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Different effects of palmitic and oleic acid on LPS induced nitric oxide production and its association with intracellular lipid accumulation

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ABSTRACT

Background: Total free fatty acids (FFAs) levels were elevated in blood circulation of obese, T2DM as well as patients with cardiovascular events. Among structural differences of FFAs found in plasma, almost 60% were palmitic acid (PA) and oleic acid (OA). In previous vitro studies, PA was the most potent lipotoxin that caused apoptosis in various cells. On the other hand, OA tended to be stored as non-toxic neutral lipid droplets inside the cells. These indicated that different structures of fatty acids had different effects in cellular metabolism. Thus, this study aimed to characterize ability of palmitic acid and oleic acid in mitigating lipopolysaccharide (LPS) induced inflammation in macrophages and to investigate how lipid droplets (LD) loaded macrophages responded to LPS.

Materials and Methods: RAW 264.7 macrophages cytotoxicity of PA and OA after a two-day incubation were analyzed by MTT assay. The ability of inflammatory protection was investigated by incubating the cells with non-toxic concentration of fatty acids for 24 hr and followed by incubating the cells with 0.5 μg/mL LPS for another 24 hr. Cell supernatants were collected and nitric oxide concentrations were assayed by griess reaction. Lipid droplets formation was assessed by determining cellular triglyceride and neutral lipid oil red O staining.

Results: PA showed higher lipotoxic activity compared to oleic acid at the same concentration. OA at 200 μM mitigated LPS induced nitric oxide production in parallel with LD accumulation in macrophages, whilst PA at its non-toxic concentration (50μM) was unable to diminish inflammation and did not alter lipid accumulation.

Conclusion: Lipid loaded macrophages mediated by OA mitigated LPS induced inflammation. The association between anti-inflammation and LD formation should be further investigated.

Introduction

The underlying mechanism of metabolic disease development is complicate, but evidently involves with metabolic and inflammatory response of monocytes/macrophages, endothelial cells, muscle cells and adipocytes.[1-3] It has been suggested that free fatty acids (FFAs) could be a key linkage contributing to the cross-talk between those cells. For example, culture media taken from macrophages pre-treated with saturated fatty acid, palmitic acid, directly inhibited glucose uptake in muscle cells.[4] FFAs can be classified into 3 major groups depending on their chemical structures, unsaturated, monounsaturated and polysaturated fatty acid.[5] Total FFAs level was elevated in the blood circulation of obese, T2DM as well as patients with cardiovascular events.[6,7] Among structural different of FFAs found in plasma, palmitic acid (PA) and oleic acid (OA) represented almost 60%.[6] Accumulating data also indicated the different effects of PA and OA on cell metabolism. In vitro studies, saturated fatty acid, PA, was the most potent lipotoxin that caused apoptosis in various cells.[8-10] On the other hand, monounsaturated fatty acid, OA, tended to be stored as non-toxic neutral lipid droplets (LD) inside the cells.[10] In addition,

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there is firm belief that some fatty acids such as polyunsaturated fatty acid, oleic acid, which is abundant in Mediterranean food is health beneficial in metabolic diseases.[11] This suggested that different structures of fatty acids possessed different effects in cell metabolism.

Recent discoveries in lipid droplets biological function notified that LD was not just the simple energy storage in cells but it was a powerful factor contributing to several metabolic diseases.[12,13] For example, high fat diet induced fatty acid accumulation in liver cells, leading to steatosis. In hepatic cells culture, OA showed more steatogenic effect than PA.[14] and these was possibly because hepatocytes more tolerated under higher concentration of OA treatment than PA. In atherosclerosis, increasing LD formation in macrophages likely decelerated atherosclerotic progression since free fatty acid was toxic to both macrophages and endothelial cells. However, when persistent accumulation overwhelmed cellular triglyceride expenditure, LD could further continue tissue inflammation and fatty streak formation. According to our knowledge, how LD loaded macrophages response to its typical activator is not well defined.

Therefore, this study aimed to characterize ability of PA and OA loaded macrophage in response to LPS induced inflammation. Furthermore, this study also investigated cellular triglyceride and cholesterol after exposure of those fatty acids. As expected, our results indicated that saturated fatty acid, PA, possessed lipotoxic activity compared to monounsaturated fatty acid, OA, at the same concentration. OA also induced lipid accumulation in macrophage and mitigated LPS induced inflammation. Thus, under chronic inflammation condition, replacement of high PA with OA might help to attenuate inflammatory state. On the other hand, under healthy condition, OA replacement possibly affects the immune function.

