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Regulatory Role of Free Fatty Acids (FFAs)—Palmitoylation and Myristoylation

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ABSTRACT

Multicellular organisms use chemical messengers to transmit signals among organelles and to other cells. Relatively small hydrophobic molecules such as lipids are excellent candidates for this signaling purpose. In most proteins, palmitic acid and other saturated and some unsaturated fatty acids are esterified to the free thiol of cysteines and to the N-amide terminal. This palmitoylation process enhances the surface hydrophobicity and membrane affinity of protein substrates and plays important roles in modulating proteins' trafficking, stability, and sorting etc. Protein palmitoylation has been involved in numerous cellular processes, including signaling, apoptosis, and neuronal transmission. The palmitoylation process is involved in multiple diseases such as Huntington's disease, various cardiovascular and T-cell mediated immune disorders, as well as cancer. Protein palmitoylation through the thioester (S-acylation) is unique in that it is the only reversible lipid modification. Our study on lipopolysaccharide (LPS) and deoxynivalenol (DON) treatment to rats provides some insights to the complex role of protein palmitoylation in chemical and microbial toxicity. In contrast, myrisoylated proteins contain the 14-carbon fatty acid myristate attached via amide linkage to the N-terminal glycine residue of protein, and occur cotranslationally. The bacterial outer membrane enzyme lipid A palmitoyltransferase (PagP) confers resistance to host immune defenses by transferring a palmitate chain from a phospholipid to the lipid A component of LPS. PagP is sensitive to cationic antimicrobial peptides (CAMP) which are included among the products of the Toll-like receptor 4 (TLR4) signal transduction pathway. This modification of lipid A with a palmitate appears to both and protects the pathogenic bacteria from host immune defenses and attenuates the activation of those same defenses through the TLR4 signal transduction pathway.

Keywords: Free Fatty Acids; Palmitoylation; Myristoylation; Microbial and Chemical Toxicity; Innate Immune Response

1. Introduction

Saturated FFAs play important roles for various biological functions such as the production of hormones, cellular membrane signaling, and the stabilization processes in the body. These are the 18-carbon stearic acid, the 16-carbon palmitic acid, and the 14-carbon myristic acid. When these important saturated fatty acids are not readily available, certain growth factors in the cells and organs will not be properly aligned. This is because the various receptors, such as G-protein receptors, need to be coupled with lipids in order to provide localization of function [1,2]. A biochemical process called palmitoylation, in which the body uses palmitic acid in stabilization processes, is very important to the body by regulating G protein-coupled receptor signaling [3,4]. Myristic acid, also called tetradecanoic acid, is a saturated fatty acid. It is commonly added co-transitionally to the nitrogen terminus of glycine in receptor-associated kinases to confer the membrane localization of the enzyme. Myristic acid has a sufficiently high hydrophobicity to become incurporated into the fatty acyl core of the phospholipid bilayer of the plasma membrane. In this way, myristic acid acts as a lipid anchor in biomembranes [5]. Myristic acid is known to be a very important fatty acid which the body uses to stabilize many different proteins, including proteins that are used in the immune system and also those that fight tumors [6]. This function is called myristoylation; it occurs when myristic acid is attached to the protein in a specific position where it functions usefully

[7-10]. For example, the body has the ability to suppress production of tumors from lung cancer cells if a certain genetically determined suppressor gene is available. This gene codes for Fus-1 which is a protein that has been modified with covalent addition of the saturated fatty acid myristic acid [6]. Myristoleic acid is an omega-5-fatty acid that is biosynthesized from myristic acid by the enzyme delta-9-desaturase. This uncommon (n-9) fatty acid has been described in the retina; acylating an NH_2 terminus of a protein related to signal transduction in photoreceptors [11]. Furthermore, the presence of this fatty acid was shown to be of diagnostic value in patients with defects of long-chain fatty acid oxidation [12], and is known to be cytotoxic to tumor cells. It induces apoptosis and necrosis in human prostate cancer LNCaP cells [13].

Multicellular organisms use chemical messengers to transmit signals among organelles and to other cells. Relatively small hydrophobic molecules such as lipids are excellent candidates for this signaling purpose. The two most common modifications, myristoylation and palmitoylation, differ with respect to the type and chemical nature of fatty acid attachment to the polypeptide backbone.

