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PREDICTION OF SIGNALING PATHWAY INDUCED BY *Solanum nigrum* POLYSACCHARIDE FRACTION, SN-ppF3 IN ACTIVATING RAW 264.7 MACROPHAGE CELLS

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Abstract

Previously, the *Solanum nigrum* polysaccharide fraction, SN-ppF3 was proven to have an immunomodulatory activity by classically activating RAW 264.7 murine macrophage cell line. However, the cellular pathway induced by SN-ppF3 has not to be outlined. In the present study, we predicted the possible cellular pathways induced when macrophage cells were treated with SN-ppF3. The cells were treated with SN-ppF3 for 24 hours and microscopically observed for any morphological due to the treatment. Pinocytosis analysis was carried out to revalidate SN-ppF3 capability as an immunomodulator and also to serve as cytotoxicity evaluation. To outline the signaling pathway induced the cell lysate of 24 hours SN-ppF3-treated macrophage cells were subjected to inflammation analysis through ELISA approach. After the treatment, the morphology of RAW 264.7 cells was obviously altered and pinocytosis activity was significantly increased. In response to the treatment, several phosphorylated proteins such as IκB-α, p38, and NF-κB p65 were significantly up-regulated. Our study suggested that SN-ppF3 treatment could classically activate macrophage through NF-κB pathways which closely similar to the pathway induced by LPS.

INTRODUCTION

Solanum nigrum is an herbal plant which widely grows and distributed throughout temperate climate zones to the tropical region of Asia and Southern hemisphere, from sea level to altitudes over 3500 meters [1]. *S. nigrum* is commonly used as traditional folk medicines and is believed to have various biological activities such as anti-cancer, anti-septic, anti-dysenteric and wound healing properties [2]. In our previous studies, the *S. nigrum* polysaccharide fraction, SN-ppF3 was proven to have immunomodulatory activities where it could classically activate macrophage cells [3] and indirectly suppressed the proliferation of breast cancer cells in tumor-induced Balb/c mice [4].

Macrophages are tissue-based phagocytic cells that are derived from blood monocytes which majorly participate in both innate and adaptive immune responses. Macrophages occupied approximately 20% population of peripheral mononuclear cell fraction, much lesser as compared to the other major phagocytes population such as polymorphonuclear cells or neutrophilic granulocytes [5]. Macrophages can be activated upon interaction with microbial components such as LPS, cytokines such as interleukin-2 [6] and also the other possible immunomodulators [7]. The pattern of macrophages polarization depends on the activators surrounding its microenvironment.

Typically, macrophages are classified into two main groups, which are (1) classically activated and (2) alternatively activated [8]. The classically activated macrophage is an integral cellular component of the organism immune system. This type of macrophages commonly plays important roles in eliminating infection by the initiation of both innate and adaptive immune responses. The activation of macrophages is triggered by antigens or cytokines that bind to the cells' specific receptors, causing the cells to secrete pro-inflammatory cytokines and radical nitrogen

species [9]. There are several mechanisms indicating the activation of cell signaling pathways. Cells initiate signals in response to the surrounding environment. Signaling molecules bind to their specific receptors on the cell surface. Upon interaction, cell signaling pathways are activated and the signal is transmitted through intracellular signaling cascades into the nucleus, resulting in the alteration of gene expression [10]. Protein phosphorylation is one of the common mechanisms in the activation of signaling pathways. The inter-conversion of phosphorylation or de-phosphorylation plays a crucial role, both in metabolic and in signaling pathways. The binding of a highly charged phosphate group to a protein will affect protein conformation and initiates or terminates relevant signal transductions [11].

The identification of particular phosphorylated proteins involved in inflammation signal transduction is necessary to predict the mechanism of cells activation. The aim of the present study is to predict the signaling pathway triggered by SN-ppF3-induced macrophages through the detection of several selected phosphorylated proteins.

MATERIALS AND METHODS

Materials

Diethylaminoethyl (DEAE) anion exchanger cellulose was purchased from Whatman, Maidstone, England. Murine macrophage cell line, RAW 264.7 was purchased from American Type Culture Collection (ATCC), Manassas, VA, USA. The PathScan Inflammation Multi-Target Sandwich ELISA kit was purchased from Cell Signaling Technology, Danvers, MA, USA. All general chemicals were analytical standard grade and were purchased from Sigma-Aldrich Company, unless specifically stated. Information of instruments used for the research was provided in the paragraphs.

