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Effects of Palmitate on the Expression of Inflammatory Cytokines in Microglial Cells

Shiyu Ma

Submitted in Partial fulfillment of the requirement for the degree of Master of Science in Molecular Biology from the Department of Biological Sciences of Seton Hall University

July 2021

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SETON HALL UNIVERSITY

College of Arts and Sciences

Department of Biological Sciences

APPROVAL FOR SUCCESSFUL DEFENSE

Shiyu Ma has successfully defended and made the required modifications to the text of the master's thesis for the M.S. during Summer 2021.

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Abstract: Obesity has become a pandemic health issue for the development of more serious illnesses such as insulin resistance, cardiovascular diseases, and hypertension. Among those pathological conditions, low grade chronic inflammation triggered by saturated fatty acids (SFA) such as palmitate (PA) has been suggested to be the culprit of crimes and various signaling modulators ranging from cellular surface receptors such as toll like receptors (TLRs) and G-protein – coupled receptor 40 (GPR40) to intracellular kinases like mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) have been postulated. In this study, mouse BV2 cells were treated with different concentrations of PA and time and the mRNA expression levels of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β were examined. In order to determine how PA induced cytokine expression in BV2 cells, these cells were pre-treated with various pharmacological inhibitors followed by treatment with PA or BSA control, and the mRNA expressions of TNF-α, IL-6, and IL-1β were examined. Inhibition of PI3K and GPR40 by Wortmannin and DC260126 attenuated PA induced expression of these inflammatory cytokines, suggesting that PI3K and GPR40 might be involved in PA-induced production of inflammatory mediators in BV2 cells.

Introduction

Obesity has become a global issue in the past 50 years. The number of people with obesity has nearly tripled worldwide since 1973 (WHO 2016). In 2016, more than 650 million people were obese (WHO 2016). People who are obese tend to have higher risks for fatty liver disease, type 2 diabetes mellitus (T2DM), hypertension, stroke, dyslipidemia and cancers, thereby threatening their life expectancy and quality of life (Blüher 2019). The cause of obesity is multifactorial including environment, genetics, absence of physical activities, and diets (Hruby 2016). Imbalanced calorie absorption and consumption result in excess serum levels of saturated fatty acids (SFAs) when a high fat diet (HFD) is consumed (I S Sobczak 2019). Mounting evidence has suggested chronic low-grade inflammation triggered by SFAs such as palmitate (PA) might be an underlying cause for obesity-associated conditions (Ogden 2007). For example, chronic low-grade inflammation has been detected in the brain, adipose tissue, and liver of obese human subjects (Melo 2020 and Sun 2012). Palmitate has been reported to trigger inflammatory cytokine productions in macrophages and hepatocytes (Riera-Borrull 2017 and Joshi 2007). However, the mechanisms by which PA induces inflammation has not been well-defined.

Free fatty acids receptors (FFARs), the Class A/1 (rhodopsin-like) receptors in a subfamily of G protein coupled receptors (GPCRs), consists of a 7 transmembrane (TM) motif and at least a cytosolic domain that initiates ligand recognition and activation (Falomir 2019). Following its activation, conformational change of G proteins from GTP exchange facilitates the dissociation of Gα subunit from $G_{\beta\gamma}$ subunits allowing further intracellular signal transduction (Hilger 2018). Gα subunit can be further categorized into several types such as Gαs, Gαq/11,Gαi/o and Gα12/13. The activation of Gαs activates adenylyl cyclase (AC) which

induces the accumulation of cAMP, a secondary messenger that activates protein kinase A (PKA) allowing the initiation of downstream transcription factors such as cyclic AMP responsive element-binding (CREB) protein (Sassone 2012). Activated Gαq/11, on the other hand, activates phospholipase C, which would facilitate the formation of diacylglycerol (DAG) for further downstream signaling (Sassone 2012). FFAR1 or GPR40 is predominantly activated by medium to long chain saturated FA ranging from C10 to C18 and expressed highly in the cells of pancreas and to a lesser extent in the brain (Falomir 2019). Studies have shown activation of GPR40 could stimulate insulin secretion via Gαq/11/Ca^{2+}/PLC/DAG pathway (Burant 2013). However, function of GPR40 in obesity associated inflammation remained unknown.

DAG is a well-known activator for conventional and novel protein kinase C (cPKC/nPKC) (Korbecki 2019). However, not all PKCs are activated by the same DAG and the relative abundance of different PKCs is largely tissue-dependent. For example, PKCθ is strongly activated by all DAGs and predominantly present in skeletal muscle and lymphoid tissues (Meller 1998). PKCδ, on the other hand, is strongly activated by DAGs containing unsaturated fatty acids and highly expressed in brain, heart, spleen, lung, and liver (Wetsel 1992). Activated PKCs have been reported to activate downstream kinases such as inhibitor kappa B Kinase (IKK) and MAPK in adipocytes and skeletal muscle cells (Yang 2010 and Coll 2008). Upon activation, IKK phosphorylates inhibitor of nuclear kappa B (IκB), a protein that keeps nuclear factor kappa B (NF-κB) inactive in the cytoplasm (Liu 2017). NF-κB then migrates into the nucleus to promote the transcription of inflammatory mediators such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6) (Liu 2017). PKCs have been linked to mitogen activated protein kinases (MAPKs) for their ability to modulate the activity of raf kinase associating with raf kinase inhibitory protein (RKIP) (Macrae 2013). The phosphorylation of

RKIP by PKCs at Ser^{153} releases raf in its active form which then phosphorylates/activates mitogen-activated protein kinase kinases (MEK). MEK then activates extracellular signal-regulated kinases (ERK) (Lorenz 2003). ERK activation has been reported to induce pro-inflammatory cytokines production in chondrocytes and brain in mice (Djouad 2009 and Wang 2004).

Phosphoinositide-3-kinase (PI3K) is involved in cellular functions such as cell growth, differentiation, and intracellular trafficking (Troutman 2012). Three classes of PI3Ks have been identified. Among those classes, Class I PI3Ks are most studied and can be sub-divided into Class IA and Class IB (Troutman 2012). The Class IA PI3K consisting of p110α, p110β, and p110δ is regulated by either subunits p85α or p85β, while Class IB PI3K, p110γ is regulated by subunits p101 or p87 (Troutman 2012). Upon activation, class I PI3Ks catalyze the phosphorylation of phosphatidylinositol 4,5-bisphophate (PIP2) into phosphatidylinositol (3,4,5)-trisphophate (PIP3), a secondary messenger that recruits downstream targets with pleckstrin-homology (PH) domain or lipid-binding domains such as phosphoinositide-dependent kinase -1 (PDK1) and protein kinase B (Akt) and localizes them to the cell membrane to activate downstream signaling pathways (Yang 2019). PI3Ks have been studied extensively in cancer research (Ellis 2019 and Morgensztern 2005). Recent data revealed PI3Ks might have additional roles in regulating inflammation. PI3K/Akt signaling has been reported to increase the production of pro-inflammatory cytokine through NF-κB activation in dendritic cells (Koorella 2014).

