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The Attenuating Effects of 1,2,3,4,6 Penta-O-Galloyl- β -D-Glucose on Pro-Inflammatory Responses of LPS/IFN γ -activated BV-2 Microglial Cells through NF κ B and MAPK Signaling Pathways

Patricia Mendonca, Equar Taka, David Bauer, Renee R. Reams, and Karam F. A. Soliman*

College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, FL 32307.

Abstract

Background—Overactivated microglial cells exhibit chronic inflammatory response and can lead to the continuous production of pro-inflammatory cytokines, perpetuating inflammation, and ultimately resulting in neuronal injury. 1,2,3,4,6-Penta-O-Galloyl- β -D-Glucose (PGG), which is a naturally occurring polyphenolic compound, has exhibited anti-inflammatory effect through the inhibition of many cytokines in different experimental models, but its effect on activated microglia cells was never described. In the present study, we investigated PGG effect in proteins involved in the NF κ B and MAPK signaling pathways, which play a central role in inflammation through their ability to induce transcription of pro-inflammatory genes.

Methods—PCR arrays and RT-PCR with individual primers were used to determine the effect of PGG on mRNA expression of genes involved in NF κ B and MAPK signaling pathways. Western blots were performed to confirm PCR results.

Results—The data obtained showed that PGG modulated the expression of 5 genes from the NF κ B (*BIRC3*, *CHUK*, *IRAK1*, *NF κ B1*, *NOD1*) and 2 genes from MAPK signaling pathway (*CDK2* and *MYC*) when tested in RT-PCR assays. Western blots confirmed the PCR results at the protein level, showing that PGG attenuated the expression of total and phosphorylated proteins (*CDK2*, *CHUK*, *IRAK1*, and *NF κ B1*) involved in NF κ B and MAPK signaling.

Conclusion—These findings show that PGG could modulate the expression of genes and proteins involved in the production of pro-inflammatory cytokines in microglia cells.

Keywords

1,2,3,4,6-Penta-O-Galloyl- β -D-Glucose; Microglia cells; NF κ B and MAP kinase signaling pathways

*Corresponding Author: Karam F. A. Soliman, PhD, Distinguished Professor, College of Pharmacy & Pharmaceutical Sciences, Florida A&M University, Room 104 Dyson Pharmacy Building, 1520 ML King Blvd, Tallahassee, FL 32307, Phone 850 599 3306, Fax 850 599 3667, karam.soliman@fam.u.edu.

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Conflict of interest
None declared.

1. INTRODUCTION

Chronic microglial activation induces the overproduction of pro-inflammatory mediators, such as pro-inflammatory cytokines (Streit *et al.*, 2004), and might override the beneficial effect of these cells in the CNS (Schwartz & Baruch, 2014). In the state of prolonged inflammation, continual activation and recruitment of effector cells can establish a feedback loop that perpetuates inflammation and ultimately results in neuronal injury (Gendelman, 2002; Oh *et al.*, 2004). The activation of microglial cells is observed in brain injuries and is induced after the exposure to the Gram-negative bacterial coat component, lipopolysaccharide (LPS) (Hanisch, 2002). Microglial activation is a highly potent trigger for cytokine synthesis through Toll-like receptor 4 (TLR4) (Hanisch, 2002), interferon- γ (IFN γ), or amyloid β (A β) (Lacy & Stow, 2011; Giulian *et al.*, 1994). The released pro-inflammatory cytokines play a key role in the induction and maintenance of neuroinflammation. Numerous studies confirmed that the levels of classical pro-inflammatory cytokines are elevated in chronic neurodegenerative diseases, especially in Alzheimer's disease (AD) and significantly contribute to this disease progression (Smith *et al.*, 2012; Zheng *et al.*, 2016; Rocha *et al.*, 2012). This inflammation process is mediated by pro-inflammatory cytokines and would create a chronic and self-sustaining inflammatory interaction between activated microglia and astrocytes, stressed neurons, and amyloid β (A β) plaques (Rubio-Perez & Morillas-Ruiz, 2012). Also, A β can stimulate a nuclear factor-kappa B (NF κ B) dependent pathway which is required for cytokine production (Combs *et al.*, 2001). The subsequent activation of the extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) pathways by A β binding to the microglial cell surface induces pro-inflammatory gene expression and leads to the production of cytokines and chemokines (Ho *et al.*, 2005).

