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Palmitic acid inhibits inflammatory responses in lipopolysaccharide-stimulated mouse peritoneal macrophages

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SUMMARY

Palmitic acid (PA) is one of free fatty acids, which is found from *Gaultheria itoana Hayata* and *Sarcopyramis nepalensis.* Although PA has a variety of pharmacological effects including mediates hypothalamic insulin resistance, induces IP-10 expression, and promote apoptotic activities, the anti-inflammatory mechanism of PA in mouse peritoneal macrophages remains unclear. In this study, we showed that PA exerted an anti-inflammatory action through suppression the production of tumor necrosis factor-α, interleukin-6, cyclooxygenases-2 and nitric oxide in lipopolysaccaride-stimulated mouse peritoneal macrophages. Our study suggests an important molecular mechanism of PA, which might explain its beneficial effect in the regulation of inflammatory reactions.

Key words: Palmitic acid; Tumor necrosis factor-α; Interleukin-6; Cyclooxygenases-2; Nitric oxide

INTRODUCTION

Inflammation may have a primary causative role in many cases of preterm labor. Briefly, in the presence of infection, bacteria or bacterial products may activate resident macrophages and lymphocytes. Macrophage activation is known to play an important role in the inflammatory process (Medzhitov and Janeway, 1997; Beutler, 2000) and produce potent proinflammatory cytokines such as tumor necrosis factor (TNF)-α, and interleukin (IL)-6 which induce inflammation and recruit other immune cells, e.g., neutrophils and T lymphocytes (Medzhitov and Janeway, 1997). IL-6 is a cytokine produced by a number of cell types such as macrophages, dendritic cells and is major players in acute-phase and immune responses of the organism. Macrophages are the major cellular source for TNF-α. TNF-α regulates systemic responses to microbial infection or tissue injury and plays a prominent role in inflammatory reactions. Although TNF-α and IL-6 are beneficial to the host defense, they can also trigger pathological conditions when expressed in excess (Beutler, 1995). For example, massive stimulation of macrophages after a severe Gram-negative bacterial infection leads to excessive production of proinflammatory cytokines and the development of fatal septic shock syndrome, and multiple organ failure (Parrillo, 1993; Beutler, 1995). In addition, higher levels of proinflammatory cytokines are also implicated in a variety of chronic inflammatory diseases including

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rheumatoid arthritis, psoriasis, and Crohn's disease (Beutler, 1995).

Cyclooxygenases (COX) produce several types of prostaglandins (PGs), which have been implicated in various physiological events including the progression of inflammation, immunomodulation and the transmission of pain (Vane and Botting, 1998a). To date, 2 types of COX enzymes have been identified, COX-1, the constitutive enzyme, which makes PGs that protect the stomach and kidney from damage, and COX-2, the inducible enzyme induced by inflammatory stimuli such as cytokines, which produces PGs that contribute to the pain and swelling of inflammation (Morita *et al*., 1995; Otta and Smith, 1995; Vane *et al*., 1998).

Nitric oxide (NO) produced by the inducible NO synthase (iNOS) isoform is an essential component of the host innate immune and inflammatory response to a variety of pathogens, such as intracellular bacteria, viruses, fungi, and parasites (Harbrecht *et al*., 1992; MacMicking *et al*.,1997; Pfeilschifter and Muhl, 1999). Nevertheless, as for other components of the host inflammatory and immune response, excessive activation of iNOS results in cardiovascular (Weinberg *et al*., 1995) and organ dysfunction (Harbrecht *et al*., 1992) in clinical (Petros *et al*., 1994) or experimental situations of inflammatory disease of both septic (Vallance and Moncada, 1993) and nonseptic (Grosjean *et al*., 1999) etiology.

Palmitic acid (PA) is a one of free fatty acids, which is found from *Gaultheria itoana Hayata* and *Sarcopyramis nepalensis* (Chen *et al*., 2009; Wang *et al*., 2009). It has been reported that PA promote apoptosis (Sparagna *et al*., 2000) and its amount in blood and mitochondria increases under some pathological conditions (Belosludtsev *et al*., 2006). It also mediates hypothalamic insulin resistance by altering protein kinase C-theta subcellular localization in rodents (Benoit *et al*., 2009). However, whether and how PA modulates the inflammatory reaction in a stimulated peritoneal macrophage is not clear. The present study was designed to investigate the PA could modulate production of cytokines (TNF-α and IL-6) and expression of COX-2 and iNOS in mouse peritoneal macrophages.