The aim of the study was to examine how SFAs such as PA affect the expression of inflammatory cytokines at the mRNA level in microglia cells and to investigate potential mechanisms of PA-induced expression of these cytokines.

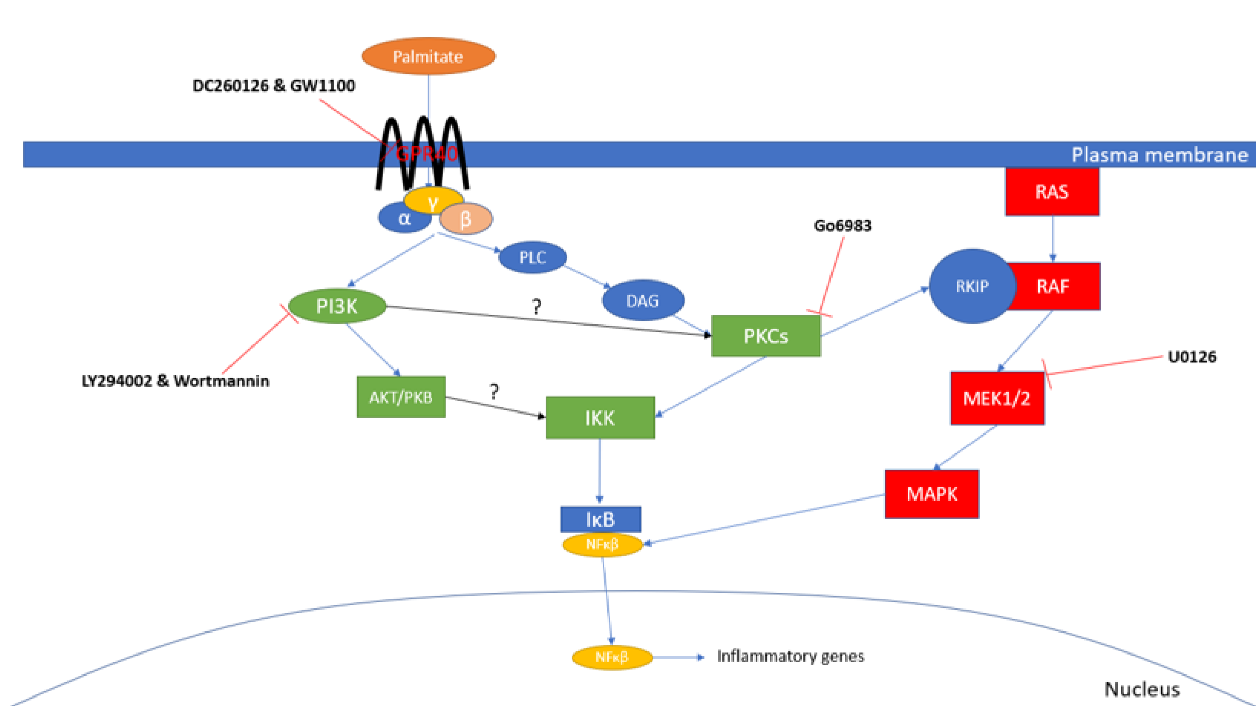

Figure 1: Diagram of potential pathways involved in PA-modulated expression of inflammatory mediators BV2 cells. Created by Shiyu Ma, (2021), Seton Hall University.

Materials and methods

Cell culture

The murine microglial cell line BV2 was kindly provided by Dr. Jau-Shyong Hong from NIH and maintained in DMEM (BioWhittaker, Walkersville, MD USA) supplemented with 10% FBS, 1% penicillin and streptomycin (Sigma, St. Louis, MO USA) at 37 °C in humidified incubator with 5% CO_2. In all experiments, cells were seeded in a 12-well plate (5×10^5 cells/well) and incubated overnight. 6 hours prior to treatment, media was removed and replaced with 0.25% FBS DMEM. Cells were at 75-80% confluency after 24 hours of treatment with ovine serum albumin (BSA) (Sigma, St. Louis, MO USA) or palmitic acid (PA) (Sigma, St. Louis, MO USA).

Total RNA isolation and RT-PCR

Total RNA was isolated using TRIzol reagent (Sigma, St. Louis, MO USA) per manufacturer's protocol. RNA was reverse transcribed with Moloney murine leukemia virus (MMLV) Reverse Transcriptase (Promega, Madison, WI USA). Briefly, total RNA concentration was quantified by spectroscopy. 1 µg total RNA was combined with 5 µL of 5X M-MLV reverse transcriptase buffer, 1.25 µL of 10mM dNTP, 0.5 µL MMLV reverse transcriptase, and nuclease-free water to bring up the final volume to 25 µL, incubated at 40 °C for 1 hour followed by 70 °C for 15 minutes. cDNA was then amplified by polymerase chain reaction (PCR) (95 ºC for 30 seconds, 56 ºC for 30 seconds, and 72 ºC for 30 seconds) with appropriate primers for *GAPDH, IL-1β, TNF-α, and IL-6 (*mGAPDH, (F) 5'-

CGAACATCATCCCTGCATCCA-3’, (R) 5’-CCCAGTGAGCTTCCCGTTCA-3’; mIL-1β, (F) 5’-GCCCATCCTCTGTGACCTCAT-3’, (R) 5’-AGGCCACAGGTATTTTGTCG-3’; mIL-6, (F) 5’-CCGGAGAGGAGACTTCACAG-3’, (R) 5’-CAGAATTGCCATTGCACAAC-3’; mTNF-α, (F) 5’-AGTCCGGGCAGGTCTACTTT-3’, (R) 5’-GGTCACTGTCCCAGCATCTT)-3’. PCR products were separated by electrophoresis in 2% agarose gel with 0.05 ug/ml ethidium bromide and visualized by FlourChem E system (Protein Sample, San Jose, California). The agarose gel images were quantitated by Image Studio Lite Ver 5.2. Relative expression of genes of interest was calculated relative to the house-keeping gene GAPDH. Data were graphically represented as fold-change relative to paired control GAPDH.

Pharmacological inhibitors treatment

Cells were pre-treated with various pharmacological inhibitors (5 µM Go6983, 10 µM LY294002, 50 nM Wortmannin, 5 µM U0126, 5 µM DC260126, and 5 µM GW1100) for 2 hours followed by treatment with 200 µM PA or with BSA vehicle for 24 hours. These concentrations were selected based on our MTT studies on microglia viability and their IC values. Total RNA was isolated and relative mRNA expression was obtained by semi-quantitative RT-PCR.

Statistical analysis

Data were analyzed by student’s t-test and one-way or two-way ANOVA using GraphPad Prism 5.

