ini adalah mengamati kemampuan dan mekanisme dari pektin yang telah direaksikan dengan enzim pektinase dalam mencegah inflamasi yang disebabkan oleh lipopolisakarida pada RAW 264.7. Sebelum digunakan, pektin direaksikan dengan enzim pektinase komersial selama 24, 48, dan 72 jam untuk menghasilkan *PET-pectin*. Hasil menunjukkan bahwa pektin yang direaksikan selama 48 jam menghasilkan aktivitas antioksidan tertinggi, sehingga pektin ini yang digunakan dalam penelitian selanjutnya. *PET-pectin* tidak menyebabkan sitotoksisitas pada sel RAW 264.7 dan dapat mereduksi sekresi nitrit sebesar 20% pada RAW 264.7 yang telah diinduksi lipopolisakarida. Ekspresi dari sitokin, termasuk iNOS, NO, COX-2, NF-κB, TNF-α, dianalisis menggunakan *western blot*. Hasil *western blot* menunjukkan bahwa ekspresi iNOS, COX-2, NF-κB, dan TNF-α menurun dengan adanya *PET-pectin*. Berdasarkan penelitian ini, *PET-pectin*mempunyai kemampuan yang menjanjikan untuk mencegah respon inflamasi. Penelitian menggunakan hewan dibutuhkan untuk menyimpulkan aktivitas *PET-pectin* dalam mencegah inflamasi.

Kata kunci: *Pektin yang direaksikan dengan enzim pectinase; inflamasi; lipopolisakarida; RAW 264.7.*

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INTRODUCTION

Inflammation is a body defense response to destructive stimulus by initiating healing process regulated by immune system; however, excessive inflammation causes chronic inflammatory conditions including arthritis, asthma, multiple sclerosis and atherosclerosis (Wang *et al*., 2016). Lipopolysaccharide (LPS) is commonly used as compound to induce inflammation in RAW 264.7 mouse macrophages cell due to its ability to trigger the secretion of pro-inflammatory cytokines (Agarwal *et al*., 1995).

Pectin is a common gelling agent used for jam and jelly making extracted from citrus peel or apple pomace. Pectin is composed of hetero-polysaccharides rich in galacturonic acid primarily found in the cell walls of terrestrial plants. Beside galacturonic acid, pectin also consists of rhamnose, arabinose, galactose and other 13 different monosaccharides (Naqash *et al*., 2017). Pectin which act as dietary fiber also has been reported to possess anti-diabetic activity (Liu *et al*., 2016).

Several methods used to hydrolyze pectin into pectic-oligosacharides which has lower molecular weight are acid, enzyme and hydrothermal hydrolyses. Pectin can be hydrolyzed by pectic enzymes into pectic enzyme-treated (PET) pectin without high temperature and extreme pH. Basically, pectic enzyme consists of three enzymes, which is deesterifying enzymes (pectinesterases), depolymerizing enzymes (hydrolases and lyases) and protopectinases (Alkorta *et al*., 1997). Previous researches have shown a promising result of PET-pectin as antioxidant and emulsifying agent (Huang *et al.*, 2011), anti-cancer (Huang *et al*., 2012; Huang *et al.*, 2018), anti-bacterial (Wu *et al*., 2014) and prebiotics (Ho *et al*., 2017). Huang *et al*. (2011) reported that hydrolyzed pectin showed higher radical scavenging activity and reducing power than untreated pectin. However, research of PET-pectin on inflammation has not been studied. Therefore, the aims of this study were to investigate the ability and study the pathway of the PET-pectin in inhibiting the inflammation of macrophage RAW 264.7 induced by LPS.

MATERIALS AND METHOD

Materials

Citrus pectin with 99% purity and 60% DE (degree of esterification) was purchased from Nacalai Tesque (Kyoto, Japan). Commercial pectic enzyme, Peclyve CP produced from *Aspergillus niger* was purchase from Lallemand, Australia. RAW 264.7 murine macrophage cell (BCRC 60001) was purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan). Other materials used in this research is analytical grade.