MATERIALS AND METHODS

Reagents

Dulbeccos Modified Eagles Medium (DMEM) and LPS were purchased from Sigma (St. Louis, MO). Anti-mouse TNF-α antibody (Ab), biotinylated anti-mouse TNF-α and recombinant mouse TNF-α were purchased from R&D Systems (Minneapolis, MN). Anti-mouse IL-6, biotinylated anti-mouse IL-6 and recombinant mouse IL-6 were purchased from Pharmingen (Sandiego, CA). Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, MI). The iNOS was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Peritoneal macrophage cultures

TG-elicited macrophages were harvested 3~4 days after i.p. injection of 2.5 ml TG to the mice and isolated, as reported previously (Narumi *et al*., 1990). Using 8 ml of HBSS containing 10 U/ml heparin, peritoneal lavage was performed. Then, the cells were distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 4-well tissue culture plates (2.5×10^5 cells/well) incubated for 3 h at 37°C in an atmosphere of 5% CO_2, washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

Cytokine assay and PGE_2 Assay

TNF-α and IL-6 secretion were measured by using a modified ELISA method as described previously (Dinarello *et al*, 2000). Briefly, 96 well plates were coated with 100 ml aliquots of anti-human TNF-α and IL-6 monoclonal Abs at a concentration of 1.0 g/ml in PBS (pH 7.4) respectively and incubated overnight at 4°C. After additional washes, 100 ml of cell medium or TNF-α and IL-6 standards were added and incubated at 37°C for 2 h. After the

wells were washed, biotinylated anti-human TNF-α and IL-6 (0.2 mg/ml) were added respectively and incubated at 37°C for an additional 2 h. Next, the wells were washed and then avidin-peroxidase was added and incubated for 30 min at 37°C. After the wells were then washed, ABTS substrate was added. Color development at 405 nm was then measured by using an automated microplate ELISA reader. In addition, a standard curve was generated for each assay plate by measuring the absorbance of serial dilutions of recombinant TNF-α and IL-6 at 405 nm. The PGE_2 level was quantified using immunoassay kits according to the manufacturer's protocols (Stressgen Biotechnologies, MI, USA).

Measurement of nitrite concentration

Peritoneal macrophages (3×10^5 cells/well) were pretreated with PA for 1 h, and then treated with LPS (10 mg/ml) for 48 h. To measure nitrite, 100 ml aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamidey/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloridey/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined in a plate reader. NO_2^- was determined using sodium nitrite as a standard. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

Western blot analysis

Peritoneal macrophages (5×10^6 cells/well) were stimulated with LPS (1 mg/ml). Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5% skim milk in PBS-tween-20 for 1 h at room temperature and then incubated with anti-iNOS. After washing in PBS-tween-20 three times, the blot was incubated with secondary Ab for 1 h and the Ab-specific proteins were visualized by the enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp. Newark).

Statistical statistical analysis

These results give a summary of the data from at least three experiments, and are presented as the mean ± S.E.M. Statistical evaluation of the results was performed by ANOVA using a Tukey post hoc test. *P* value < 0.05 was considered significant.

RESULTS

The effects of PA on cytokine production in mouse peritoneal macrophages

To investigate the effect of PA on TNF-α and IL-6 production from LPS -stimulated mouse peritoneal macrophages, ELISA was performed. As shown in

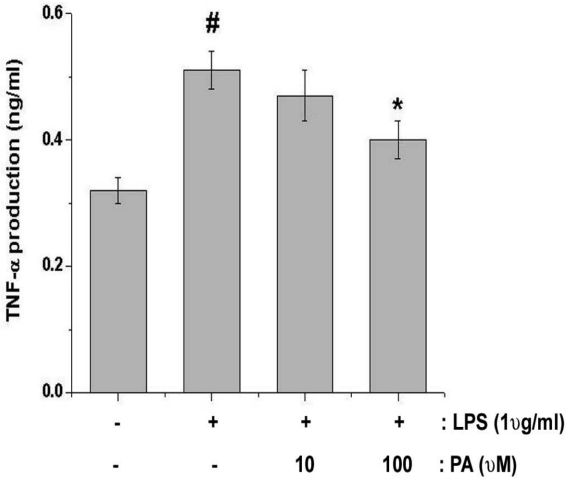

Fig. 1. Effects of PA on TNF-α production in mouse peritoneal macrophages. Cells (3×10^5) were pretreated with PA (10 - 100 mM) for 1 h and stimulated with LPS (10 mg/ml) for 24 h. TNF-α concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, significantly different from the un-stimulated cells. $^{*}P < 0.05$, significantly different from the LPS-stimulated cells (non-treated with PA).