1.1. Myristoylation

After the initiating methionine is removed, the 14-carbon fatty acid myristate is attached via amide linkage to the N-terminal glycine residue. The reaction occurs cotranslationally and is catalyzed by the soluble enzyme N-myristoyl transferase (NMT). NMT exhibits strict specificity for an N-terminal glycine and mutation of this glycine to alanme abrogates myristoylation [14].

FFAs are known to be markers of cellular membrane degradation through lipid peroxidation and are substrates for the production of reactive oxygen species (ROS) [15]. Since the generation of ROS during the metabolism of arsenic is thought to be involved in arsenic toxicosis, understanding the deleterious effects caused by ROS that attack the vital molecules like DNA, has become important [16].

Ross *et al.* [17] have investigated the FFAs profile from the rats treated with sodium arsenite (NaAs O_2), a single oral dose, in water, and in a lipid medium, to pregnant rats on gestational day (GD) 10, a time point at mid-organogenesis. $NaAsO_2$ was administered in deionized water (AsH_2O) or in half and half dairy cream (AsHH) at a dose of 41 mg $NaAsO_2$/kg body weight. Control animals were treated with either dairy cream (HH) or deionized water (H_2O). The animals were sacrificed on GD 20.

The study reveals an elevation of FFAs in the maternal liver and brain, and the fetal brain. In the fetal brain, myristic and stearic acids concentrations were higher in animals treated with AsHH vs. AsH_2O (**Figure 1**). This could be due to the aggregation of NaAs with HH which could delay its metabolism and excretion as compared to NaAs in H_2O that can be readily cleared by the kidneys. The fact that NaAs crosses the blood-brain barrier as well as the placental barrier freely [18-20], the NaAs-HH complex could be retained longer in the brain as compared to NaAs and AsH_2O which are washed out of the brain easily by the efflux system of the brain [21]. This prolonged presence of AsHH in the cells probably induces the process of myristoylation and palmitoylation in the cellular signaling pathways. The increase of oleic and arachidonic acid in the liver of AsH_2O vs. H_2O group, indicates a direct effect of NaAs on these fatty acids. The depletion of myristoleic acid in the maternal brain, as indicated in **Figure 2**, could be due to the active participation of myristoylation against the toxic effect of NaAs in the maternal brain. Myristoylation is an irreversible process and normally precedes the process of palmitoylation [14]. The palmitoylation process is, in contrast, reversible and palmitic acid can be substituted by fatty acids

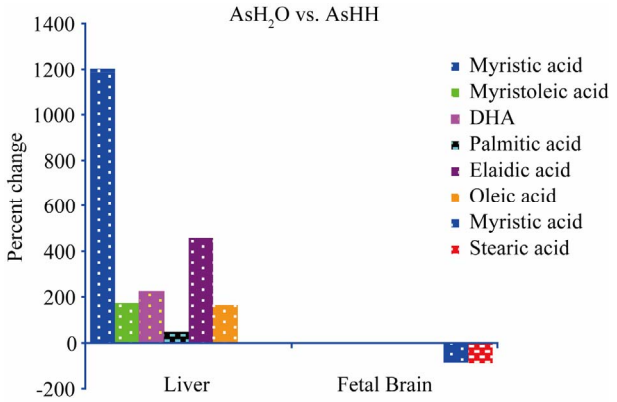


Figure 1. The formula used for the calculation of percent changes in Figure 1 is as follows: [{(AsH_2O − AsHH)/AsHH} × 100].

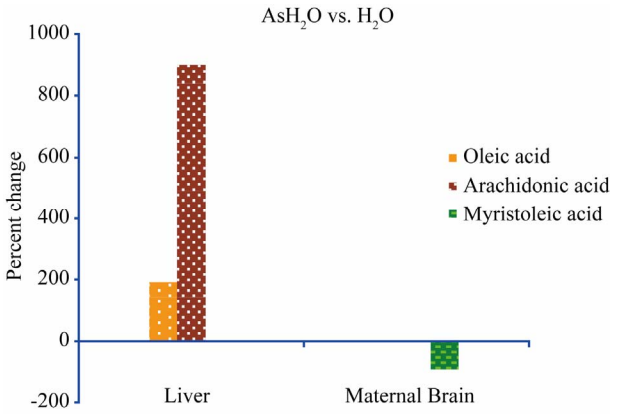


Figure 2. The formula used for the calculation of percent changes in Figure 2 is as follows: [{(AsH_2O − H_2O)/H_2O} × 100].