Results

Effects of different concentrations of PA treatment on mRNA expression levels of cytokines

To investigate whether PA exhibited dose-dependent effects on the expression of different cytokines, BV2 cells were starved with low serum (0.25% FBS) media for 6 hours followed by treatment with 100, 200, or 300 μM PA or equal volume of BSA as control in complete media (10%FBS) for 24 hours. Total RNA was then extracted, and the mRNA expression of IL-1β, TNF-α, and IL-6 was analyzed by semi-quantitative RT-PCR. As shown in Figure 2, PA induced IL-1β mRNA expression in a dosage-dependent manner (Figure 2A). 24 hours of 100 μM PA appeared to decrease the mRNA expression of TNF-α while 200 μM PA appeared to increase the mRNA expression of TNF-α as compared to BSA control (Figure 2B). Both 200 and 300 μM PA were able to stimulate significant IL-6 mRNA expression ($P<0.01$), while 100 μM PA failed to have noticeable impact on IL-6 mRNA expression as compared to BSA control (Figure 2C).

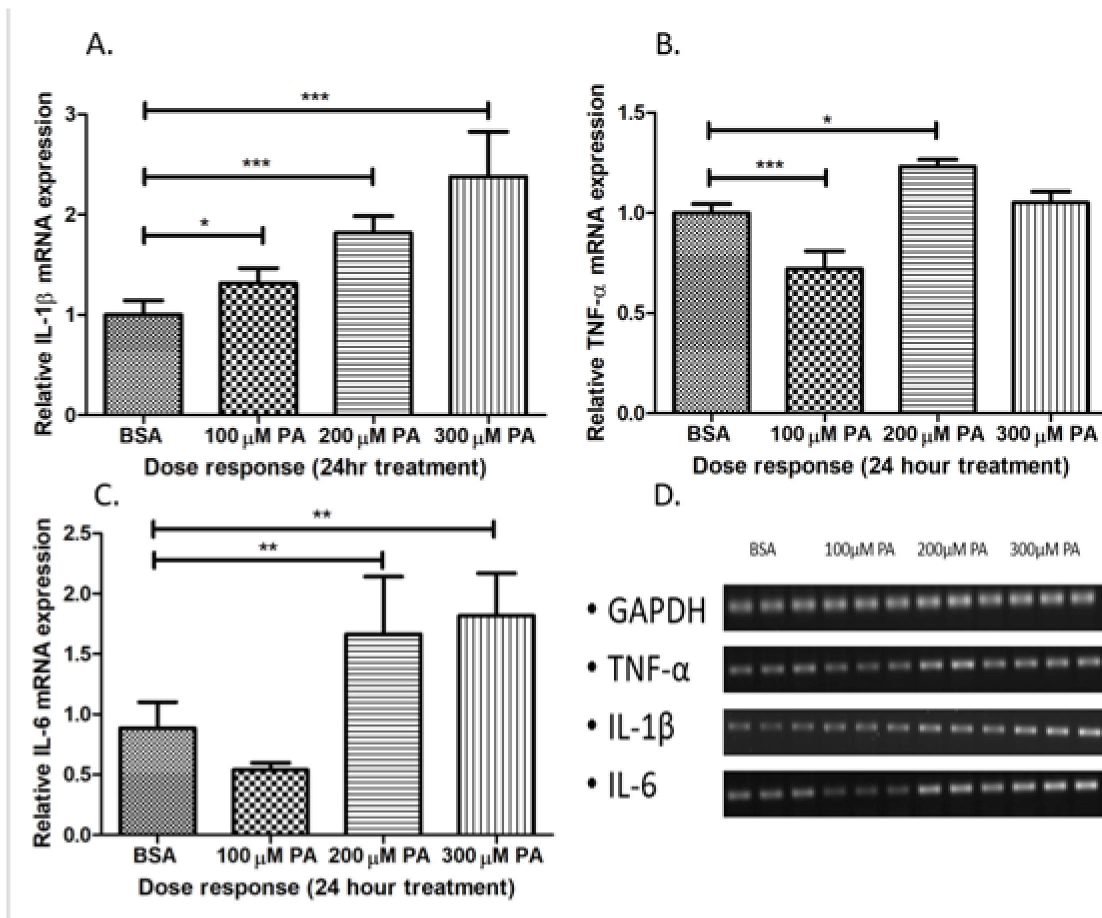

Figure 2: mRNA expression of IL-1β, TNF-α, and IL-6 in BV2 cells at 24 hours following treatment with varying concentrations of palmitate or vehicle as measured by semi-quantitative RT-PCR. (A) Relative mRNA expression of IL-1β normalized by GAPDH. (B) Relative mRNA expression of TNF-α normalized by GAPDH. (C) Relative mRNA expression of IL-6 normalized by GAPDH. Data shown as mean ± SD. N=3. (D) Agarose gel images of TNF-α, IL-1β, and IL-6 RT-PCR results.

Time-dependent effects of PA treatment on mRNA expression of cytokines

To determine time-dependent effects of PA treatment on mRNA expression of cytokines, BV2 cells were treated with 200 μM PA for 6, 24 and 48 hours. RNA was isolated and relative mRNA expression of IL-1β, TNF-α, and IL-6 expression was measured by semi-quantitative RT-PCR. Analysis of data revealed that treatment with 200 μM PA significantly increased the mRNA expression of IL-1β and IL-6 at 24 hour ($P<0.001$), but not at 6 hour or 48 hour following PA exposure (Figure 3A and 3C). TNF-α mRNA expression was significantly increased at 6 hours ($P<0.001$) and 24 hours ($P<0.05$) following 200 μM PA treatment, but not at 48 hours post-treatment (Figure 3B).