The expression of NF κ B transcription factors is abundant in the brain. The basal levels of NF κ B expression have been identified in the brain where their amounts are higher than those of peripheral tissues (Shih *et al.*, 2015). Studies of postmortem brain tissue from patients with Alzheimer's disease (AD) have revealed increased NF κ B activity in cells involved in the neurodegenerative process. p65 immunoreactivity increased in neurons and astrocytes in the immediate vicinity of amyloid plaques in brain sections from AD patients, consistent with NF κ B activation in those cells (O'Neill & Kaltschmidt, 1997). Immunohistochemical analyses of brain sections from Parkinson's patients revealed a 70-fold increase, relative to age-matched controls, in the proportion of dopaminergic neurons in the substantia nigra exhibiting nuclear p65 immunoreactivity (Hunot *et al.*, 1997). Spinal cords of patients with ALS show increased NF κ B activation in astrocytes associated with degenerating motor neurons (Migheli *et al.*, 1997). Another evidence supporting a role for NF κ B in chronic neurological disorders comes from studies of multiple sclerosis. Analyses of neural tissue from patients with multiple sclerosis indicated that NF κ B is activated at high levels in microglia of active plaques (Bonetti *et al.*, 1999). Therefore, this pathway seems to be an essential target for anti-inflammatory compounds.

Natural compounds offer a great source of novel bioactive compounds and development into drugs for treating inflammatory diseases with fewer side effects and more accessible costs (Gautam & Jachak, 2009). 1,2,3,4,6-Penta-O-Galloyl- β -D-glucose (PGG) is a naturally

occurring polyphenolic compound highly enriched in some medicinal herbs such as *Rhus chinensis* Mill and *Paeonia suffruticosa* (Cao *et al.*, 2014). These plants have been reported as a traditional Chinese medicine (Djakpo & Yao, 2010). PGG studies have reported its anti-angiogenesis, anti-cancer, and anti-diabetic effects (Yu *et al.*, 2011; Bruno *et al.*, 2013; Hu *et al.*, 2008; Zhang *et al.*, 2009). In our laboratory, we have established that PGG is a very potent inhibitor of hLDH-A, which is a therapeutic target for cancer, given its role in tumor initiation, progression, and metastasis (Deiab *et al.*, 2015).

Previous studies showed that PGG also exhibits anti-aggregation effects on Alzheimer's amyloid β proteins *in vitro* and *in vivo*. Low concentrations of PGG (IC₅₀=3 μ M) could inhibit A β aggregation and promote its destabilization. PGG oral intake reduced A β plaque burden and A β peptide content in brain tissue in transgenic mice, suggesting that PGG could be a potential therapeutic drug for AD patients with mild cognitive impairment (Fujiwara *et al.*, 2009). Studies involving pro-inflammatory cytokines showed PGG activity through the inhibition of the expression of TNF- α in LPS-stimulated human peripheral blood mononuclear cells (Feldman *et al.*, 2011), and the secretion of TNF- α and IL-6 induced by IL-1- β in rat synoviocytes (Wu & Gu, 2009). It also inhibited IL-8 mRNA expression and secretion in human monocytic U937 cells stimulated with PMA or TNF- α (Oh *et al.*, 2004). PGG studies in peritoneal and colonic macrophages reduced activation of NF κ B and MAPK signaling pathways by directly interacting with the MyD88 adaptor protein, having a potential to ameliorate inflammatory diseases, such as colitis (Jang *et al.*, 2013). PGG also suppressed gene expression and secretion of pro-inflammatory cytokines in a dose-dependent manner and blocked the activation of NF κ B, indicating its potential for the treatment of inflammatory diseases through the down-regulation of NF κ B mediated activation of human mast cells (Lee *et al.*, 2007).

In our previous study, we demonstrated that PGG inhibited the expression of the pro-inflammatory cytokines: MCP-5 and Pro MMP-9, which may be associated with the formation of senile plaques and neurofibrillary tangles in AD disease (Mendonca *et al.*, 2017). Therefore, the present study was designed to determine the effect of PGG in genes and proteins of the NF κ B and MAPK signaling pathways, which are known to have a crucial role in inflammatory processes through their ability to induce transcription of pro-inflammatory genes.

2. MATERIAL AND METHODS

2.1. Chemicals and Reagents

Dulbecco's Modified Eagle Medium-High Glucose Medium (DMEM) and Heat Inactivated Fetal Bovine Serum (FBS-HI) were obtained from Atlanta Biologicals (Flowery Branch, GA, USA). Penicillin/streptomycin and Hank's Balanced Salt Solution (HBSS) were obtained from GIBCO (Waltham, MA, USA). Dimethyl sulfoxide (DMSO), 1,2,3,4,6-Penta-O-Galloyl- β -D-glucose (purity 96.8%), lipopolysaccharide from *Salmonella enterica* (LPS), interferon γ (IFN γ), and other general reagents were all purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and VWR International (Radnor, PA, USA). NF κ B and MAPK signaling pathways arrays, Bradford protein assay and PCR reagents were bought from Bio-Rad (Hercules, CA, USA). Protease and Phosphatase inhibitors were obtained from G-

Biosciences (Saint Louis, MO, USA). Anti-goat IgG-horseradish peroxidase and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and primary antibodies from Santa Cruz Biotechnology, Cell Signaling (Danvers, MA, USA), and Thermo Fisher Scientific (Waltham, MA, USA), as listed below.