Methods

Sample preparation

Fresh plants of *Solanum nigrum* L. *nigrum* (**Figure 1**) were purchased from the local market in Lembah Pantai, Kuala Lumpur, Malaysia. The plants were identified and authenticated by Dr. Sugumaran Manickam from Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia, and a voucher specimen was deposited at the Rimba Ilmu Herbarium (Herbarium number: KLU 47872). *S. nigrum* polysaccharides were extracted according to the previously described method [3]. Briefly, polysaccharide was extracted from dried, ground stems of *S. nigrum* by refluxing the sample with 2 L of petroleum ether (60°C–80°C), followed with 2 L of 80% ethanol. The residue was then boiled in 2 L of 95°C water for 5 hours. The polysaccharide in the filtrate of the boiled mixture was precipitated out with an equal volume of 70% ethanol, overnight at 4°C. After the precipitate was collected and dried, sample was subjected to ion exchange purification through a diethylaminoethyl cellulose column (ø20 mm × 250 mm), where polysaccharides were eluted with a linear gradient of 0–1.5 M sodium chloride in 5 mM of sodium phosphate buffer. Fraction SN-ppF3 was collected, dialyzed and freeze-dried before it was stored at -20°C.

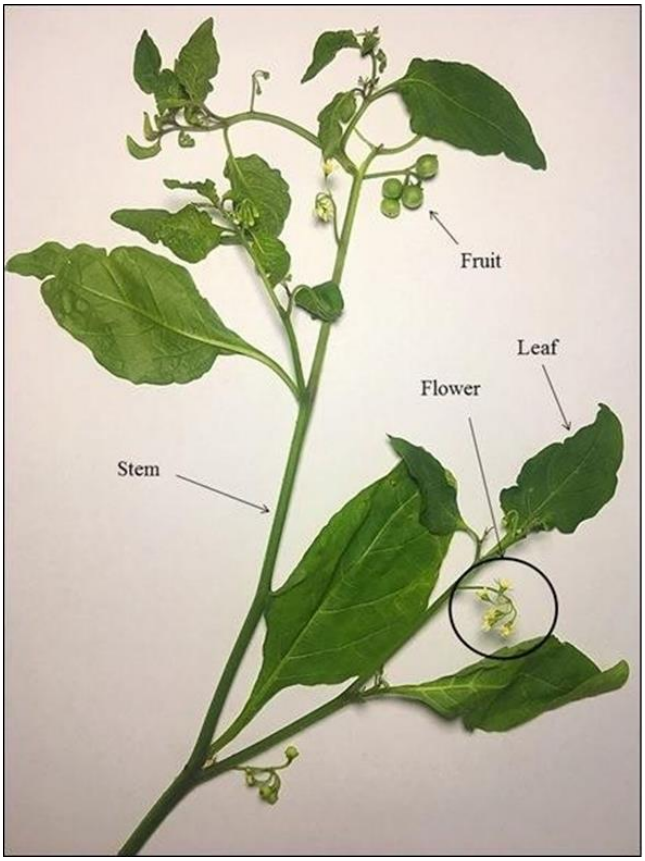

Figure 1. The appearance of *Solanum nigrum* L. *nigrum.*

Cell line

Murine macrophage cell line, RAW 264.7 was purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% fetal bovine serum, 2% of 100× penicillin/streptomycin and 1% of 100× amphotericin B. The cells were cultured in a 5% CO2 and 100% humidified incubator (CelCulture CO2, Esco Technologies, Hatboro, PA, USA) at 37°C, and the culture was passaged every 2 or 3 days.

Morphological observation

To observe the morphological condition of activated RAW 264.7 cell line, cells (5×10^5 cells/mL) were seeded in a six-well culture plate (Orange Scientific, Braine-I'Alleud, Belgium) and incubated for 24 hours. The cells were then treated with polysaccharide samples, SN-ppF3 at a sample concentration of 100 µg/mL for 24 hours. LPS was used as a positive control at the same concentration. Then, cells were observed and photographed using an inverted microscope attached with a camera (Leica). Magnification power used was ×200. The observation focused on changes of cell morphology after treatment, as compared to the non-treated cells.