Enzyme-Hydrolyzed Pectin Preparation

Citrus pectin was firstly added into water to reach 1% (w/v) concentration of pectin. Afterwards, 5% (v/v) pectic enzyme was added and reacted at 45°C for three different durations, which were 24, 48, and 72 h. Resulted products were freeze dried and analyzed for ABTS radical scavenging activity at different concentrations based on method described by Re et al. (1999). Chosen parameter to produce PET-pectin was used for further analysis.

RAW 264.7 Cell Culture and Viability Determination

RAW 264.7 murine macrophage cell was cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin, and then incubated at 37°C in a 5% CO_2 incubator (NU-4500, NuAire, MN, USA). The cells were grown as monolayer and subcultures were performed with 1.5 ml of 1× trypsin in phosphate buffered saline (PBS). The morphology of RAW 264.7 cells was observed using an inverted microscope (CK30-F100, Olympus, Tokyo, Japan). Firstly, RAW 264.7 cells were seeded in a 96 well-plate at concentration of 1×10^4 cells/well and incubated at 37°C overnight to let the cell adhere to the well-plate. Afterwards, cells were treated with various concentrations of PET-pectin (0, 400 and 800 µg/ml) and lipopolysaccharide or LPS (1 µg/ml) for 24 h at 37°C. MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to assess the cell viability. Cells were added with 10 µL of MTT solution (5 mg/ml) and 100 µL

medium. The reaction between cell and MTT was conducted at 37°C for 4 h. The crystal formed by viable cell was then dissolved in 100 µL of DMSO for 10 min (Wang et al., 2016). The absorbance were measured at 570 nm by microplate spectrophotometer (Bio-Tek, VT, USA).

Nitrite Determination

Nitrite released was determined by Griess reagent as described by Gunawardena et al. (2013). Firstly, cells were cultured in a 96 well plate with $1x10^4$ cells/wall. After letting it to adhere overnight, LPS (1 µg/ml) together with different concentration (0, 400, and 800 µg/ml) of PET-pectin were added into cells and incubated for 24 h. Supernatant after incubation (80 µl) was taken to new 96 well plate and added with 80 µl of Griess reagent (1% sulfonamide and 0.1% naphthylethylenediamine in 5% HCl) for 10 min. The absorbance determined at 540 nm using microplate spectrophotometer (Bio-Tek, VT, USA).

Western Blot Analysis

Western blot analysis were used to determine cytokines production on LPS induced RAW 264.7 cell according to the method carried out by Kim et al. (2016). Cytokine observed were STAT-3, NF-κB, COX-2, iNOS, and TNF-α, inflammatory related cytokines. After treated with PET-pectin at the concentration (0, 400 and 800 µg/ml) on LPS induced RAW 264.7 cell, control (untreated cells) and different PET-pectin treatment on LPS induced cell were collected and washed with phosphate-buffered saline (PBS). The proteins were then extracted using Halt^{TM} protease inhibitor cocktail (100x): 0.5M EDTA solution (100x): RIPA (radioimmunoprecipitation) lysis and extraction buffer with ratio 1:1:100 at 4°C for 10 min. Equivalent amounts of proteins were separated by 10% SDS-PAGE, and then electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Schwalbach, Germany). After blocking with 5% skim milk, the membranes were incubated with primary antibodies (β-actin and detected cytokines) at 4 °C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for an hour. Finally, signals were detected by an enhanced chemiluminescence system (G:Box, Syngene, MD, USA).

RESULTS AND DISCUSSION

The antioxidant activity observed as ABTS radical scavenging activity of produced pectic-enzyme treated pectin (PET-pectin) was observed in different pectin concentration and reaction time in 1% pectinase enzyme solution. The results of the antioxidant activity were shown in Table 1.