Materials and methods

Cell Culture

The mouse macrophage cell line, RAW 264.7, was kindly provided by Assoc. Prof. Dr. Siriwan Ongchai. The cells were maintained in Dulbecco's modified Eagle's medium Dulbecco's modified Eagle's medium (DMEM) supplement with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000 units penicillin/streptomycin) at 37 °C in 5% CO_2 incubator.

Free fatty acid preparations and treatments

FFA preparations and treatments were performed using the procedures previously described with slightly modification.[15] In brief, FFAs were dissolved in 0.1% NaOH in DMSO at 55 °C to prepare the 500 mM stock concentration then the stock concentrations were dissolved in DMSO for the 50 mM working stock concentration preparation and stored at -20 °C.

The 50 mM stock concentration were added to 10% FBS DMEM culture media to prepare the final FFA treatment concentrations at 50 and 200 μM. FFAs at 50 and 200 μM concentrations were be complexed with approximately 0.25% albumin in media at the FFA/albumin molar ratio at 1.3 and 5.3 respectively.

Cytotoxicity of free fatty acids

The cells were seeded into 96-well plate at a concentration of $5x10^4$ cells/well overnight and exposed to PA and OA at various concentrations for 24 hr. After free fatty acids incubation, cell viability was tested by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Twenty μL of 5 mg/mL MTT dye was added into each well. After 4 hr incubation at 37 °C in 5% CO_2 incubator, cell supernatant was discarded and 200 μL of DMSO was added to dissolve the MTT formazan product. The absorbance was assessed at 570 nm. The cell viability was represented as percentage using 0.4% DMSO as vehicle control.

Effect of free fatty acids on LPS induced inflammation

After the cells were treated with various concentrations of free fatty acids for 24 hr, 0.5 μg/mL of LPS was added to induce inflammation for 24 hr. Culture supernatants were collected and immediately determined NO level.

Nitric oxide determination

Nitric oxide (NO) in culture supernatants were measured by using Griess reagent. One hundred μL of culture supernatants were transferred into 96-well plate. Then, the same volume of Greiss reagent was also added to the plate in order to measuring NO. Sodium nitrite at the concentrations of 0-100 μM was used as standard. The absorbance was read at 562 nm.

Effect of free fatty acids on cellular lipid droplet accumulation

Macrophages were seeded at 3.5 x 10^6 cells density in petri dishes for overnight. After that the cells were exposed to 50 μM PA and 200 μM OA for 24 hr. Then, the cells were 3 time washes with ice cold sterile PBS.

To determine cellular triglyceride content, cellular proteins were collected in ice cold PBS by using cell scrapper. Collected proteins were sonicated at 40 °C, 100% amplitude, for 30 second and kept in -20 °C until use. Ten μL of sonicated cellular protein in PBS was added into 96-well plate. Intracellular triglyceride was quantified by enzymatic assay according to the manufacturer instruction. The absorbance was assessed at 550 nm. Cellular proteins were also quantified (Pierce™ BCA protein assay kit.)

For quantitative neutral lipid content in macrophages, the cells were fixed with 10% formalin for 30 min, after that the cells were washed 2 times with sterile water. Four mL of 60% Isopropanol were added into the petri dish and incubated for 1 min, then discarded Isopropanol. Three mL of 300 mg oil red O in 60% Isopropanol were added and incubated for 30 min. Then, the cells were washed with sterile water for 6 times. One mL of absolute isopropanol was added to dissolve the lipid staining. The absorbance was assessed at 520 nm.

Statistical analysis

Data are shown as mean±SD of 3 independent experiments. The data set was tested for normality by Shapiro-Wilk test and then differences between groups were assessed by one way ANOVA. $p<0.05$ was taken as significance.

Results

Palmitic acid was more toxic than oleic acid to Macrophages RAW 264.7

Mouse macrophages, RAW264.7, were exposed to increasing concentration of PA and OA for 24 hr. As expected, PA showed the higher toxicity compared to OA at the same concentration with IC50 of 293±9.6 μM, whereas, IC_{50} of OA was more than 400 μM (Figure 1). Non-cytotoxic concentrations of each FAs, PA at 50 μM and OA at 200 μM, which caused the cell survival more than 80%, were used in all the next experiments.