2.2. Cell Culture

BV-2 microglial cells were generously provided by Dr. Elizabetta Blasi (Blasi *et al.*, 1990). Cells were cultured in DMEM media supplemented with 10% Heat Inactivated Fetal Bovine Serum (FBS-HI) and 1% penicillin/streptomycin (100U/ml penicillin and 0.1mg/ml streptomycin) in a humidified atmosphere of 5% CO₂ at 37°C in an incubator. Plating media for each experiment consisted of DMEM, 2.5% of FBS-HI, and no penicillin/streptomycin-penicillin. LPS was prepared in water at 1 mg/ml, and IFN γ in PBS at 200ng/ml; both stored at –20°C. For experiments, LPS and IFN γ were added to the culture media at a working concentration of 0.2 μ g/ml and 0.2ng/ml, respectively. PGG was dissolved in DMSO before dilution in the media and the final concentration of DMSO did not exceed 0.1% (Elsisi *et al.*, 2005). The concentration of PGG used in all the experiments was 25 μ M, which was established by cell viability in our previous study (Mendonca *et al.*, 2017). The experiments were set in T-75 flasks, and the concentration of cells was 5 $\times$ 10⁵/ml.

2.3. PCR

2.3.1 RNA Extraction—BV-2 cells were treated with PGG and then activated or not with LPS/IFN γ after 1 h. The cells were harvested after 24 h and then washed twice with PBS. Cell pellets were lysed with the 1ml Trizol reagent. Chloroform (0.2 ml) was added to the lysed samples; the tubes were shaken, incubated at 15–30°C for 2–3 min and centrifuged at 10,000 g for 15 min at 2–8°C. The aqueous phase was transferred to a fresh tube, and the RNA precipitated by mixing 0.5 ml of isopropyl alcohol. After incubation, samples were centrifuged, the supernatant was removed, and the RNA pellets were washed with 75% ethanol. The samples were mixed before being centrifuged at 7,500 g for 5 min at 2–8°C. The RNA pellet was dried and dissolved in RNase-free water and incubated for 30 min on the ice before use. Finally, RNA purity and quantity were determined using Nanodrop (Thermo Fischer Scientific - Wilmington, DE, USA).

2.3.2 cDNA Synthesis—The cDNA strand was synthesized from the mRNA using iScript advanced reverse transcriptase from Bio-Rad. A solution of 4 μ l of the 5X iScript advanced reaction mix (containing primers), 1 μ l of reverse transcriptase, 5 μ l of the sample (250ng/5 μ l) for RT-PCR assay and 7.5 μ l of the sample (1.5 μ g/7.5 μ l) for RT-PCR with individual primers, and water were added to 0.2ml tubes, in a total of 20 μ l. The Reverse Transcription thermal cycling program included two steps: 42°C for 30 min and then 85°C for 5 min.

2.3.3 RT-PCR Array (NF κ B and MAPK Signaling Pathway) and RT-PCR (individual primers)—Real-time PCR amplification was performed following the manufacturer protocol (BioRad). A 10 μ l of the sample (10ng cDNA/reaction) plus 10 μ l of master mix were added to each well for the array assays, and a 1 μ l of the sample (200ng cDNA/reaction), 10 μ l of master mix, 1 μ l of primer and 8 μ l of water were added to each tube

for the individual RT-PCRs. The thermal cycling process including the initial hold step at 95°C for 2 min and denaturation at 95°C for 5 secs, followed by 40 cycles of 60°C for 30 secs (annealing/extension), and 60°C for 5 sec/step (melting curve) using Bio-Rad CFX96 Real-Time System (Hercules, CA, USA). The PCR arrays were specific to genes that play a role in the NFRB, and MAPK signaling pathways and the selected primers were specific to each gene of interest.

The UniqueAssay ID for each primer is described as follows:

BIRC3 (UniqueAssayID: qMmuCID0027290); *CHUK* (UniqueAssayID: qMmuCID0005344); *IRAK1* (UniqueAssayID: qMmuCID0005129); *NFKB1* (UniqueAssayID: qMmuCID0005357); *NOD1* (UniqueAssayID: qMmuCID0015768); *CDK2* (UniqueAssayID: qMmuCID0017791); *MYC* (UniqueAssayID: qMmuCID0006528)

2.4. Western Blot

2.4.1 Protein Assay—BV-2 cells were treated with PGG and then activated or not with LPS/IFN γ after 1 h. The cells were harvested after 24 h and then washed twice with PBS. Lysis buffer containing protease inhibitor cocktail and/or phosphatase inhibitor cocktail was added to the pellet. Protein concentration was determined using the Bio-Rad Bradford protein assay. A series of concentration standards ranging from 0 – 2 mg/ml were prepared using bovine serum albumin (BSA). The standards and samples (5 μ l), and Bio-Rad Bradford protein assay reagent (200 μ l) were loaded into a 96-well plate. Protein concentrations were quantified at 595 nm wavelength with a microplate reader Infinite M200 (Tecan Trading AG).