such as stearic, oleic, and arachidonic acids for the palmitoylation process to regulate the cellular functions [22]. As shown in the fetal brain (**Figure 3**), the enhancement of myristic, stearic, and arachidonic acids in the AsHH group could be a protective effect due to the slow clearance of the AsHH from the fetal brain. This, in turn, could be explained by differences in the sensitivity of membrane functions for signal pathways during the developmental stages of the fetal brain, as compared to the maternal brain. Taken together, findings of different levels of myristic and myristoleic acids in the maternal and fetal brains and the other organs indicate that the antitumoric activity against $NaAsO_2$ treatment is more pronounced in the brain than in the kidney and liver.

1.2. Palmitoylation

In contrast, palmitoylated proteins contain the 16-carbon fatty acid palmitate attached via throester linkage to one or more cysteme residues. Palmitoylation is a posttranslational reaction that appears to be mediated by a membrane-bound palmitoyl acyl transferase. Unlike myristoylation, whrch is generally a relatively stable modification, palmitoylatron can be reversed by the action of thioesterases.

In most proteins, palmitic acid and some other fatty acids are esterified to the free thiol of cysteines and to the N-amide terminal as shown in **Figure 4**. This palmitoylation process enhances the surface hydrophobicity and membrane affinity of protein substrates and play important roles in modulating proteins' trafficking, stability and sorting. Since this linkage between the palmitate and protein is readily cleaved, cycles of palmitoylation and depalmitoylation occur in a regulated manner for many proteins [23].

Protein palmitoylation has been involved in numerous cellular processes, including signaling, apoptosis, and neuronal transmission [24]. The palmitoylation process is also involved in multiple diseases such as Huntington's disease, various cardiovascular and T-cell mediated immune disorders, as well as cancer. Protein palmitoylation through the thioester (S-acylation) is unique in that it is the only reversible lipid modification.

- Palmitoylation is involved in the process of protein trafficking between organelles and in the segregation or clustering of proteins in membrane compartments [25-27].
- Palmitoylation increases the hydrophobicity of proteins to promote protein-membrane association [28, 29].
- Modification of proteins to control protein-protein interaction [30-32], lipid raft targeting [33] and intracellular trafficking [34,35].
- The palmitoylation process is involved in multiple diseases such as Huntington's disease, various cardiovascular and T-cell mediated immune disorders, as well as cancer [36].

1.3. The Role of Palmitoylation in Microbial and Chemical Toxicity: Signal Pathways, Protein Binding and Trafficking

Lipopolysaccharide (LPS)

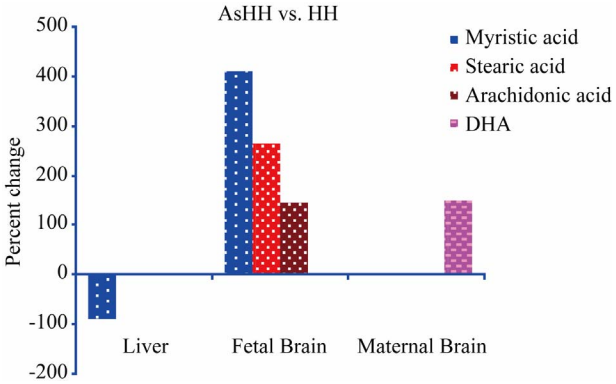


Figure 3. The formula used for the calculation of percent changes in Figure 3 is as follows: [{(AsHH − HH)/HH} × 100].