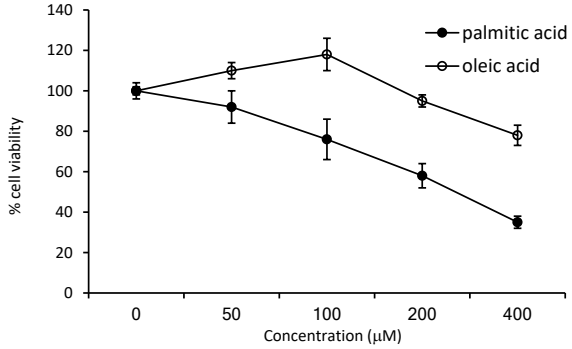

Figure 1. *Palmitic (PA) and oleic acid (OA) lipotoxicity. RAW 246.7 macrophages were incubated with 0-400 μM PA (close circle) or OA (open circle) for 48 hr. Cell viabilities were determined by MTT assay. The toxicity study was performed in triplicates from three independent experiments. Data are average of percentage of cell viability ±SD.*

Oleic acid induced neutral lipid accumulation

PA at the concentration of 50 μM did not alter intracellular lipid storage when determined by quantitative oil red O staining (Figure 2A) as well as TG assay (Figure 2B). On the other hand, the cells incubated with OA at the concentration of 200 μM increased the absorbance of neutral lipid staining approximately 25% compared to vehicle control (Figure 2C) and the increased of TG accumulation in the cells was also observed (control =573.5±96.7, OA =866.7±44.6 μg TG/mg protein) (Figure 2D). There were no interfering effects of reducing property of both PA and OA on TG assay when each of the treatment media containing FAs were directly incubated with TG reagent (OD of control =0.086±0.003 , PA 0.087±0.002 = and OA =0.093±0.002)

Oleic acid mitigated LPS induced NO production

To investigate whether different structures of FAs effect the LPS induced NO production, the cells were incubated with PA and OA for 24 hr, then followed by exposed the cells with 0.5 μg/mL LPS. Incubation the cells with PA and OA alone did not increase NO production compared to the control, suggesting that both PA and OA at their non-toxic concentrations did not induce inflammation.

When the cells exposed to PA for 24 hr and then further co-incubated with LPS for the next 24 hr, there was no difference in NO concentration compared to the cells that exposed to LPS alone (Figure 3A). The cells pre-incubated with OA for 24 hr before being co-exposed with LPS showed the reduction of NO level compared to LPS control group (Figure 3B), suggesting that the increasing LD in macrophage had ability to mitigate inflammation.