2.4.2 Western Blot—Cell lysates were separated by electrophoresis on 10% SDS-polyacrylamide gels and then transferred to Immobilon-P PVDF membranes. Blots were blocked for 1h with 5% BSA in Tris-buffered saline with 0.05% Tween 20 in PBS (PBST) and then incubated overnight at 4°C with the specific antibody (dilution 1:1000). Next day membranes were washed with PBST and incubated with anti-goat IgG-horseradish peroxidase in PBST for 4h. Protein loading was monitored in each gel lane by probing the membranes with anti-GAPDH antibodies. Immunoblot images were obtained using a Bio-Rad VersaDoc Imager, and the densitometry analyzed with Quantity One Software (Bio-Rad Laboratories, Hercules, CA).

2.4.3 ProteinSimple Western Assay—Phosphorylated proteins were determined using automated Wes Simple Western Analysis (ProteinSimple, San Jose, CA, USA). All reagents were supplied by ProteinSimple, and the analysis was performed according to the user manual. Protocols were optimized for each antibody and protein loading. In brief, protein extracts were mixed with a master mix to give a final concentration of 0.2 mg/ml total protein, 1x sample buffer, and 1x fluorescent molecular weight markers and 40 mM dithiothreitol. Samples were heated at 95°C for 5 min. Samples, blocking solution, primary antibodies (dilution: 1:5 to 1:25), horseradish peroxidase-conjugated secondary antibodies, chemiluminescent substrate, and separation and stacking matrices were loaded into designated wells in a microplate. After plate loading, fully automated electrophoresis and immunodetection took place with the capillary system. Target proteins were identified using

a primary antibody and immunosorbed using an HRP-conjugated secondary antibody and chemiluminescent substrate. Chemiluminescence was captured by a charge-coupled device camera, and the digital image was analyzed and quantified using ProteinSimple Compass software.

2.5. Data Analysis

Statistical analysis was performed using Graph Pad Prism (version 6.07). All data were expressed as a mean $\pm$ standard error from at least 3 independent experiments, and the significance of the difference between the groups was assessed using a one-way ANOVA, followed by Dunnet multiple comparison test. STRING (Search Tool for the Retrieval of Interacting Genes/Proteins - string-db.org) and KEGG (Kyoto Encyclopedia of Genes and Genomes - genome.jp/kegg) databases were used to analyze protein function and interaction.

3. RESULTS

To determine the effect of PGG on mRNA expression of 89 genes from NF κ B and 88 genes from MAPK signaling pathways in BV-2 microglia cells (Figure 1A and 1B), the total RNA of LPS/IFN γ -treated BV-2 cells with and without PGG pre-treatment were subjected to real-time PCR. Results showed that among the studied genes, 7 were significantly modulated by PGG (6 negatively and 1 positively) (Table 1A and 1B).

The 6 genes down-regulated by PGG were: *BIRC3*, *CHUK*, *IRAK1*, *NFRB1*, *NOD1* and *CDK2*; and the one up-regulated was *MYC* (see the description of genes in Figure 1A and 1B). Most of them belong to the NF κ B signaling pathway, except *CDK2* and *MYC*, which are involved in the MAPK signaling pathway (Table 1A and 1B). RT-PCR with individual primers was performed with the genes modulated by PGG to validate the results from the arrays. The results corroborated with the ones found in the PCR array. PGG caused a change in the expression of the genes *BIRC3*, *CHUK*, *IRAK1*, *NFRB1*, *NOD1*, *CDK2*, and *MYC* involved in the NF κ B and MAPK signaling pathways (Figure 2 and 3).

To confirm PCR results at the protein level, Western blots were performed with specific antibodies against BIRC3, CHUK, IRAK1, NF κ B1, NOD1, CDK2, and MYC. The results showed that LPS/IFN γ induced the expressions of these proteins in BV-2 microglial cells, but this induction was significantly reduced when PGG was added 1h before LPS/IFN γ treatment.

The protein expression of normalized intensity expressed as a percentage of control showed that PGG inhibited the expression of BIRC3 (16.5%), CHUK (80.5%), IRAK1 (26.8%), NF κ B1 (77.1%), NOD1 (23.1%), and CDK2 (49%). The opposite occurred with the gene MYC from the MAPK signaling pathway, which was highly expressed (46%) when the cells were treated with PGG. The results corroborated with the data obtained from the RT-PCR assays (Figure 4A and 4B). The effect of PGG in the expression level of phosphorylated proteins was measured by using ProteinSimple Western assay. CDK2, CHUK, IRAK1, and NF κ B1 were chosen based on the greatest activity of PGG on the inhibition of the total protein levels. The activation of BV-2 microglial cells by LPS/IFN γ increased the expression of phosphorylated CDK2, CHUK, IRAK1, and NF κ B1. However, the pre-treatment with

PGG attenuated the expression all the examined proteins, following the same pattern as seen when the total proteins were tested (Figure 5).