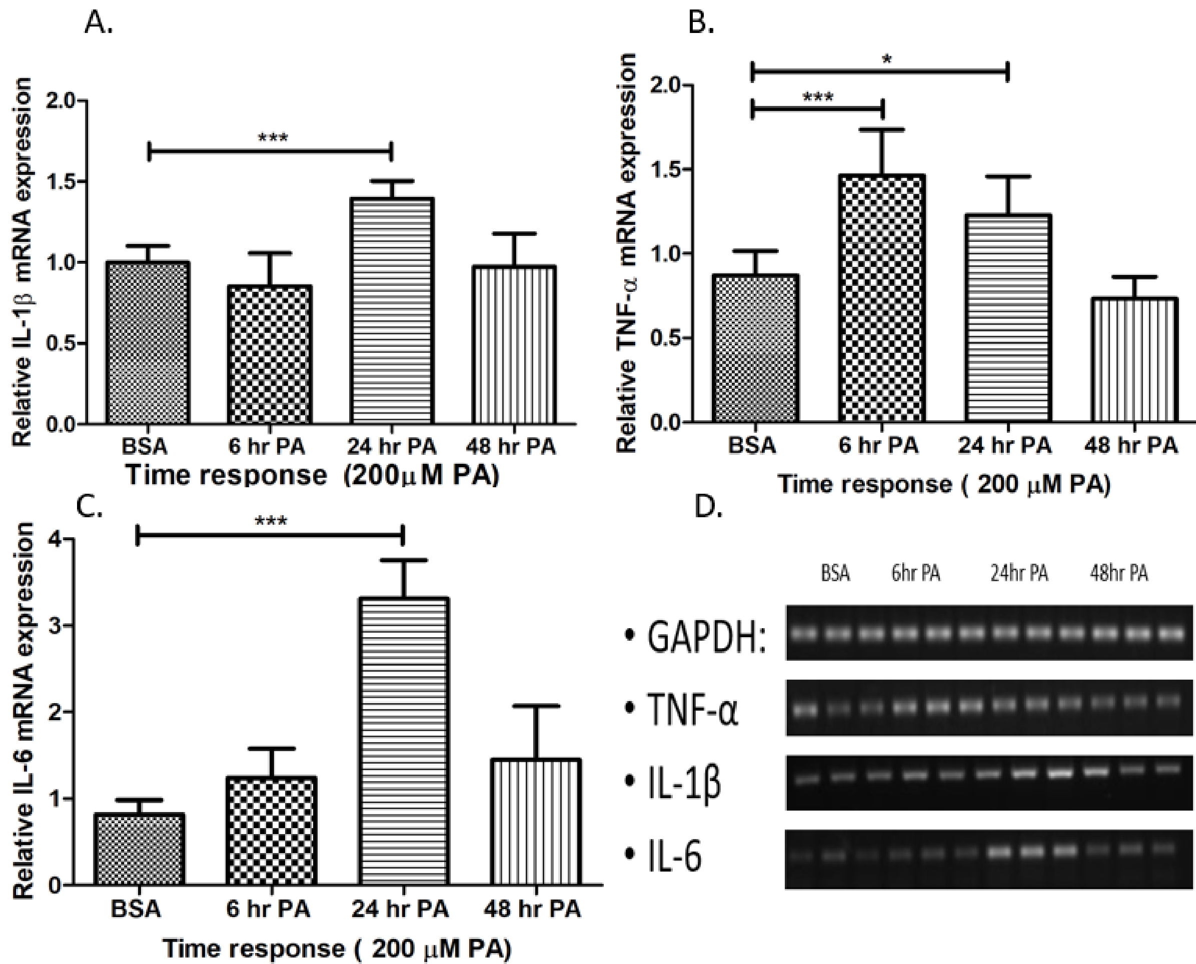

Figure 3: mRNA expression of IL-1β, TNF-α, and IL-6 in BV2 cells treated with 200 μM PA or vehicle for different periods of time as measured by semi-quantitative RT-PCR. (A) Relative mRNA expression of IL-1β normalized by GAPDH. (B) Relative mRNA expression of TNF-α normalized by GAPDH. (C) Relative mRNA expression of IL-6 normalized by GAPDH. (D) Agarose gel images of TNF-α, IL-1β, and IL-6 RT-PCR results.

Effects of Go6983 on mRNA expression of IL-1β, TNF-α, and IL-6

To investigate the role of PKCs in the effects of PA on cytokine expression, BV2 cells were pre-treated with 5 µM Go6983, a broad-spectrum PKC inhibitor, for 2 hours and followed by treatment with 200 µM PA or BSA control for 24 hours. The mRNA expression of IL-1β, TNF-α, and IL-6 were measured by semi-quantitative RT-PCR. Treatment with 200 µM PA for 24 hours significantly increased IL-1β and IL-6 mRNA expression ($P<0.001$). The administration of 5 µM Go6983 significantly attenuated IL-6 and TNF-α mRNA expression in both PA and BSA-treated cells (Figure 4B and 4C), while PA induced IL-1β mRNA expression was not attenuated by treatment with Go6983 (Figure 4A).

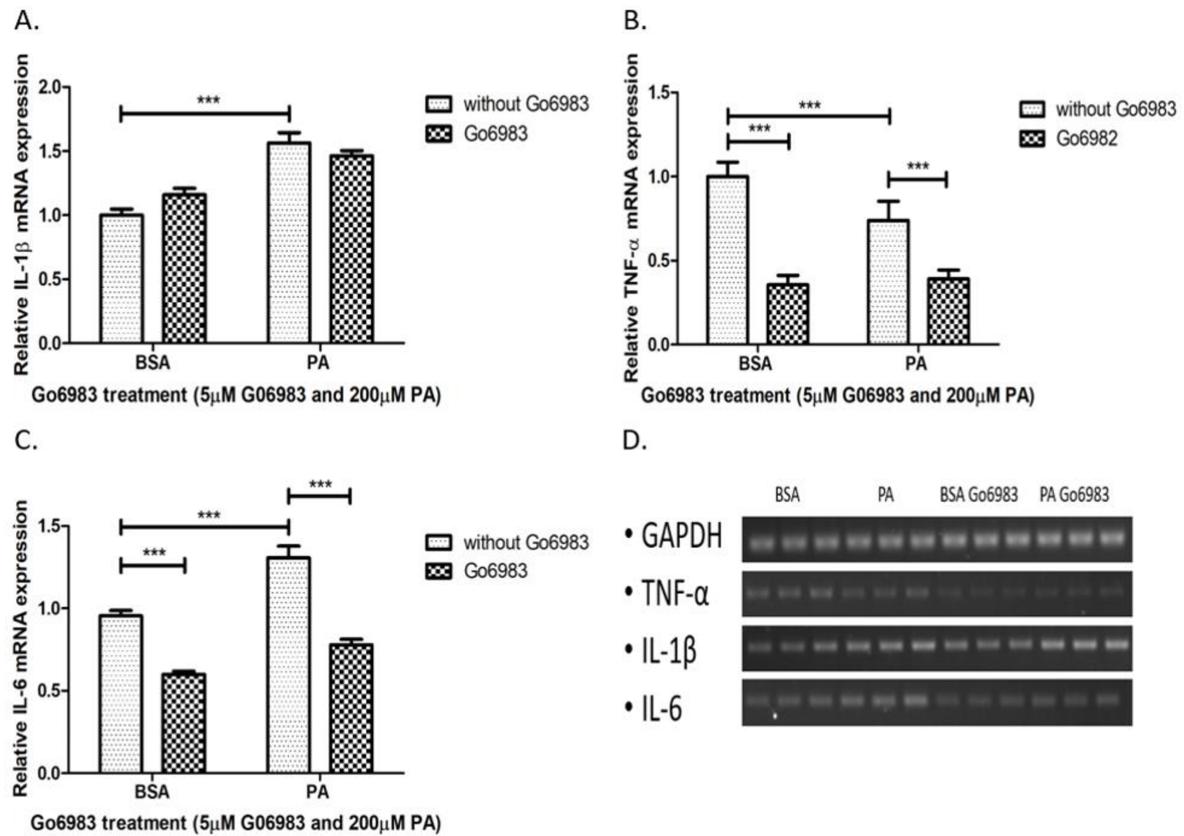

Figure 4: Relative mRNA expression of IL-1β, TNF-α, and IL-6 in BV2 cells pre-treated with 5 μM Go6983 or vehicle followed by 24 hours of treatment with BSA or 200 μM PA as measured by semi-quantitative RT-PCR. (A) Relative mRNA expression of IL-1β normalized by GAPDH. (B) Relative mRNA expression of TNF-α normalized by GAPDH. (C) Relative mRNA expression of IL-6 normalized by GAPDH. (D) Agarose gel images of TNF-a, IL-1β, and IL-6 RT-PCR results.