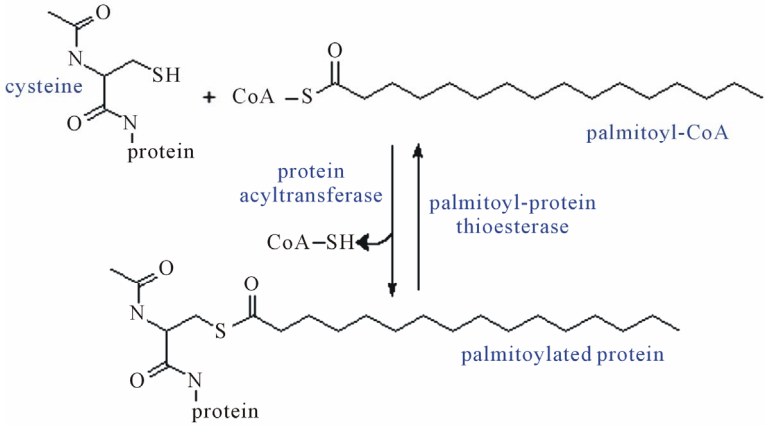


Figure 4. Protein S-palmitoylation—the thioester linkage of long-chain fatty acids to cysteine residues in proteins.

An essential virulence property of bacterial pathogens is the ability to sense the environment within the host tissues and to coordinate the expression of the virulence factors that promote the bacterial survival and replication strategies.

- The infected host also senses the presence of invading bacteria and responds by activation of the innate immune system. In Gram-negative bacteria, Lipid A is the region of LPS (**Figure 5**), also known as endotoxin, which is responsible for the immunostimulatory activity of LPS.
- The inflammatory response is necessary to eliminate most infections and, at the same time, responsible for some of the main pathophysiological symptoms associated with persistent infections. Bacterial pathogens can coexist with their hosts in part because they modify the structure of lipid A to attenuate the inflammatory response and evade immune recognition (**Figure 6**) [37].
- Palmitoylated lipid A can both protect pathogenic bacteria from host immune defenses and attenuate the activation of those same defenses through the Toll-like receptor 4 (TLR4) signal transduction pathway (**Figure 7**).

Deoxynivalenol (DON)

Several DON contamination episodes have caused the FDA to establish regulatory measures needed to control DON in foods [38].

- Exposure to sublethal levels of trichothecenes can stimulate or suppress immune parameters such as lymphocyte proliferation, host resistance, cell-mediated immunity, and humoral immune function in a variety of animal and cell culture models depending on dose, exposure frequency, and timing of exposure [39].
- LPS-induced nitric oxide (NO) production by RAW264 cells was dose-dependently inhibited by DON [40].

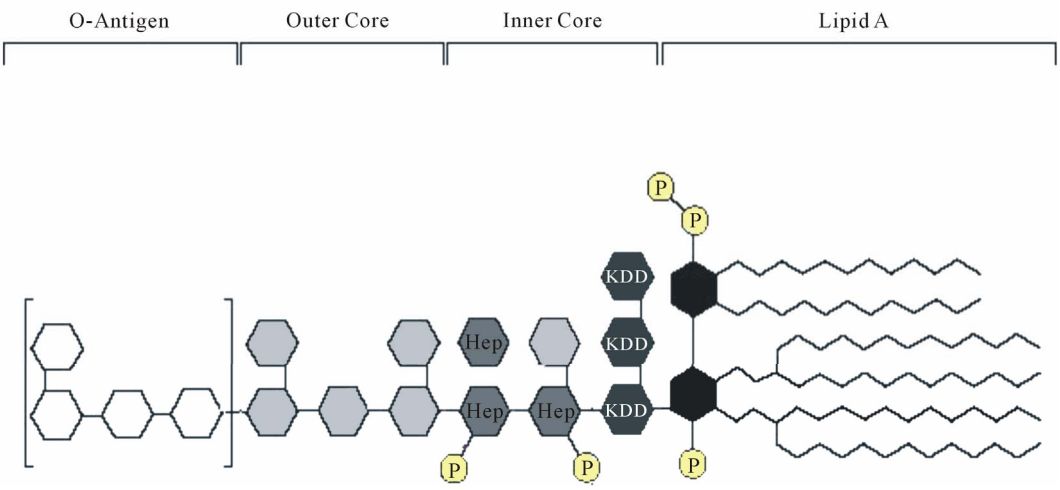


Figure 5. Lipopolysaccharide—the main component of the outer leaflet of the bacteria outer membrane is the immunodominant antigen of most Gram-negative pathogen.