To study the functional protein association among CDK2, CHUK, IRAK1, and NF κ B1, we used bioinformatics tools. STRING database was used to analyze the association of the selected proteins (Figure 6A). The results obtained show that CDK2 and CHUK only interacted with IRAK1 and NF κ B1, while IRAK1 and NF κ B1 show association with all the other two proteins. The highest interaction was found between NF κ B1 and CHUK, and NF κ B1 and IRAK1, suggesting a functional link between them (Figure 6C). Besides the association among these four proteins, several others also interacted with them, maximizing connections in different signaling pathways (Figure 6B).

Gene ontology annotation achieved from the STRING database showed that these genes are placed into 5 biological processes and have 2 molecular functions (Table 2A and 2B). They are also involved in 35 KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (Table 3). *CDK2*, *CHUK*, *NF κ B1*, and *IRAK1* were described as associated with many signaling pathways that lead to cytokine production, including NF κ B, MAPK, Chemokine, Toll-like receptor, and Nod-like Receptor signaling pathways (Table 3; Figures 7).

These results show that PGG modulates the expression of genes and proteins involved in the NF κ B and MAPK signaling pathways, which play a key role in cytokines release in the inflammatory process.

4. DISCUSSION

The expression of proinflammatory genes associated with neurodegenerative diseases is regulated by transcriptional mechanisms (Glass *et al.*, 2010). A possible mechanism for the increase in cytokines seen in AD is via A β stimulation of NF κ B activity in microglia (Combs *et al.*, 2001). Suppression of NF κ B in microglia results in decreased neurotoxicity (Chen *et al.*, 2005) and NF κ B regulatory elements lie upstream of the APP protein, which is necessary for plaque formation (Grilli *et al.*, 1996). The hypothesis that inflammation and NF κ B activation is the underlying cause of AD is further supported by the observations that chronic LPS injections accelerate AD progression (Lynch *et al.*, 2003) and patients with systemic infection exhibit increased rates of cognitive decline (Holmes *et al.*, 2003). Taken together, the evidence suggests that NF κ B plays a role in plaque formation and even a more important role in inflammation and cytokine signaling in AD progression (Tilstra *et al.*, 2011). Another important signaling route is the MAPK signal transduction pathway, which plays a crucial role in many aspects of immune-mediated inflammatory responses (Hommes *et al.*, 2003). Given the critical role of MAPK pathways in regulating cellular processes that are affected in Alzheimer's disease (AD), the importance of MAPKs in disease pathogenesis is being increasingly recognized. All MAPK pathways, such as ERK, c-Jun N-terminal kinase (JNK) and p38 pathways, are activated in vulnerable neurons in patients with AD, suggesting that MAPK pathways are involved in the pathophysiology and pathogenesis of AD (Zhu *et al.*, 2002).

Previous studies have shown that PGG inhibited LPS-induced IRAK1 degradation and phosphorylation, IKK β phosphorylation, I κ B α degradation and translocation of the p65 subunit of NF κ B into the nucleus. IRAKs work in the signaling cascades of TLRs, which, unregulated activation may lead to pathological situations ranging from chronic inflammation to the onset of autoimmune diseases (Gottipati *et al.*, 2007) through the induction of pro-inflammatory transcription factors, such as NF κ B (Lukiw *et al.*, 1998; Ringwood & Lil, 2008; Keating *et al.*, 2007; Meylan & Tschopp, 2008). Many genomic studies show that AD brains have a significant disruption in the homeostatic expression of essential brain genes and a progressive up-regulation of inflammatory gene expression, driven in part by over-activation of transcription factor NF κ B. This supports both, the development and progression of neurodegenerative disease processes (Colangelo *et al.*, 2002; Lukiw *et al.*, 2000b; Lukiw & Pogue, 2007; Lukiw, 2004; Loring *et al.*, 2001; Bazan & Lukiw, 2002; Lukiw *et al.*, 2005; Cui *et al.*, 2007). Indeed, the TLR-IRAK-NF κ B signaling axis is substantially over-stimulated in AD brain (Ringwood & Lil, 2008, Colangelo *et al.*, 2002; Lukiw *et al.*, 2000b; Lukiw & Pogue, 2007; Lukiw, 2004) and plays a regulatory role in mediating neuropathological effects of the A β peptide since A β activation further modulates the release of inflammatory cytokines (Li, 2004; Tan *et al.*, 2008; Tahara *et al.*, 2006;). Regarding neuroinflammation, NF κ B activation has been shown to mediate cytokine expression (Jana *et al.*, 2002; Nakajima *et al.*, 2006). It has been suggested that NF κ B plays an important role in sustaining the vicious cycle of inflammatory response and in neuroglial interactions in neurons and astrocytes (Pugazhenthir *et al.*, 2013). Many reports have been shown that PGG has the potential to suppress the activation of NF κ B via inhibition of IKK activity. PGG inhibited I κ B kinase activity in activated macrophages RAW 264.7 cells activated by LPS, blocked phosphorylation of I κ B from the cytosolic fraction, and inhibited NF κ B activity in activated macrophages (Pan *et al.*, 2000). Also, by using LPS-stimulated peritoneal macrophages, PGG activity was investigated in several intracellular signaling proteins. PGG treatment reduced the phosphorylation of p-TAK1, p-IKK β , and p-I κ B α at a concentration of 5 μ M (Jang *et al.*, 2013).