Effects of LY294002 and Wortmanin on the mRNA expression of IL-1β, TNF-α, and IL-6

To determine whether PI3K signaling pathway was involved in the effects of PA on cytokine expression, BV2 cells were pre-treated with 10 μM LY294002 and 50 nM Wortmannin for 2 hours and then treated with 200 μM PA or equal volume of BSA for 24 hours. The RNA was harvested and mRNA expression of IL-1β, TNF-α, and IL-6 was determined using semi-quantitative RT-PCR. Analysis of data revealed that 200 μM PA was able to increase significant IL-1β and IL-6 mRNA expression except for TNF-α expression at 24 hours (Figure 5). Treatment with 10 μM LY294002 significantly decreased the basal level of IL-6 expression and attenuated PA-induced increase in IL-6 mRNA expression (Figure 5C). In contrast, treatment with 10 μM LY294002 did not significantly affect basal IL-1β mRNA expression or PA-induced increase of IL-1β (Figure 5A).

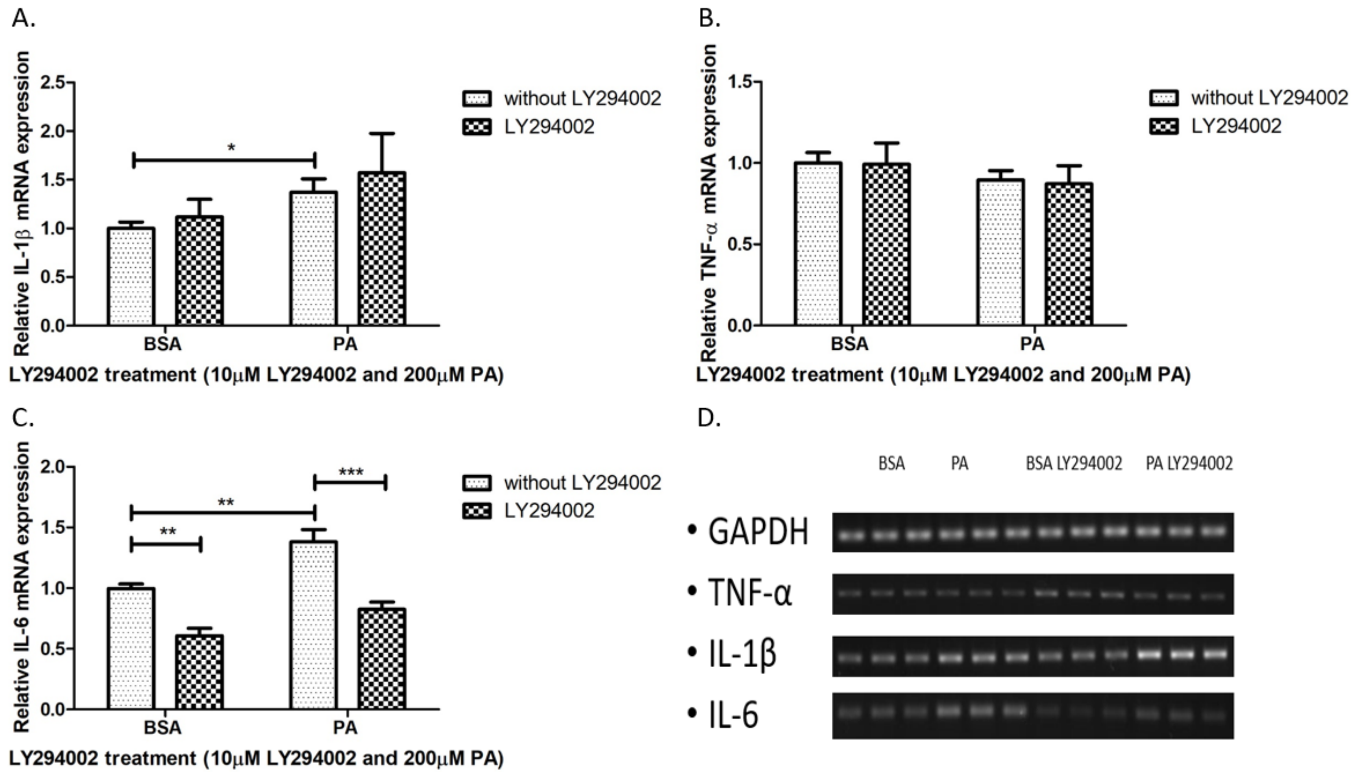

Figure 5 : Relative mRNA expression of IL-1β, TNF-α, and IL-6 in BV2 cells pre-treated with 10 μM LY294002 or vehicle followed by 24 hours of treatment with BSA or 200 μM PA. (A) Relative mRNA expression of IL-1β normalized by GAPDH. (B) Relative mRNA expression of TNF-α normalized by GAPDH. (C) Relative mRNA expression of IL-6 normalized by GAPDH. Data shown as mean ± SD. N=3. (D) Agarose gel images of RT-PCR results of TNF-a, IL-1β, and IL-6.

Consistently, pretreatment with 50 nM Wortmannin significantly diminished PA induced IL-6 mRNA expression but failed to have any impact on PA induced IL-1β mRNA expression (Figure 6C and 6A). Neither 200 μM PA nor 50 nM wortmannin had any significant effects on the mRNA expression of TNF-α (Figure 6B).