Pinocytosis analysis

To activate the cells, RAW 264.7 cells (5×10^5 cells/mL) were seeded in a 96-well culture plate (Orange Scientific) and incubated for 24 hours. The cells were then treated with 100 μg/mL of SN-ppF3 for 24 hours. At the end of the incubation period, the medium was replaced with a medium containing 50 μg/mL NR dye and incubated for another 4 hours. After the incubation period, the medium was discarded and the cells were washed with a washing solution. To evaluate the pinocytosis activity, the NR dye was eluted out from the cells by incubating the cells with 200 μL of resorbing solution for 30 minutes at room temperature with rapid agitation on an LT BioMax 500 microplate shaker (Thermo scientific). The absorbance at 540 nm was measured by a Multiskan Go microplate spectrophotometer (Thermo Scientific).

Detection of phosphorylated signaling protein

1. Preparation of cell lysate
RAW 264.7 cells (5×10^5 cells/mL) were seeded in a six-well cell culture plate and treated with polysaccharide fraction SN-ppF3 at 100 μg/mL for 24 hours. LPS was used as a positive control at the same concentration. The cells were harvested using a scraper, washed with 10 mM Tris- sucrose buffer, pH 7.0 and centrifuged at 1,000 ×*g* at room temperature for 5 minutes. The cells were lysed with 2 mL of 2-D protein extraction buffer III (GE Healthcare) for 15 minutes and then pipetted several times on the ice. The cell lysate was collected by centrifuging the lysed cells at 42,000 ×*g* at 4°C for 10 minutes.

2. ELISA analysis
To predict the pathways taken place in activated macrophages, a PathScan Inflammation Multi-Target Sandwich ELISA kit (Cell Signaling) was used. All reagents and solutions required for this assay were provided in the kit. Cell lysate from non-treated and treated RAW 264.7 cells was prepared as described in Section 1. One-hundred-microliter of cell lysate solution was pipetted into a 96-wells microplate coated either with anti-NF-κB p65, anti-phosphorylated-NF-κB p65, anti-phosphorylated-SAPK/JNK, anti-phosphorylated-p38, anti-phosphorylated-IκB-α or anti-phosphorylated-STAT3 antibodies and plate was incubated overnight at 4°C. Then, the medium was discarded, and the wells were washed four times with 200 μL of 1× washing buffer. The washing buffer was then discarded and the plate was blotted onto a fresh paper towel to remove the excess washing buffer. Then, 100 μL of detection antibodies respective to each coated wells was added and the plate was incubated for 1 hour at 37°C. The solution was discarded, and the wells were washed again before 100 μL of horseradish peroxidase-streptavidin-linked secondary antibody solution was added into each well. The plate was incubated for 30 minutes at 37°C. After the final wash, 100 μL TMB One-Step substrate reagents was added to each well, and the plate was incubated at 37°C for another 10 minutes in the dark. Lastly, 50 μL of stop solution was added into each well to stop the color development, and the absorbance at 450nm was immediately measured by a multiskan Go spectrophotometer (Thermo Scientific).

RESULTS AND DISCUSSION

Sample preparation

S. nigrum polysaccharide fraction number 3, SN-ppF3 was previously purified and its physicochemical properties have been characterized. Based on our previous documented data, the fraction contained a polysaccharide with the size of 109.42 kDa. Through the monosaccharide analysis using HPLC-RID, SN-ppF3 was shown to comprise of three neutral monosaccharides namely rhamnose, glucose and galactose, with a molecular ratio of 1.00:0.92:0.86. Thus it was suggested that SN-ppF3 belonged to either rhamnogalacturonan, homogalacturonan or rhamnose hexose type of peptic polysaccharide group. The FT-IR analysis showed that SN-ppF3 displayed a typical spectrum absorption peak of polysaccharide [3-4].

Morphological observation of RAW 264.7 cell line treated with SN-ppF3

Observation of cellular changes is the primary qualitative evaluation of the interaction between cells and treatment samples, in conjunction with the reaction of macrophages biochemical activities. Referring to **Figure 2**, morphological alteration of macrophage cells was clearly observed when cells were treated with SN-ppF3 (**Figure 2, panel B**) and LPS (**Figure 2, panel C**) as compared to non-treated (**Figure 2, panel A**) cells. One of the common morphological changes of classically activated macrophages is the cells shape alteration, where larger and irregular cell shape was observed instead of smaller round or circular shape of resting macrophage cells [12]. The morphological alteration is mediated by the increase in actin filaments, as a response towards pro-inflammatory factor such as LPS or IFN-γ [13]. As a result, an extension formation of filopodia, lamellipodia and membrane ruffles was observed in this study, reflecting the actin cytoskeletal reorganization [14] as preparation for macrophages function enhancement, especially for phagocytosis [15].