Using LPS/IFN γ -stimulated BV-2 microglia cells, in this study, we investigated the effect of PGG on several genes and proteins involved in the signaling cascade of NF κ B and MAPK pathways. The results show that PGG may be able to modulate the level of expression of different cytokines that are transcribed in the cellular nucleus, by modulating the expression of genes and proteins that participate in the NF κ B and MAPK signaling activation. Although the controls for some genes presented a high basal activity, by comparing “LPS/IFN γ ” and “PGG + LPS/IFN γ ” treatments, it is clear that PGG was able to attenuate the expression of the genes in a significant way. The results also showed that PGG activity could be seen not only in the transcription but also in the protein level.

Our data showed that PGG down-regulated the expression of *NF κ B1*, *CDK2*, *CHUK*, and *IRAK1*. All four genes are involved in signaling pathways that lead to the production of inflammatory cytokines through the activation of NF κ B and MAPK pathways. The previous report studying the deregulation of the NF κ B pathway in peripheral mononuclear cells (PBMC) of AD showed that the expression level of the NF κ B1 (p105/50Kd) gene was significantly higher in AD on adult age-matched controls. Also, expression of various genes and both NF κ B p50 and NF κ B p65 DNA binding activity were increased in PBMC from

AD patients in comparison with those from age-matched controls (Ascolani *et al.*, 2012). The CDKs (Cyclin-dependent kinases) are known to be responsible for the progression of the cell cycle (Kang *et al.*, 1997; Boonstra, 2003), and play roles in cell differentiation, cell death (especially apoptosis), transcription and neuronal function (Knockaert *et al.*, 2002). Previous studies also have suggested that CDK inhibitor drugs may have potential as a novel anti-inflammatory and pro-resolution agents (Chen *et al.*, 2003). CHUK, also known as IKK- α or IKK1, is involved in the NF κ B activation. Activation of the transcription factor NF κ B by cytokines is rapid, mediated through the activation of the IKK complex with subsequent phosphorylation and degradation of the inhibitory I κ B proteins (Adli *et al.*, 2010). I κ B phosphorylation by the I κ B kinase (IKK) complex is a critical regulatory step in the NF κ B activation pathway (Gilmore, 2006; Scheidereit, 2006), and it involves kinases, including IKK1/IKK α as the catalytic component of this complex. When the pathway is activated, phosphorylated NF κ B dimers are released and translocated to the nucleus, bind to the DNA sequences RB, and induce transcription of target genes (Scheidereit, 2006; Zandi *et al.*, 1997). By down-regulating the expression of these genes and proteins, PGG may be modulating the activation of signaling pathways that lead to the release of pro-inflammatory cytokines, which consequently induce neuroinflammation.

Although PGG present gallic acid in its structure, the literature shows that its potential activity is not exclusively because of the presence of this component. Bruno and colleagues (2013) investigated the inhibitory potential of gallic and tannic acid, and the structure related compound PGG, on the aggregation of islet amyloid polypeptide, amylin (IAPP). Results showed that while both, tannic and gallic acids, appear to be poor inhibitors of IAPP-based aggregation, PGG appears to be a strong inhibitor of IAPP-based aggregation. PGG functioned in a concentration-dependent manner to inhibit fiber formation and Thioflavin T (ThT) binding under extreme conditions known to promote IAPP aggregation. The authors stated that it remains to be determined why PGG functions as a strong inhibitor of IAPP aggregation, while its structural relatives, tannic and gallic acids, are not (Bruno *et al.*, 2013). Therefore, the potent properties of PGG listed in our paper may not be the same as the ones exhibited by gallic acid.

5. CONCLUSION

Our findings show that PGG can modulate several genes and proteins involved in the NF κ B and MAPK signaling pathways, which play a key role in neuroinflammation. PGG attenuating effect may be able to regulate TLR-IRAK-NF κ B signaling pathway, which, unregulated activation may lead to chronic inflammation (Gottipati *et al.*, 2007). By modulating the expression of CDK2, CHUK, IRAK1, and NF κ B1, PGG may regulate the production of pro-inflammatory cytokines, and therefore, it could explain the mechanism by which PGG exhibited an inhibitory effect on the expression of MCP-5 and Pro MMP-9 pro-inflammatory cytokines in activated microglia cells (described in our previous paper (Mendonca *et al.*, 2017)).