Table 1. Radical scavenging activity of pectic-enzyme treated pectin (PET-pectin) produced from different treatment time and concentrations

Reaction time (hour)	PET-pectin concentration in reaction (mg/ml)		
	25	50	100
24	36.92±0.19^{g}	52.50±0.50^{e}	76.69±0.34^{b}
48	37.25±0.91^{g}	59.30±0.48^{c}	82.89±0.33^{a}
72	38.62±1.08^{f}	58.09±0.25^{d}	77.13±0.33^{b}

$^{a-g}$Data expressed as mean ± standard deviation that do not share a letter are significantly different observed by one-way analysis of variance with Duncan Multiple Range Test post-hoc.

Results showed that there was a higher increase in antioxidant activity found in the PET-pectin made with higher pectin concentration, while lower changes found in PET-pectin made from different reaction time. From the findings, 100 mg/ml pectin concentration and 48-hours-reaction time was chosen as the optimum condition to produce PET-pectin as it showed significantly higher antioxidant activity.

The higher the concentration of pectin represented higher substrate provided in the reaction to be degraded by pectinase enzyme. Pectin is a complex polysaccharide consisting galacturonic acid (sugar

acid) as main component. Degrading pectin into shorter component may increase reducing sugar end, which in turn increased antioxidant activity. The reducing end of sugar derivative can reduce other components by oxidizing its carbonyl end into carboxyl group (Wade, 2013). This finding is supported by the research done by Alrahmany and Tsopmo (2012) showing that carbohydrases (viscozyme, celluclast, and amyloglucocidase) treatment on oat bran increased its radical scavenging activity.

The anti-inflammatory activity of PET-pectin was observed on RAW 264.7 macrophage cells. Figure 1a showed the cell viability of RAW 264.7 cells treated with different concentration of enzyme-hydrolyzed pectin. It showed that enzyme-hydrolyzed pectin caused no inhibition on the RAW 264.7 cell growth, which also refers that there is no toxicity caused by enzyme-hydrolyzed pectin on RAW 264.7 cells. Even at the PET-pectin concentration of 200 µg/ml, there was significant increase of cell viability, which showed that at low concentration, PET-pectin can be used as growth substrate of RAW 264.7 cells.