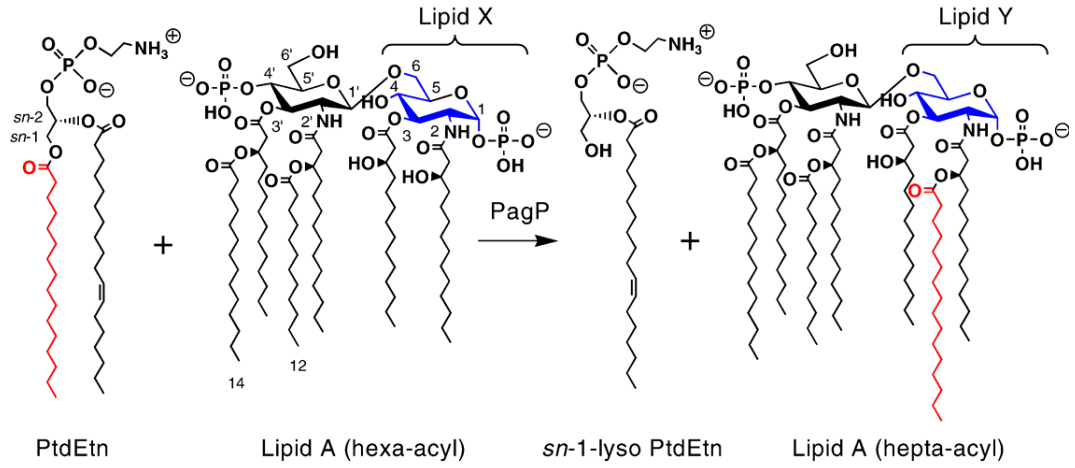


Figure 6. A palmitate chain from a phospholipid is incorporated into lipid A by an outer membrane enzyme PagP.

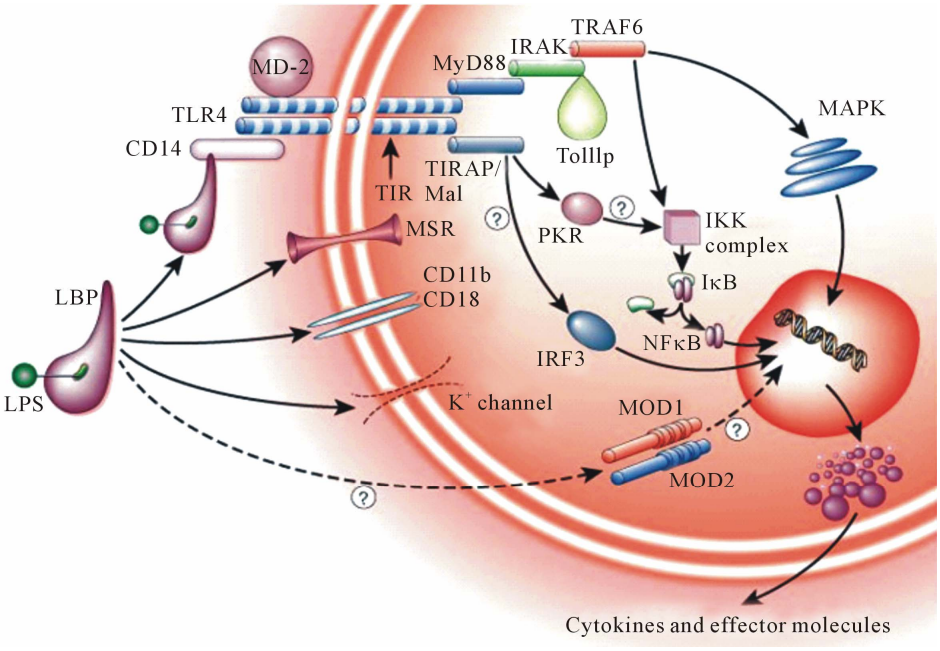


Figure 7. A group of proteins that comprise the TLR4 family of receptors detects invading pathogen and mount a rapid defensive response.

- DON has also suppressed LPS-induced inducible NO synthase (iNOS) promoter activity and the expression of iNOS protein in a similar concentration range to that of the inhibitory effect on NO production [41].
- DON induced IgA nephropathy in mice by upregulating IL-6 expression, which is suppressed by the consumption of some of the polyunsaturated fatty acids [42].