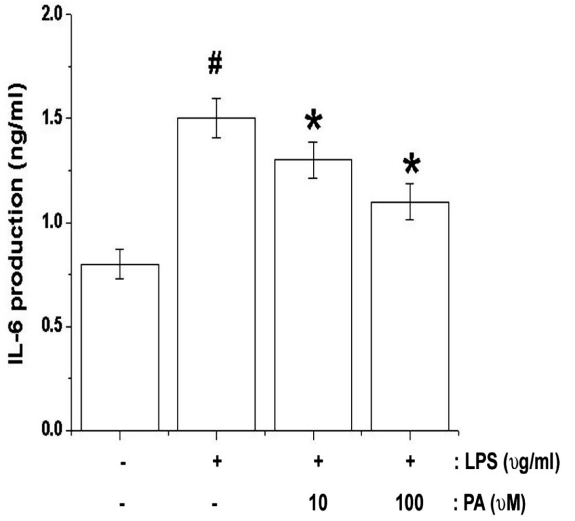

Fig. 2. Effects of PA on IL-6 production in mouse peritoneal macrophages. Cells (3×10^5) were pretreated with PA (10 - 100 mM) for 1 h and stimulated with LPS (10 mg/ml) for 24 h. IL-6 concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, significantly different from the un-stimulated cells. $^{*}P < 0.05$, significantly different from the LPS-stimulated cells (non-treated with PA).

Figs. 1 and 2, TNF-α and IL-6 production in response to LPS was inhibited by pre-treatment with PA in a dose-dependent manner. The maximal inhibition rate of TNF-α and IL-6 production by PA (100 mM) was 30.11 ± 2.8%, 35.31 ± 2.1%, respectively.

The effects of PA on PGE_2 production and COX-2 expression

We investigate the effect of PA on PGE_2 production in LPS-stimulated mouse peritoneal macrophages. As shown in Fig. 3A, PGE_2 production was enhanced in response to LPS. However, this increase was significantly inhibited by pretreatment with 10 - 100 mM of PA. The maximal inhibition rate of PGE_2 production by PA (100 mM) was 42.31 ± 3.4% ($P < 0.05$).

PGE_2 was regulated by COX-2, therefore, we evaluated if PA exerted an effect on COX-2 expression. LPS enhanced the level of COX-2 expression when compared to the unstimulated

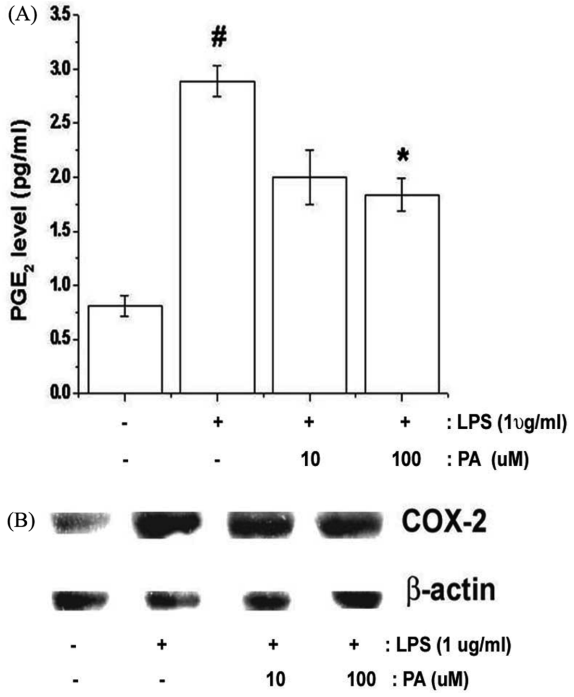

Fig. 3. Effects of PA on PGE_2 production and COX-2 expression in mouse peritoneal macrophages. (A) Cells (3×10^5 cells/ml) were pretreated with PA (10-100 mM) for 1 h, and then stimulated with LPS (1 mg/ml) for 24 h. The amount of PGE_2 production was then measured by using immunoassay kits. (B) Cells (5×10^6 cells/ml) were pretreated with PA (10 - 100 mM) for 1 h, and then stimulated with LPS (1 mg/ml) for 24 h. The protein extracts were then assayed by Western blot analysis for COX-2. All data represent the mean ± S.E.M. of 3 independent experiments. $^{\#}P < 0.05$, significantly different from the unstimulated cells. $P < 0.05$, significantly different from the LPS-stimulated cells.

cells. However, PA inhibited the increased level of COX-2 in a dose-dependent manner (Fig. 3B).

The effects of PA on NO production and iNOS expression in mouse peritoneal macrophages

To investigate the effect of PA on LPS-induced NO production, cells were pretreated with PA for 2 h and then treated with LPS for 48 h. The result showed that PA inhibited NO production in dose-dependent manner. The maximal inhibition rate of NO production by PA (100 μM) was 31.21 ± 3.3 (Fig. 4A).