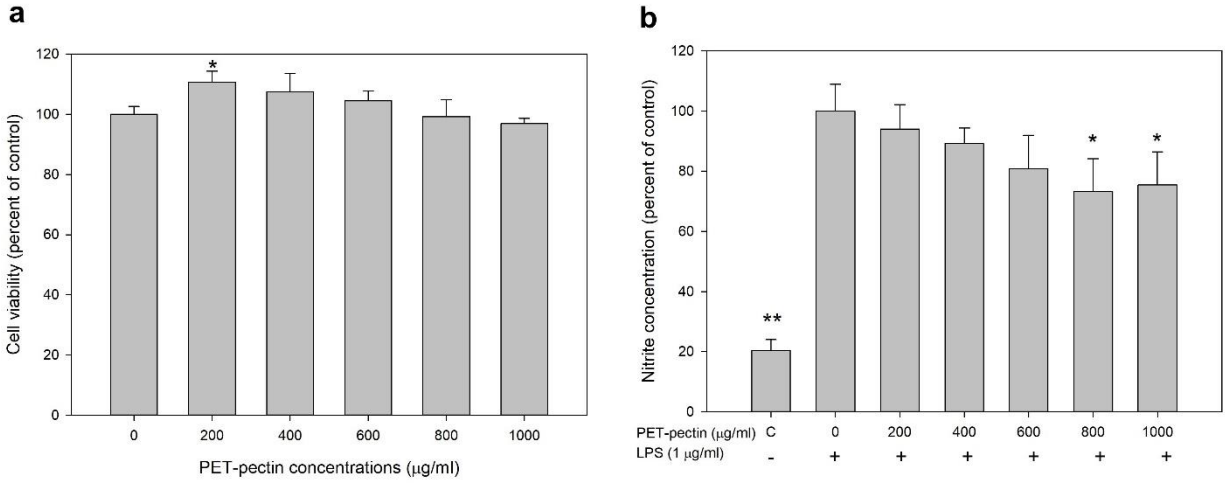

Figure 1. The cell viability (a) and nitric oxide production of lipopolysaccharide (LPS) induced injury (b) on RAW 264.7 cell after treated with different concentrations of pectic-enzyme treated pectin (PET-pectin). *Significantly different at 95% confidence and **significantly different at 99% confidence to 0 µg/ml PET-pectin determined by independent t-test.

Nitrite oxide (NO), a short-lived free radical, is cytokines activated molecule produced by macrophage cells as inflammatory response whose production is associated with iNOS via activated NF-κB signaling pathways (Tripathi et al., 2007). Figure 1b showed the nitric oxide production of lipopolysaccharide (LPS) injured RAW 264.7 cells. Result showed that LPS caused an excessive increase in nitric oxide produced by RAW 264.7 cells. However, PET-pectin added into LPS injured RAW 264.7 cells decreased of the produced nitric oxide.

The morphology of the RAW 264.7 can be seen in Figure 2. It showed the condition of RAW 264.7 before (Figure 2a) and after (Figure 2b) stimulated with LPS without PET-pectin. After stimulated with LPS, the morphology of RAW 264.7 cells changed from round form into irregular form with pseudopodia formation. This irregular form and pseudopodia formation were found lesser on the cell co-treated with PET-pectin (Figure 2c and d), indicating that PET-pectin can prevent the damage cause by LPS on RAW 264.7 cells as observed from the morphology of the cells.