Pinocytosis analysis of RAW 264.7 cell line treated with SN-ppF3

One of the parameters commonly used to determine activation of macrophages is the increase in pinocytosis activity. Macrophages are well capable to infuse a massive volume of fluid phase materials by pinocytosis. The pinocytosis activity of activated RAW 264.7 cells was analyzed by evaluating the uptake of NR solution. Commonly, macrophages are able to infuse extracellular fluids with small particles from the surrounding environment into the cytoplasm by a non- specific endocytic process. However, it was reported that the activity of pinocytosis remarkably increases when cells were treated with an inflammatory factor such as LPS [16]. In this analysis, the result suggested that the treatment of RAW 264.7 cells with SN-ppF3 significantly increased the pinocytosis activity as compared to that of non-treated cells, although not as high as LPS-treated cells (**Figure 3**). The constitutive process of pinocytosis could continuously occur and it is enhanced with an activator such as LPS. Previous research stated that pinocytosis activity in

macrophage is significantly enhanced only when the cell was exposed to LPS with a concentration of 100 ng/mL or higher. Since phagocytosis preferably induced by the binding of opsonizing C3b or IgG to their receptors on macrophages, pinocytosis can act as additional support to phagocytosis in eliminating various types of antigens [17]. Aside from that, the uptake of NR solution by SN-ppF3-treated cells suggested that the treatment is non-toxic to the cells, as most cells were viable and capable of absorbing NR solution after 24 h treatment.

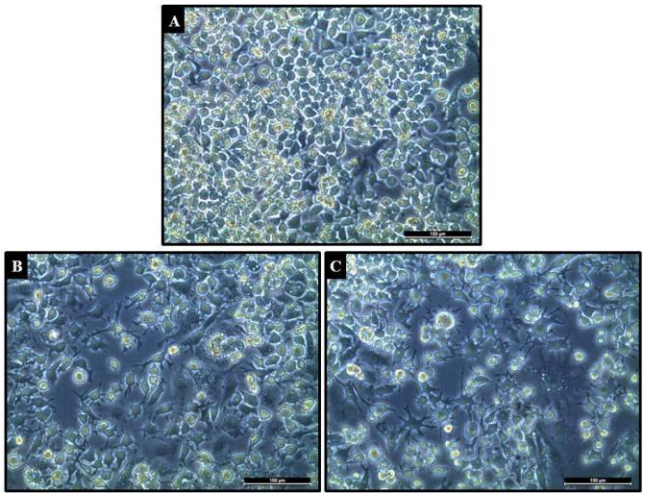

Figure 2. Morphological observation of (A) non-treated, (B) SN-ppF3-treated and (C) lipopolysaccharide-treated RAW 264.7 murine macrophage cell line. Cells were treated with SN-ppF3 or LPS for 24 hours, observed and photographed using a light microscope attached with a camera (Leica). Magnification power used was ×200. The observation focused on changes of cells morphology after treatment, as compared to the non-treated cells. The formation of thread-like extension and cell shape alteration were observed after cells were treated with SN-ppF3 and LPS.

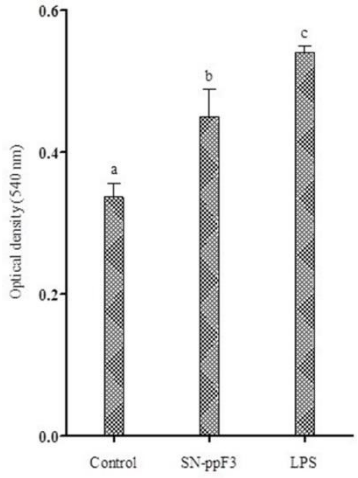

Figure 3. NR uptake of control, SN-ppF3, and LPS-treated RAW 264.7 murine macrophage cell line for pinocytosis evaluation. Macrophages cells were treated with SN-ppF3 and LPS at 100 µg/mL for 24 hours and incubated with the neutral red solution for at least 4 hours. The pinocytosed NR by the cells was measured at absorbance of 540 nm. LPS-treated cells showed the highest OD followed by SN-ppF3-treated cells, indicating the activation of the cells as compared to control. Data presented were mean ± standard deviation (n=3). Different letters (a–c) indicate the significant difference at p<0.05.