To examine the role of palmitoylation in animals treated with lipopolysaccharide and deoxynivalenol individually and concurrently at doses that alone would not cause overt toxicity, the following study has been carried out and presented at the Society of Toxicology Annual Meetings [43,44].

2. Methods

Male Harlan Hsd:Sprague-Dawley rats (virus and antibody-free), 9 weeks of age, were acclimated to their environment for approximately one week. The animals were housed individually in polycarbonate cages in a temperature controlled environment (24˚C - 26˚C), and a relative humidity of 40% - 70%. A light-dark cycle was maintained with lights on at 7:00 AM and off at 7:00 PM. After a period of acclimation, the rats were randomly assigned to the different treatment groups of eight animals each. The animals were fed ground Purina rodent chow 5002 (Purina Mills, Inc., Richmond, IN) and water from an in-house water system ad libitum throughout the study. The rats were divided into 4 groups. Group 1 was administered an IP dose of 10 mg DON/kg; group 2: 83 µg LPS/kg; group 3: 10 mg DON and 83 µg LPS/kg; group 4: 1 mL saline/kg. Animals were sacrificed at 3, 24, and 72 hr after dosing. The liver and brain were immediately harvested and frozen in liquid nitrogen. The specimens were then stored at −80˚C until the fatty acids were extracted. Extraction of free fatty acids and analysis by gas chromatography were described in Ross *et al.* [17].

3. Statistical Analysis

All response parameters were tested using the Shapiro-Wilk Test to determine whether the response parameter follows a normal distribution. This information was used to determine whether a parametric or non-parametric analysis was used for subsequent analysis. Time differences for each level of DON and LPS (that is, presence or absence) were tested by a one-way Analysis of Variance (ANOVA). If the ANOVA was statistically significant ($p < 0.05$), a protested LSD t-test was used for pairwise comparison of 3, 24 and 72 hours. For each time period and level of DON, a t-test was used to compare presence and absence of LPS. For each time period and level of LPS, a t-test was used to compare presence and absence of DON. Time differences for each level of DON and LPS were analyzed by the nonparametric Kruskal-Wallis test. If this test was significant ($p < 0.05$), the Mann-Whitney test was used for pair wise comparison of 3, 24 and 72 hours. For each time period and level of DON, the Mann-Whitney test was used to compare presence and absence of LPS. For each time period and level of LPS, the Mann-Whitney test was used to com-

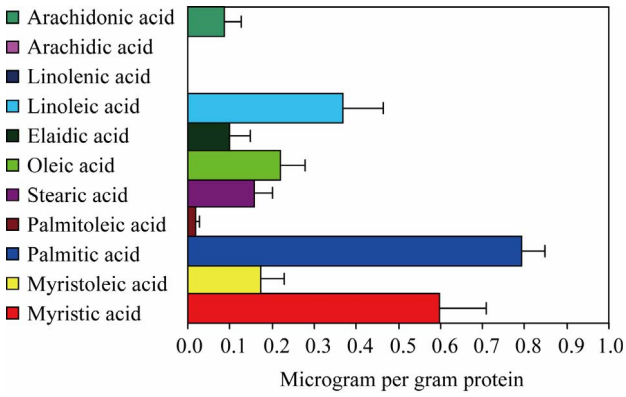
pare presence and absence of DON.

4. Results

The individual and concurrent administration of LPS and DON had no effect on the fatty acids of the brain and the glutathione levels of the brain and liver (data not shown). In the liver of animals treated with DON, there was a significant decrease in palmitic acid at 24 hours (**Figure 8(b)**) compared to 3 hr (**Figure 8(a)**) and an increase at 72 hr (**Figure 8(c)**) compared to 24 hr (**Figure 8(b)**) after dosing. This profile is an indication of palmitic acid utilization between 3 and 24 hours and its restoration by 72 hr, when the mild toxic effect was neutralized. In the liver of animals treated with LPS, elaidic acid was significantly decreased at 24 hr (**Figure 8(e)**) and at 72 hr **Figure 8(f)**) when compared to 3 hr (**Figure 8(d)**). Stearic acid was significantly decreased at 72 hr (**Figure 8(f)**) compared to 3 hr (**Figure 8(d)**) after dosing. The minimally toxic doses of LPS and DON concurrent administration produced no changes in the fatty acids of the liver (**Figures 8(g)-(i)**).