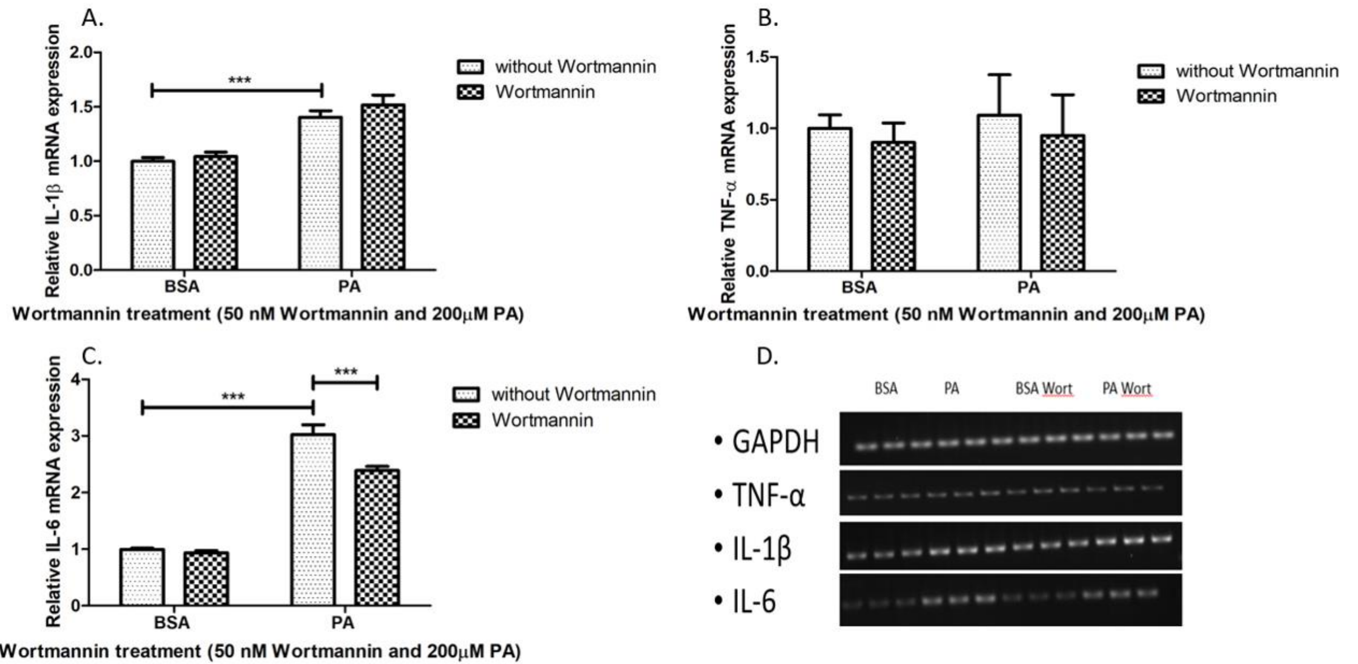

Figure 6: Relative mRNA expression of IL-1β, TNF-α, and IL-6 in BV2 cells pre-treated with50nM Wortmannin or vehicle followed by 24 hours of treatment with BSA or 200 μM PA as measured by semi-quantitative RT-PCR. (A) Relative mRNA expression of IL-1β normalized by GAPDH. (B) Relative mRNA expression of TNF-α normalized by GAPDH. (C) Relative mRNA expression of IL-6 normalized by GAPDH. Data shown as mean ± SD. N=3. (D) Agarose gel images of RT-PCR results of TNF-a, IL-1β, and IL-6.

Effects of U0126 on the mRNA expression of IL-1β, TNF-α, and IL-6

To probe potential involvement of MAPK in the effects of PA on cytokine expression, BV2 cells were pre-treated with a MEK inhibitor (U0126) for 2 hours and subsequently treated with 200µM PA or equal volume of BSA for 24 hours. The mRNA expression of IL-1β, IL-6 and TNF-α was determined by semi-quantitative RT-PCR. Significant increase in the mRNA expression of IL-1β, IL-6 and TNF-α was detected when treated with 200µM PA (Figure 7). The administration of 5µM U0126 significantly attenuated TNF-α and IL-6 expression in both PA and BSA treated cells ($P<0.001$) (Figure 7B and 7C). Surprising, 5µM U0126 upregulated IL-1β mRNA expression when treated with BSA and PA induced IL-1β mRNA expression cannot be altered by U0126 (Figure 7A).

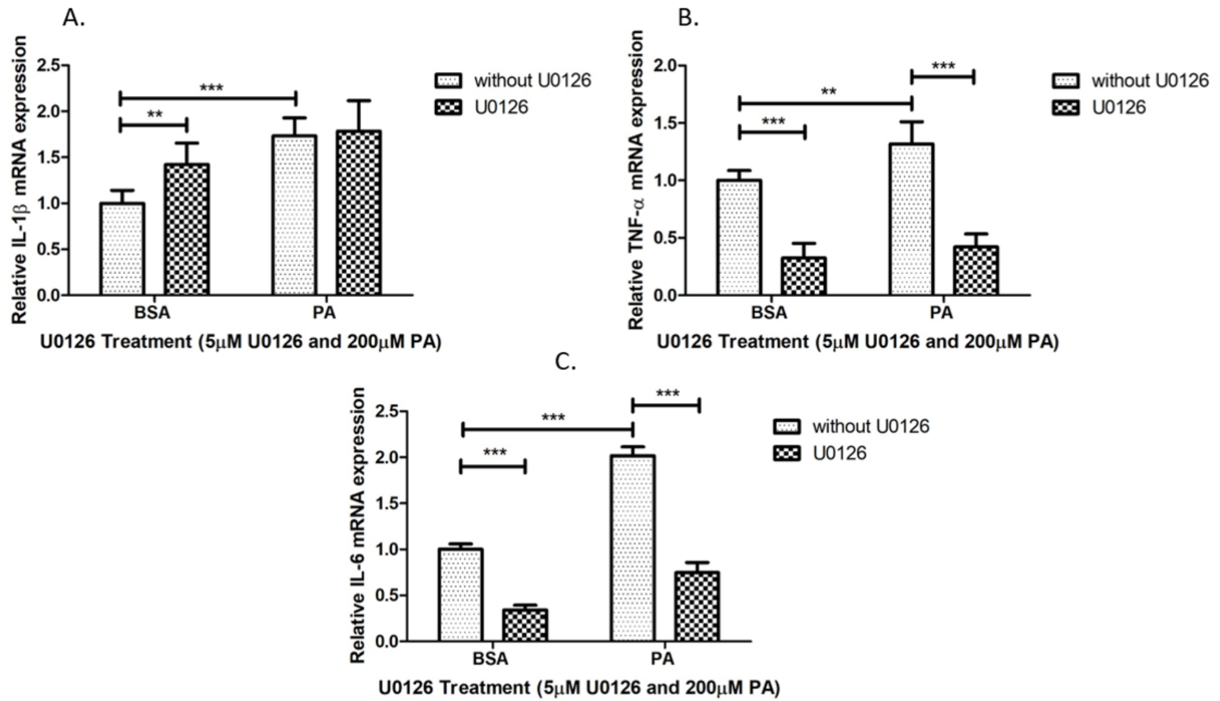

Figure 7: Relative mRNA expression of IL-1β, TNF-α, and IL-6 in BV2 cells pre-treated with 5 μM U0126 or vehicle followed by 24 hours of treatment with BSA or 200μM PA as measured by semi-quantitative RT-PCR. (A) Relative mRNA expression of IL-1β normalized by GAPDH. Data shown as mean ± SD. N=3. (B) Relative mRNA expression of TNF-α normalized by GAPDH. Data shown as mean ± SD. N=3. (C) Relative mRNA expression of IL-6 normalized by GAPDH.

Effects of DC260126 and GW1100 on the mRNA expression of IL-1β, TNF-α, and IL-6

To investigate how GPR40 might influence PA induced inflammation, BV2 cells were pre-treated with GPR40 inhibitors, 5 µM DC260126 or 5 µM GW1100, for 2 hours and followed by 200 µM PA or BSA treatment for 24 hours. The relative mRNA expression of IL-1β, TNF-α, and IL-6 was determined by semi-quantitative RT-PCR. The mRNA expression of IL-1β, TNF-α, and IL-6 was significantly increased by 200µM PA treatment as compared to BSA controls (Figure 8). Treatment with 5µM DC260126 significantly reduced PA-induced increase in the mRNA expressions of IL-1β, TNF-α, and IL-6 (Figure 8).