Prediction of the signaling pathway induced by SN-ppF3 in RAW 264.7 cells

The determination of specific phosphorylated protein associated with inflammation is one of the mechanisms to predict the possible signaling pathways triggered by SN-ppF3 to induce classically activated macrophages. Based on our previously reported data on NO and cytokines production [3], it is a hint that the treatment of macrophage cells with SN-ppF3 triggered NF-κB signaling pathway, as closely similar to the pathway triggered by LPS. The present study showed that the phosphorylations of selected signaling proteins such as p38 MAP kinase, IκB-α, STAT3, and NF-κB p65 were significantly higher in SN-ppF3-treated cells as compared to that of non- treated cells (**Table 1**).

Table 1. Phosphorylation of selected signalling proteins in the inflammation pathway

	Absorbance value at 450 nm		
Protein	**Non-treated**	**SN-ppF3**	**LPS**
NF-κB p65	3.744 ± 0.030	3.793 ± 0.020	3.745 ± 0.040
Phosphorylated NF-κB p65	0.455 ± 0.001[a]	0.514 ± 0.010[b]	0.621 ± 0.040[c]
Phosphorylated SAPK/JNK	0.089 ± 0.003	0.095 ± 0.023	0.098 ± 0.013
Phosphorylated p38	2.044 ± 0.050[a]	2.213 ± 0.090[b]	2.458 ± 0.030[c]
Phosphorylated IκB-α	0.119 ± 0.006[a]	0.144 ± 0.020[b]	0.175 ± 0.002[b]
Phosphorylated STAT3	0.453 ± 0.002[b]	0.487 ± 0.014[c]	0.415 ± 0.001[a]

LPS: Lipopolysaccharide. Data expressed were mean ± standard deviation (n=3). Different superscripted letters (a–c) in each row indicate the significant difference at p<0.05.

In a well-outlined inflammation signaling pathway, LPS was able to be recognized by most mammalian macrophage cells membrane molecules through CD14 and TLRs, especially TLR4 [18]. Signal transduction by TLR4 initiates the activation of IRAK through an adaptor protein of MyD88. This subsequently activates TRAF6 and IKK, leading to phosphorylation of IκB-α. Phosphorylated IκB-α detaches from NF-κB p65/p50 subunit and is degraded. The degradation of IκB-α allows the phosphorylated-p65/p50 dimeric NF-κB transcription factor to translocate into the nucleus and induces the expression of several pro-inflammatory mediators such as cytokines, iNOS and COX-2 [12,19]. Higher expression of phosphorylated NF-κB p65 in SN-ppF3 and LPS-treated cells suggested the induction of this pathway. The treatment of macrophage cells with SN-ppF3 also triggered significant phosphorylation of p38 MAPK, as compared to non-treated cells. Phosphorylated p38 MAPK is commonly detected in LPS stimulated macrophages [20] and responsible signal for IL-1 and TNF-α production in most mammalian monocytes, and actively participates in a signalling cascade; which controls the cellular responses to pro-inflammatory cytokines and other cellular stresses [21]. Thus, the expression of phosphorylated p38 MAPK reflects the result of TNF-α production in the treated macrophage cells. The expression of

phosphorylated STAT3 protein in SN-ppF3 treated cells was significantly up-regulated, which was in contrast to that of LPS treated cells. The STAT3 pathway is commonly mediated by IL-6, which leads to the phosphorylation of STAT3 and subsequently induces diverse biological responses [22]. The low expression of phosphorylated STAT3 in LPS treated cells observed was expected. The previous study by Chen et al., (2012) demonstrated that the phosphorylated STAT3 peaked after 8 hours of treatment with LPS and the concentration decreased in the subsequent incubation period [23]. Since signaling of STAT3 involves the translocation of phosphorylated STAT3 into the nucleus, different time frame analysis would give a better picture to determine the difference between these two treatments. Overall, it could be suggested that the pathway triggered by SN-ppF3 was similar to that of LPS. However, more detail studies are required in order to deduce a better understanding of the signaling pathway.

CONCLUSION

In this present study, it was suggested that macrophages cells were classically activated when the cells were treated with SN-ppF3. After 24 hours of treatment, cells morphology was obviously altered and the pinocytosis activity was significantly increased, as compared to the non-treated cells. As for the analysis of the signaling proteins, the increase in NF-κB p65, p38, IκB-α, and STAT3 phosphorylation were observed. It was suggested that the treatment caused the activation of NF-κB and STAT3 pathways which leads to the pro-inflammatory activities of the cells. Through the data obtained, it was clearly shown that the polysaccharide fraction SN-ppF3 was able to activate macrophage cells, which can possibly act as an important proxy against immune-associate diseases.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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