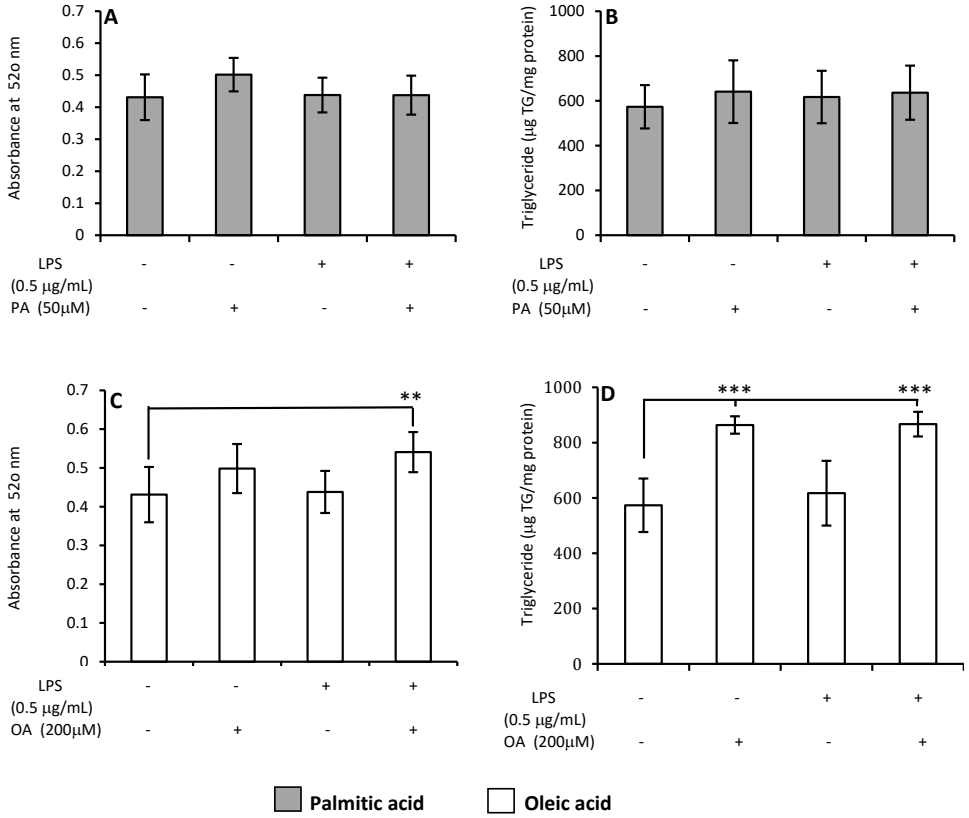

Figure 2. *Effects of Palmitic acid (PA, A and B) and Oleic acid (OA, C and D) on intracellular lipid contents. Macrophages, RAW 264.7, were treated with 50 μM PA and 200 μM OA for 24 hr. Intracellular lipid contents were assessed by quantitative Oil Red O staining (A and C) and Triglyceride accumulation (B and D). Cellular protein levels were also determined and there was no significantly different between each experimental condition. The difference of lipid contents were assessed by one way ANOVA test (** p<0.01; *** p<0.001).*

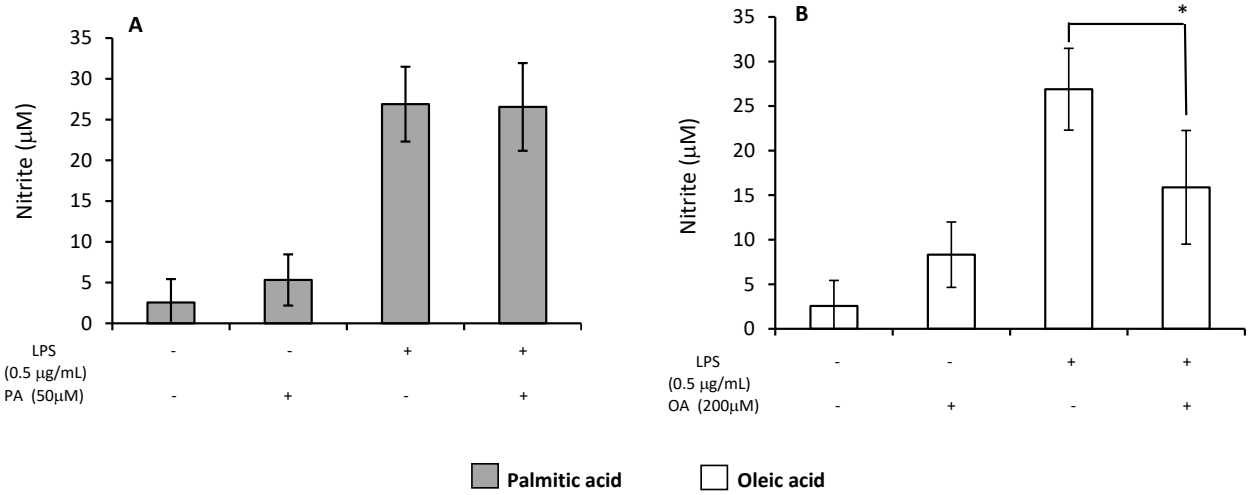

Figure 3. *Effects of Palmitic acid (PA, A) and Oleic acid (OA, B) on LPS induced NO production. Macrophages, RAW 264.7, were treated with 50 μM PA and 200 μM OA for 24 hr. Then the cells were exposed to 0.5 μg/mL of LPS for another 24 hr. No levels in cell supernatants were immediately measured. The difference of NO were assessed by one way ANOVA test (* p<0.05).*