Treatment with 200 µM PA increased significant IL-1β, TNF-α, and IL-6 mRNA expression. Exposure with 5µM GW1100 significantly diminished PA induced IL-1β, TNF-α, and IL-6 mRNA expression with an exception that GW1100 also attenuated TNF-α mRNA expression in BSA-treated cells (Figure 9).

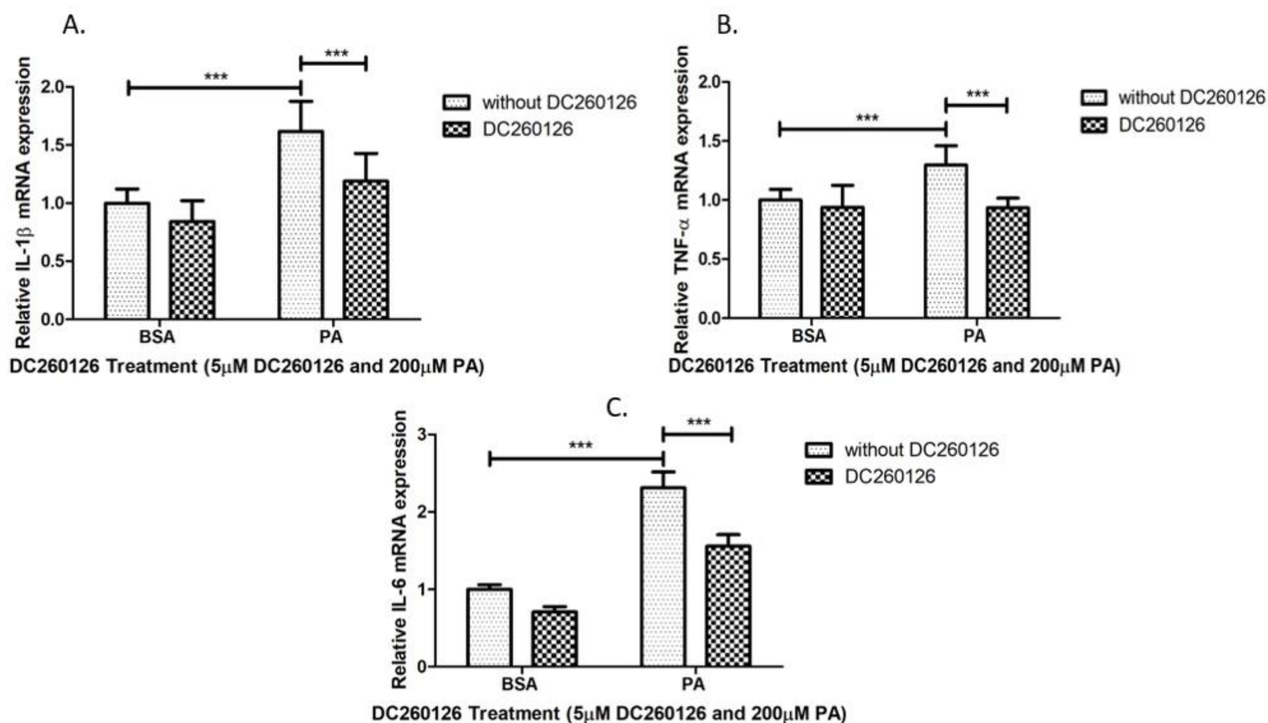

Figure 8: Relative mRNA expression of IL-1β, TNF-α, and IL-6 in BV2 cells pretreated with 5 μM DC260126 followed by 24 hours of treatment with BSA or 200μM PA as measured by semi-quantitative RT-PCR. (A) Relative mRNA expression of IL-1β normalized by GAPDH. Data shown as mean ± SD. N=3. (B) Relative mRNA expression of TNF-α normalized by GAPDH. Data shown as mean ± SD. N=3. (C) Relative mRNA expression of IL-6 normalized by GAPDH. Data shown as mean ± SD. N=3.

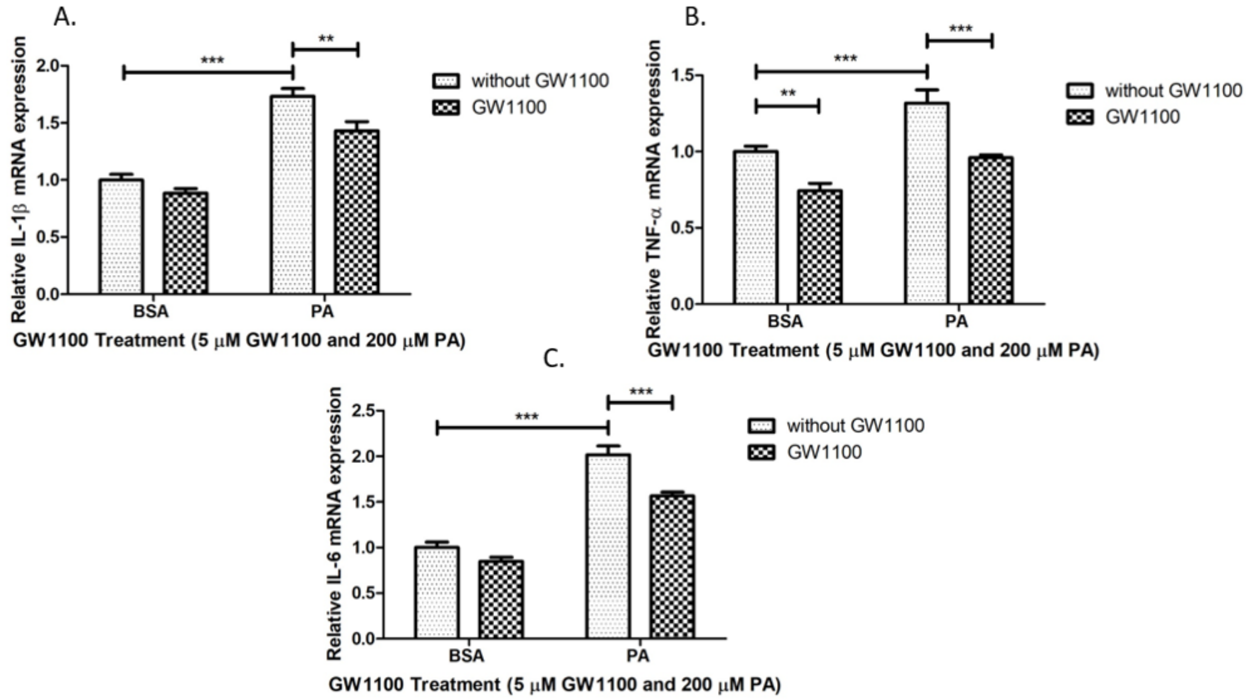

Figure 9: Relative mRNA expression of IL-1β, TNF-α, and IL-6 in BV2 cells pretreated with 5 μM GW1100 or vehicle followed by 24 hours of treatment with BSA or 200 μM PA as measured by semi-quantitative RT-PCR. (A) Relative mRNA expression of IL-1β normalized by GAPDH. Data shown as mean ± SD. N=3. (B) Relative mRNA expression of TNF-α normalized by GAPDH. Data shown as mean ± SD. N=3. (C) Relative mRNA expression of IL-6 normalized by GAPDH. Data shown as mean ± SD. N=3.