Figure 8 shows a diagram of the proposed mechanism of PGG attenuating effects in the production of pro-inflammatory cytokines through the inhibition of CDK2, CHUK, IRAK1, and NF κ B1, consequently reducing neuroinflammation.

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List of Abbreviations

AD	Alzheimer's Disease
Aβ	Amyloid β
CDKs	Cyclin-dependent kinases
CHUK	Conserved Helix-Loop-Helix Ubiquitous Kinase
ERK	Extracellular signal-regulated kinase
IFNγ	Interferon γ
IRAK1	Interleukin-1 receptor-associated kinase 1
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
NFKB	Nuclear factor-kappa B
PBMC	Peripheral mononuclear cells
PGG	1,2,3,4,6-Penta-O-Galloyl- β -D-glucose
TLR4	Toll-like receptor 4

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Highlights

- PGG modulated the expression of genes and proteins involved in the NF κ B and MAP kinase signaling pathways.
- PGG down-regulated the expression of CDK2, CHUK, IRAK1, and NF κ B1 in the transcriptional and protein level in LPS/IFN γ -activated BV-2 microglial cells.
- By down-regulating the expression of these genes and proteins, PGG may be modulating the activation of transcription factors that are responsible for the production of pro-inflammatory cytokines, which consequently induce neuroinflammation.

Figure 1: NFRB (A) and MAPK (B) PCR array layout. Figures **A** and **B** show the tested genes in each one of the arrays. The red circles show the genes down or up-regulated after PGG pre-treatment (1 h before) in LPS/IFN γ activated BV-2 microglia cells.

Figure 2: RT-PCR Assay (individual primers): Proteins from NFκB Signaling.

The effect of PGG on the expression of genes involved in the NFκB signaling pathway: *BIRC3* (A), *CHUK* (B), *IRAK1* (C), *NFKB1* (D), and *NOD1* (E). Graph bars show control, PGG (25μM), LPS/IFNγ, and PGG pre-treatment (PGG (1 h before) + LPS/IFNγ). Data represent protein expression as the mean ± S.E.M (n=3). Statistical significance of the difference between control and different treatments was determined by a one-way ANOVA, followed by a Dunnett multiple comparison test. *p 0.05, **p 0.01, ***p 0.001 and ****p 0.0001, ns: not significant.

Figure 3: RT-PCR Assay (individual primers): Proteins from MAPK Signaling.

The effect of PGG on the expression of genes involved in the MAPK signaling pathway: *CDK2* (A) and *MYC* (B). Graph bars show control, PGG (25 μ M), LPS/IFN γ , and PGG pre-treatment (PGG (1 h before) + LPS/IFN γ). Data represent protein expression as the mean $\pm$ S.E.M (n=3). Statistical significance of the difference between control and different treatments was determined by a one-way ANOVA, followed by a Dunnett multiple comparison test. *p 0.05, **p 0.01, ***p 0.001 and ****p 0.0001, ns: not significant.

Figure 4: Western Blot Assay.

The effect of PGG on the expression of total proteins involved in the NF κ B (CHUK, IRAK1, NF κ B1, and NOD1) and MAPK signaling pathways (CDK2 and MYC). Bands (**A**) represent control, PGG (25 μ M), LPS/IFN γ , and PGG pre-treatment (PGG (1 h before) + LPS/IFN γ) after 24 hours of treatment. **B** shows the protein expression of normalized intensity, expressed as % of control of the proteins inhibited by PGG treatment (mean $\pm$ S.E.M. n=3).

Figure 5: ProteinSimple Western Assay.

The effect of PGG in the expression of the phosphorylated proteins: IRAK1, CHUK, NFκB1, and CDK2. Bands represent control, PGG (25μM), LPS/IFN γ , and PGG pre-treatment (PGG (1 h before) + LPS/IFN γ) after 24 hours of treatment (n=3).

Figure 6: Protein Association Network.

A- Interactions among IRAK1, CHUK, NFκB1, and CDK2. **B-** Interaction among IRAK1, CHUK, NFκB1, and CDK2 and other proteins in the same network. **C-** Table showing the protein interactions and the suggested score for the functional link between them, according to STRING database.

Figure 7: Chemokine (A) and Toll-like Receptor (B) Signaling Pathways.

The figure shows the activation pathway for cytokine production, including the steps of receptor activation, gene transcription, and cytokine production. Red stars show where CHUK, IRAK1, and NF-κB1 play their role (KEGG Pathway).

Figure 8:

Proposed effects of PGG on the NFκB and MAPK signaling proteins involved in the release of MCP-5 and Pro MMP-9 cytokines.

Antibody	Catalog #	Vendor
BIRC3	SC-7944	Santa Cruz
CHUK	SC-10760	Santa Cruz
IRAK1	SC-7883	Santa Cruz
NFKB1	SC-7178	Santa Cruz
NOD1	SC-99163	Santa Cruz
CDK2	SC-748	Santa Cruz
MYC	SC-40	Santa Cruz
GAPDH	SC-25778	Santa Cruz
Phospho IRAK1	PA5-38631	ThermoFisher Scientific
Phospho CHUK	C84E11	Cell Signaling
Phospho NFKB1	SC-271908	Santa Cruz
Phospho CDK2	SC-101656	Santa Cruz

Table 1:
NF κ B (A) and MAPK (B) PCR array results.