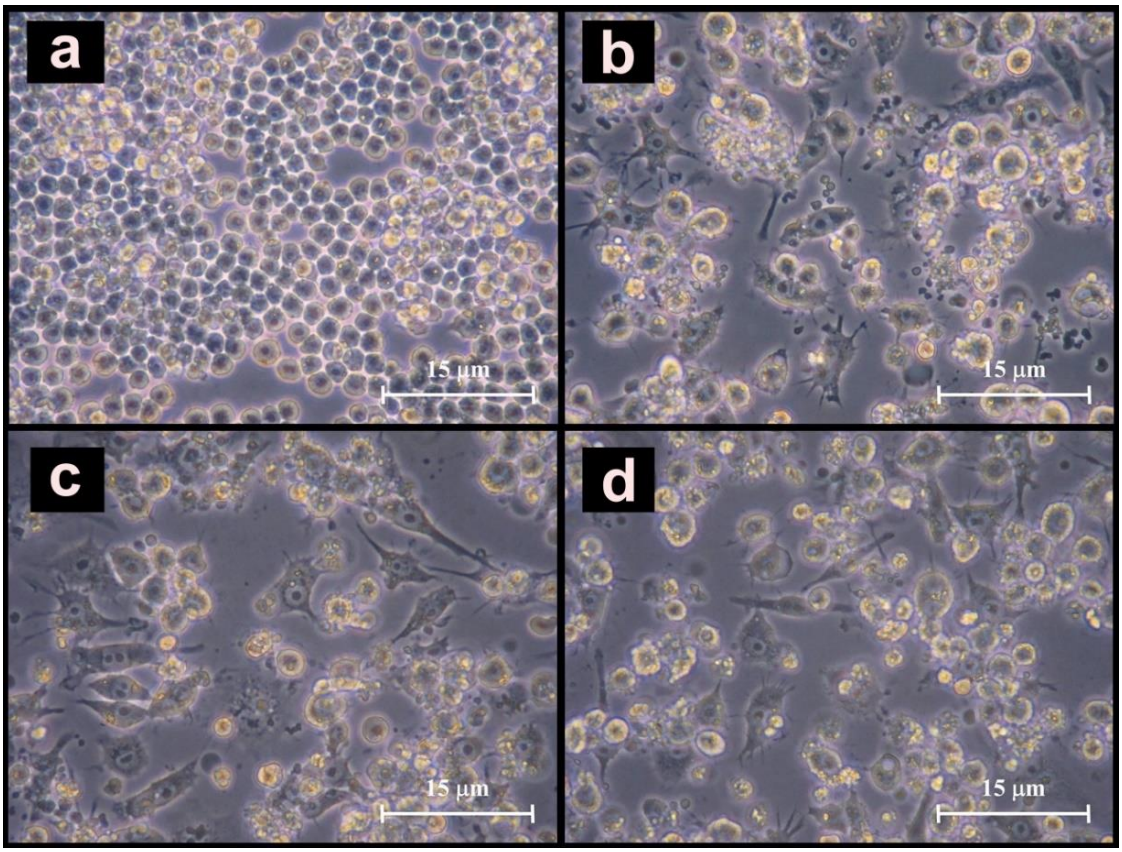

Figure 2. RAW 264.7 Cell morphology at different PET-pectin concentrations. (a) Control (b) 1 µg/ml lipopolysaccharide (LPS) without PET-pectin (c) 1 µg/ml LPS with 400 µg/ml PET-pectin (d) 1 µg/ml LPS with 800 µg/ml PET-pectin.

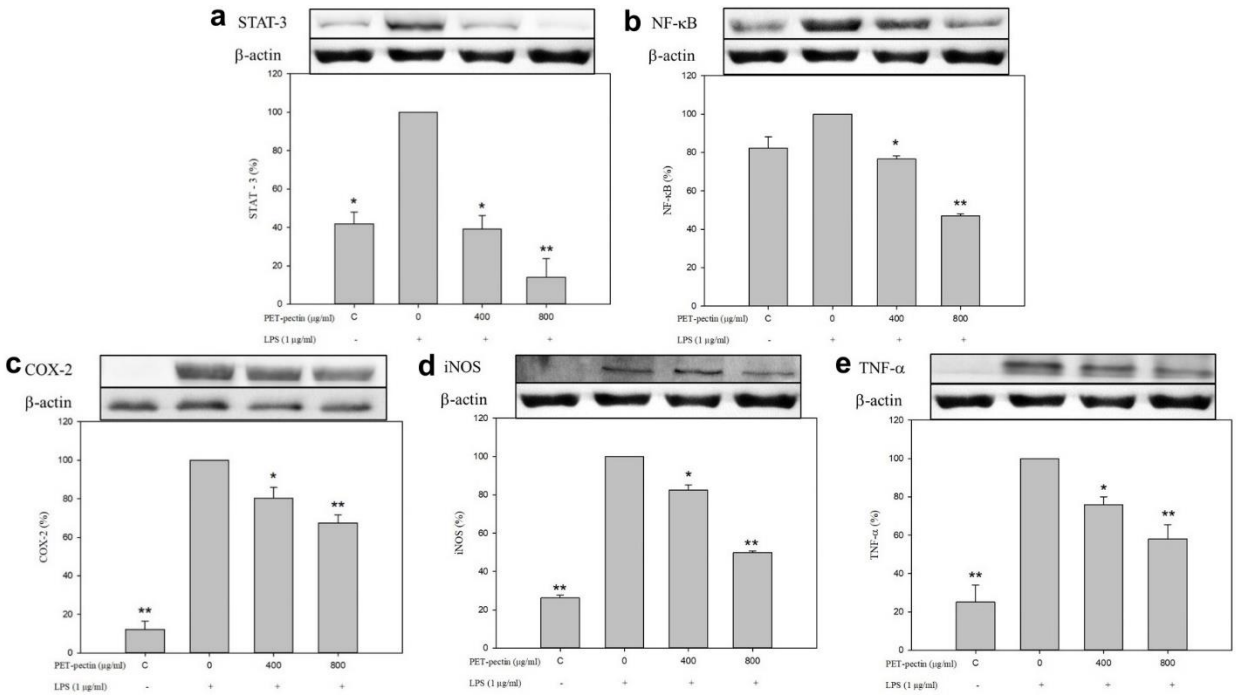

Figure 3. Protein expression of inflammation related response (a) STAT-3, (b) NF-κB, (c) COX-2, (d) iNOS, and (e) TNF-α observed by western blot methods. *Significantly different at 95% confidence and **significantly different at 99% confidence to 1 µg/ml lipopolysaccharide (LPS) stimulated without PET-pectin determined by independent t-test.