Discussion

Replacing dietary oil containing high ratio of saturated fatty acid with unsaturated fatty acid in order to cardiovascular disease prevention are widely recommended by health care professional.[16] However, numerous data reported the contradict effects of long term consumption of PUFAs. Some studies showed that high PUFAs diets decreased risk of metabolic dysregulation, on the other hand, some studies showed the disagreement results.[17] These controversy effects could be explained by the study designs which the majority of articles reported about the association between SAFs, MUFAs, PUFAs and metabolic abnormalities were long term observational cohort studies which many confounding factors, such as total energy controlling, were difficult to manage. Thus, studies of the individual fatty acids on cellular activities were important in order to providing more information regarding their real effects. Our study focused on the effect of PA and OA which are the main fatty acids found in macrophages in diabetes patients.[18]

There are accumulating data affirmed that PA was lipotoxic in various cells.[19-21] Our results also reaffirmed that PA (C16:0) caused cell death in mouse macrophages, whilst monounsaturated fatty acid, OA (C18:1) showed less toxicity.

Previous studies also indicated that combination treatment of PA with OA prevented the hepatocyte.[19] and embryonic fibroblasts from apoptosis, suggesting the protective role of OA in saturated fatty acids induced lipotoxicity.[18,22] Interestingly, those effects seemed to associate with LD formation. Our study observed that cells treated with PA alone did not alter intracellular triglyceride content, whereas OA increased intracellular triglyceride at their non-toxic concentration. Listenberger LL and his colleagues showed that PA at its apoptotic induced concentration (700 μM) did not change triglyceride content in Chinese hamster ovary cells. They suggested that PA dominantly underwent the generation of reactive oxygen species and ceramide which consequently activated the caspase activation rather than triglyceride synthesis.[21] Unlike PA, OA activated PPARα as well as diacylglycerol acyltransferase (DGAT) activity which accelerated triglyceride production in cells.[21,23] However, recent study reported that knockdown DGAT neither enhanced PA induced apoptosis nor inhibited the protective role of OA against PA.[24] Thus, mechanism of OA involved in lipotoxic prevention is still unclear.

Apart from the association between LD formation and lipotoxicity, LD induction also affected several cellular metabolisms. Over-expression of DGAT in adipocytes caused induction of intracellular triglyceride accumulation in mouse adipocytes and the metabolic abnormalities such as hyperglycemia and inhibition of insulin signaling in hepatocytes were also observed.[25] Induction of Intracellular triglycerides in skeletal muscles of zinc transporter 7 knockout mice caused mitochondria enlargement and initiated mitochondria dysfunction.[26] However, according to our knowledge, how the accumulated intracellular triglyceride affected macrophage activity was unwell addressed. In our study, as expected, we found that OA induced triglyceride accumulation in macrophages at its non-toxic concentration. Thus, we further challenged the lipid loaded macrophages with its typical instigator, LPS, and the levels of nitric oxide production were observed as a marker of inflammatory response. We found that both PA and OA at their non-toxic concentration did not increase NO levels. Treatment the cells with PA for 24 hr did not induced intracellular triglyceride accumulation and when PA treated macrophage was further exposed to LPS, NO level was not altered, compared to LPS treatment in vehicle control macrophages. Whilst, the association between the inflammatory diminishing effect and induction of cellular triglyceride accumulation was observed in OA treatment. Cellular triglyceride levels were increased when macrophages were incubated with OA. Challenging the high intracellular triglyceride macrophages induced by OA with LPS showed the reduction of NO in culture media, indicating the anti-inflammatory activity of OA.

Recent study showed the anti-inflammatory effects of OA in adipocytes derived from human subjects by lowering TNFα and IL-6 in non-obese subjects compared with the

cells taken from obese subjects. It also showed the induction of adiponectin after 24 hr OA treatment.[27] In macrophages, there was an experiment which macrophages, J774 cell line, were both incubated with pre-and post LPS exposure. They found that the combination treatment of OA and PA at the FFA/albumin ratio of 2:1 after LPS exposure worsened the inflammatory state by increasing TNFα. Interestingly, the 5 days combination treatment prior to LPS exposure reduced TNFα mRNA level compared to LPS alone.[28] However, they did not mention about the lipid accumulation in the cells. Our study was conducted to study the individual effect of each free fatty acid in response to LPS. We emphasized that OA had ability to mitigate LPS induced inflammation by reducing NO production. The association between anti-inflammation and LD formation should be further investigated.

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Disclosures

None

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