Discussion

There is increasing evidence that chronic inflammation triggered by elevated free fatty acids (FFAs) such as palmitate (PA) (C16:0) might be mechanistically involved in obesity-associated conditions such as type 2 diabetes and cardiovascular diseases (Pi-Sunyer 2009). Various upstream and downstream targets including TLRs (Huang 2012), GPR40 (Korbecki 2019), PKC (Jové 2005), PI3K (Cianciulli 2016), MAPK (Li 2018) and ROS (Ly 2017 and Sadeghi 2017) have been postulated and examined to potentially elucidate FFA associated inflammation. This study primarily focused on the effects of PA on the mRNA expression of inflammatory cytokines in BV2 cells.

Time and dose-dependent effects of PA on cytokines expression was first examined**.** We did not detect significantly increased mRNA abundance of IL-1β, IL-6, and TNF-α in BV2 cells treated with 100µM PA for 24 hours (Figure 2). However, significantly increased expression of IL-1β and IL-6 was detected in cells treated with 200µM and 300µM PA (Figure 2). Because 300µM PA treatment was caused extensive cell death, we chose 200µM PA treatment for future experiments. Interestingly, 100µM PA seemed to downregulate TNF-α mRNA expression at 24 hour treatment exhibiting an anti-inflammation event (Figure 2B). Currently, it is unknown what contributed to that event. Next, Significant induction of IL-1β and IL-6 by PA was detected only at 24 hour treatment, while TNF- α expression was significant as early as 6 hours and remained significant until at least 24 hours (Figure 3). 6 hour and 48 hour duration were both unfavorable conditions because 6 hour PA exposure was insufficient to increase IL1β and IL-6 expression and 48 hour PA exposure caused too much cell death. Thus, 200 µM PA treatment for 24 hours was chosen for investigating the effects of PA on the mRNA expression of IL-1β, IL-6, and TNFα. (Figure 2 and 3).

The possible involvement of PKC, PI3K, GPR40 and MAPK in PA-induced expression of cytokines was examined by using pharmacological inhibitors. Pharmacological inhibition of PKC with Go6983 significantly suppressed PA-induced IL-6 and TNF-α mRNA expression but did not affect PA-induced IL-1β expression (Figure 4), suggesting that PKC may be involved in PA-induced inflammation. Studies have reported that 16:0-DAG can activate PKCs and therefore stimulate NF-κB activation resulting in pro-inflammatory cytokine production (Korbecki 2019). Due to the range of PKCs expressed in microglial cells, however, a broad-spectrum PKC inhibitor - Go6983 might not be capable of identifying a specific PKC group responsible for PA induced inflammation. Basal level gene expression of IL-6 and TNF-α was attenuated significantly by Go6983 when treated with BSA (Figure 4B and 4C), suggesting PKC might inhibit the basal level of NFκB activation. Future investigation should focus on finding a specific PKC inhibitor for a specific PKC group to examine how that PKC isoform would affect PA induced inflammation in BV2 cells.

Pharmacological inhibition of PI3K with Wortmannin and LY294002, PI3K inhibitors that irreversibly and reversibly inhibit PI3K by covalently modifying catalytic subunit and competing with ATP for the active site of catalytic subunit p110 (Sun 2014), significantly suppressed PA-induced mRNA expression of IL-6 (Figure 5C and 6C). However, both inhibitors failed to suppress PA-induced IL-1β expression (Figure 5A and 6A). The role of PI3K in inflammation has been controversial. Current study that the suppression of IL-6 expression by LY294002 and Wortmannin (Figures 5 & 6) was consistent with those studies suggesting that PI3K might positively regulate inflammation (Ojaniemi 2003 and Li 2003). However, other studies have shown contradicting results that suppression of PI3K would exacerbate

inflammation (Troutman 2012 and Schabbauer 2004). The discrepancy in the role of PI3K might be due to the usage of different pharmacological regulators and varying dosage (Troutman 2012).

Pharmacological inhibition of MEK with U0126 attenuated PA induced TNF-α and IL-6 mRNA expression but failed to reduce PA induced IL-1β expression. Moreover, U0126 significantly attenuated TNF-α and IL-6 mRNA expression and upregulated IL-1β mRNA expression in BSA-treated cells (Figure 7). The Ras/Raf/MEK/ERK pathway has been studied extensively and numerous inhibitors along the pathway have been tested both in vivo and in vitro (Yang 2019 and Liu 2017). Recent evidence has indicated that MEK/ERK pathway might also involve in neuronal death and neuro-inflammation. Inhibition of MEK/ERK had shown signs of neuroprotection in the cell models of oxidative stress (Satoh 2000), mechanical trauma (Mori 2002) and reduction in microglial activation and inflammatory cytokine production (Lu 2007). The attenuation of PA-induced TNF-α and IL-6 mRNA expression by U0126 in Figure 7 suggested MEK1/2 might be involved in PA induced inflammation.

Pharmacological inhibition of GPR40 with DC260126 and GW1100 attenuated PA induced IL-1β, TNF-α, and IL-6 expression (Figure 8 and 9). Some studies revealed that activated GPR40 could attenuate β cell dysfunction and improve insulin secretion (Verma 2014, Burant 2013, and Ryota 2019). Others have shown that GPR40 antagonists might be beneficial for the well-beings of β cells (Sun 2013, Meidute 2008, Wu 2012, and Natalicchio 2013). As for inflammation associated with obesity, GPR40 agonists alleviated inflammation in mice with a high fat diet (HFD) (Dragano 2017). However, current study revealed opposite results that the suppression of inflammatory cytokines by GPR40 antagonists might be beneficial for alleviating PA induced inflammation. Meanwhile, some studies have reported similar results that activated GPR40 could lead to phosphorylation and degradation of IκB and eventually cause activation of

NF-κB via PI3K/Akt and PKC/MAPK pathways (Mena 2016). As a result, it remains unclear what underlies different manifestations in GPR40. Further investigation is required to define the role of GPR40 in PA-induced inflammation.

It should be noted that TNF-α expression has been inconsistent throughout the study. Based on the time-course studies shown in Figure 2, PA induced TNF- α expression in a time-dependent manner such that 6 hours of PA exposure would induce the high level of TNF-α expression with lower induction at 24 hours. Future experiments related to TNF-α expression should focus more time points including at 6 hours of treatment. Since TNF-α can be activate various kinases like MAPK (Sabio 2014), the possibility of paracrine stimulation that early production of TNF-α at 6 hour might stimulate IL-1β and IL-6 production at 24 hour is worthwhile to test.

In summary, this study investigated the effects of palmitate on the mRNA expression of pro-inflammatory cytokines in BV2 cells. Among all inhibitors we have experimented, Wortmannin and DC260126 successfully inhibited inflammatory cytokine expression, suggesting the involvement of PI3K and GPR40 in PA-induced inflammation.

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