To determine the effect of PA on LPS-induced

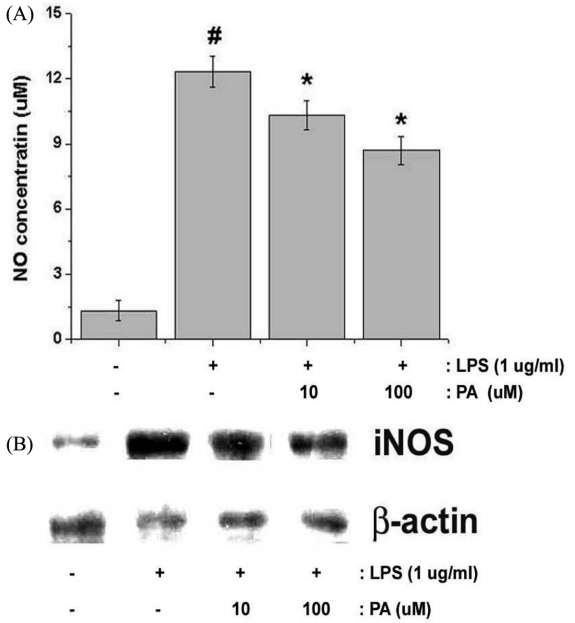

Fig. 4. Effects of PA on NO production and iNOS Expression. (A) Cells (3×10^5 cells/ml) were pretreated with PA (10 - 100 mM) for 1 h, and then treated with LPS (1 mg/ml) for 48 h. NO production in the medium was measured by the Griess reaction. The amount of NO production was quantitatively assessed using $NaNO_2$ as a standard. (B) Cells (5×10^6 cells/ml) were pretreated with PA (10 - 100 mM) for 1 h, and then stimulated with LPS (1 mg/ml) for 24 h. The protein extracts were assayed by western blot analysis for iNOS. All data represent the mean ± S.E.M. of four independent experiments. $^{\#}P < 0.05$, significantly different from the unstimulated cells. $^{*}P < 0.01$, significantly different from the LPS-stimulated cells.

iNOS expression in peritoneal macrophages, Western blot anaylsis was performed. Data in Fig. 4B, LPS caused a significant increased of iNOS expression, but PA suppressed the iNOS expression.

DISCUSSION

PA is one of free fatty acids, which is found from *Gaultheria itoana Hayata* and *Sarcopyramis nepalensis* (Chen *et al*., 2009; Wang *et al*., 2009) and has a variety of pharmacological effects. In this study, we showed that PA regulates the inflammatory response in mouse peritoneal macrophages.

Macrophage activation is known to play an important role in the inflammatory process (Janeway and Medzhitov, 1997; Beutler, 2000) and produce potent proinflammatory cytokines such as TNF-α and IL-6 which induce inflammation (Stevens *et al*., 1992) and recruit other immune cells. These cytokines are implicated in the initiation and progression of human labor and delivery. In this study, we showed that LPS can lead to the overproduction of TNF-α and IL-6, but these increases were significantly inhibited by pretreatment with PA. Therefore, these results suggest that PA has anti-inflammatory effects through regulation of inflammatory cytokine production. COX, another key enzyme in inflammation, is the rate-limiting enzyme that catalyzes the formation of PGs from arachidonic acid. Levels of PGs increase early in the course of the inflammation (Wallace *et al*., 1999). COX also exists in both constitutive (COX-1) and inducible (COX-2) forms. It is well known that the COX-1 is a housekeeping protein in most tissues and it catalyzes the synthesis of PGs for normal physiological functions. In constant, inducible isoform, COX-2, is rapidly stimulated by tumor promoters, growth factors, cytokines and pro-inflammatory molecules (Minghetti *et al*., 1998) and responsible for the production of the high levels of PGs in several pathological conditions such as inflammation. Since, COX-2 is induced by stimulation in inflammatory cells, inhibitors of COX-2 induction might candidates for the new type of nonsteroidal anti-inflammatory drugs. We observed the increased production of COX-2 protein by macrophages exposed LPS. However, LPS in combination with PA led to a significant reduction in COX-2 protein expression. Although NO played an important role in the host defense against various pathogens, the overproduction of NO can be harmful and result in septic shock, rheumatoid arthritis, and autoimmune diseases (Leiro *et al*., 2004). Therefore, therapeutic agents that inhibition of iNOS may be useful for the relieving these inflammatory conditions. In this study, PA inhibited iNOS expression in dose dependent manner. We suggest that the anti-

inflammatory action of PA may be associated with the reduction of iNOS expression.

In conclusion, these findings demonstrate that PA effectively attenuates the production of pro-inflammatory cytokines (TNF-α, IL-6) and expression of COX-2, and iNOS, and enhance our understanding of the anti-inflammatory properties of PA.

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