To observe the mechanism of PET-pectin on anti-inflammatory activity, western blot was conducted to observe different protein expression involved in inflammation reaction. The protein observed were STAT-3, Nf-κB, COX-2, iNOS and TNF-α as shown in Figure 3a-e.

All the protein expression increased after stimulated with 1 µg/ml LPS. For the cell co-treated with LPS and PET-pectin, the protein expression was significantly lower compared to the cell without PET-pectin treatment in a dose-dependent manner.

The mechanism of action observed in this study was shown in Figure 4. LPS acted as stimuli to trigger inflammation caused the increase in STAT-3 and NF-κB protein expression. STAT-3 and NF-κB, two key proinflammatory pathways control essential tumor-promoting functions in various malignancies, including cell survival, cell proliferation, and suppression of an immune response (Bollrath and Greten, 2009). Afterwards, the expression TNF-α, COX-2, and iNOS increased. Inducible isoform of NOS or iNOS then caused an increase in NO expression. PET-pectin can cause reduction in inflammation as shown in the reduction of STAT-3, NF-κB, iNOS, COX-2, and TNF-α expressions.

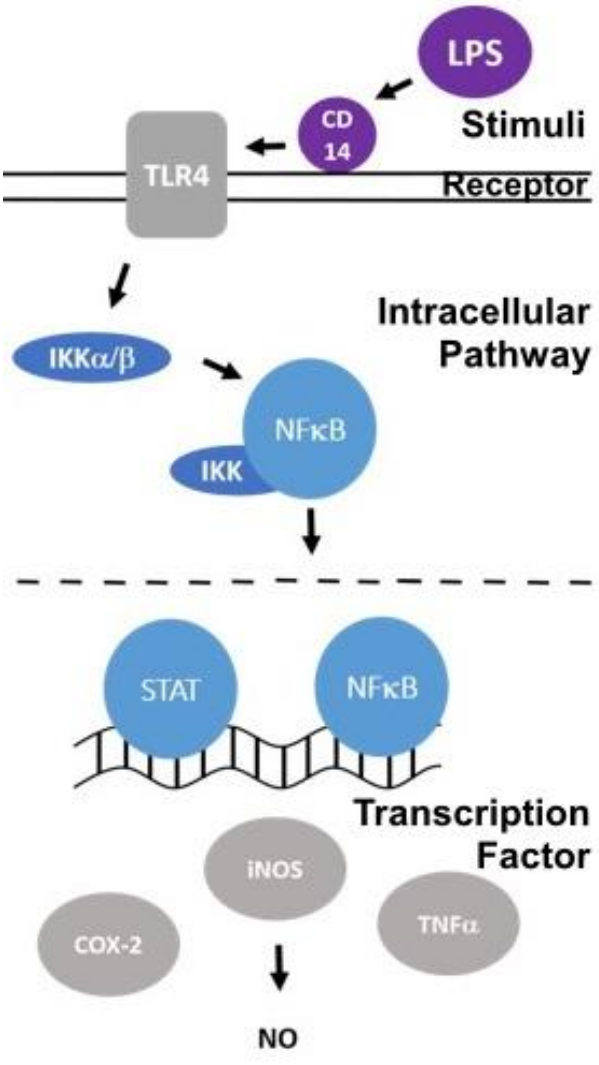

Figure 4. The pathway of mechanism of action

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