The table shows the difference in gene expression in LPS/IFN γ vs. PGG pre-treatment (PGG (1 h before) + LPS/IFN γ) BV-2 microglia cells. **A-** NF κ B and **B-** MAPK signaling pathways. Arrows indicate down or up-regulation of the genes. Statistical significance was evaluated by a Student's t-test, $p < 0.05$ ($n=3$).

A	Symbol	Description	Fold Change	Function
	BIRC3	Baculoviral IAP Repeat Containing 3	↓ 1.8	Cellular inhibitor of Apoptosis, upregulated in activated microglia (Kavanagh et al., 2014)
	CHUK	Conserved Helix-Loop-Helix Ubiquitous Kinase	↓ 4.5	Regulates the release of NF κ B transcription factor (Adli et al., 2010)
	IRAK1	Interleukin 1 Receptor Associated Kinase 1	↓ 9.7	Protein activated by TLR. Regulates inflammatory Gene expression (Gottipati et al., 2007)
	NFKB1	Nuclear Factor Kappa B Subunit 1	↓ 3.2	Regulates the release of NF κ B transcription factor (Lappas et al., 2002; Yoon et al., 2009)
	NOD1	Nucleotide Binding Oligomerization Domain Containing 1	↓ 1.7	Pathogen recognition Cytoplasmic receptors (Caruso et al., 2014)
B	Symbol	Description	Fold Change	Function
	CDK2	Cyclin Dependent Kinase 2	↓ 3.8	Regulation and Progression of Cell Cycle (Kang et al., 1997; Boonstra, 2003)
	MYC	Myelocytomatosis Viral Oncogene Homolog	↓ 6.9	Pro-oncogene Increased in many Neurodegenerative diseases (Ferrer et al., 2001)

Table 2:
Biological Processes and Molecular Functions.

Table **A** shows 5 biological processes and Table **B** shows 2 molecular functions in which CDK2, CHUK, IRAK1, and NF κ B1 are involved (STRING database).

A	BIOLOGICAL PROCESSES				
	<i>Pathway Description</i>				
Proteins	<i>I-KappaB Kinase/NFκB Signaling</i>	<i>Intracellular Signal Transduction</i>	<i>NIK/NFκB Signaling</i>	<i>Positive Regulation of Transcription</i>	<i>Cellular Response to Interleukin-1</i>
IRAK1		X	X	X	X
CHUK	X	X		X	
NF κ B1	X	X	X	X	X
CDK2		X		X	
B	MOLECULAR FUNCTIONS				
	<i>Pathway Description</i>				
Proteins	<i>Protein Heterodimerization activity</i>		<i>Protein-Serine-Threonine Kinase Activity</i>		
IRAK1	X		X		
CHUK	X		X		
NF κ B1	X				
CDK2			X		

Table 3:
KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway.

The table shows the 35 pathways by which *CDK2*, *CHUK*, *IRAK1*, and *NFKB1* are involved. The ones in red correspond to the main pathways associated with cytokine production.

Pathway Description	<i>IRAK1</i>	<i>CHUK</i>	<i>NFKB1</i>	<i>CDK2</i>
Measles	X	X	X	X
Epstein-Barr virus infection	X	X	X	X
NF-kappa B signaling pathway	X	X	X	
Apoptosis	X	X	X	
Prostate cancer		X	X	X
Small cell lung cancer		X	X	X
Toll-like receptor signaling pathway	X	X	X	
Chagas disease (American trypanosomiasis)	X	X	X	
Toxoplasmosis	X	X	X	
Hepatitis B		X	X	X
Herpes simplex infection		X	X	X
Pathways in cancer		X	X	X
PI3K-Akt signaling pathway		X	X	X
NOD-like receptor signaling pathway		X	X	
Acute myeloid leukemia		X	X	
RIG-I-like receptor signaling pathway		X	X	
Cytosolic DNA-sensing pathway		X	X	
B cell receptor signaling pathway		X	X	
Adipocytokine signaling pathway		X	X	
Pertussis	X		X	
Leishmaniasis	X		X	
Pancreatic cancer		X	X	
Chronic myeloid leukemia		X	X	
T cell receptor signaling pathway		X	X	
TNF signaling pathway		X	X	
Neurotrophin signaling pathway	X		X	
Osteoclast differentiation		X	X	
FoxO signaling pathway		X		X
Hepatitis C		X	X	
Tuberculosis	X		X	
Chemokine signaling pathway		X	X	
Viral carcinogenesis			X	X
Ras signaling pathway		X	X	
MAPK signaling pathway		X	X	
HTLV-I infection		X	X	