5. Discussion

The administration of LPS (83 μg/kg BW) activated the innate immune response of the Sprague-Dawley rat and induced a classical but reversible (48 - 72 hr) sickness syndrome response with clear evidence of inflammation [45]. Liver histopathology of this study revealed early mild hepatotoxicity following IP dose of 10 mg/kg DON [46]. The fatty acid profile of this study indicates utilization of palmitic acid in the liver of animals treated with DON (**Figure 8(b)**) and the utilization of stearic and elaidic acids in the liver of animals treated with LPS (**Figure 8(f)**). This is indication of the involvement of these fatty acids in the palmitoylation process. The decrease in palmitic acid at 24 hr with DON treatment is indication of the utilization of palmitic acid by the early exposure of the cells to the toxin. After this mild toxic effect was counteracted, the level of palmitic acid was restored. The decrease of stearic (C18:0) and elaidic acids (C18:1 t) in the liver of animals treated with LPS could be due to substitution of these fatty acids for palmitic acid. LPS administration intraperitoneally induced hepatic vascular cell adhesion molecule (VCAM) mRNA as early as 0.5 hour after dosing [47]. Therefore, it is possible that palmitic acid (C16:0) was depleted and restored before our first sampling time of 3 hour after dosing, and by this time stearic and elaidic acids were substituted for palmitic acid. There were no changes in the free fatty acids of animals treated with LPS and DON concurrently. This lack of fatty acid activity may be due to the competition of DON and LPS for binding sites, and also to the combination of activities induced by DON



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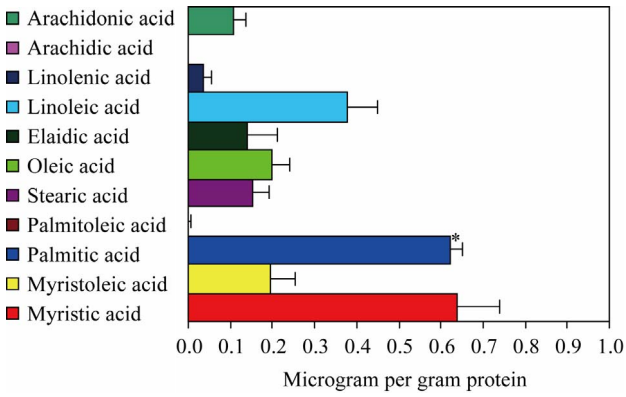

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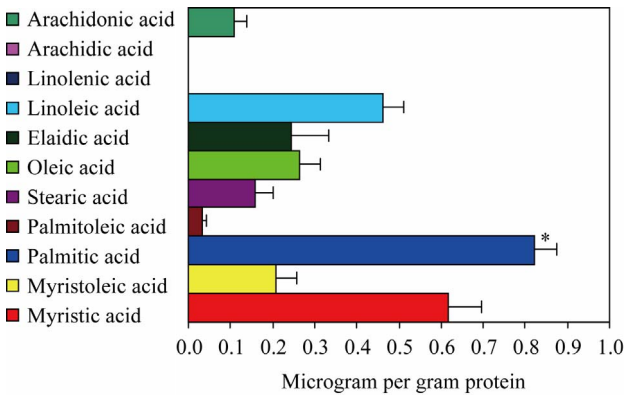

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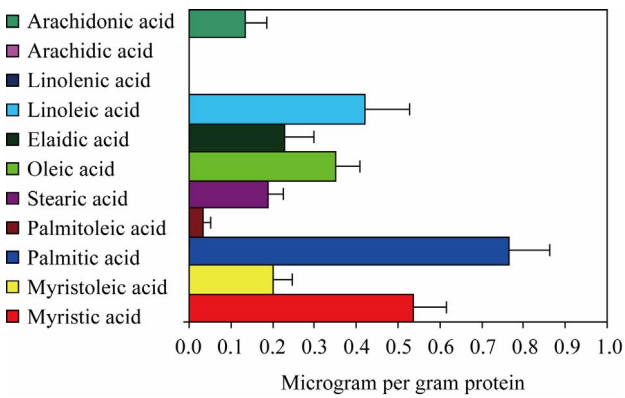

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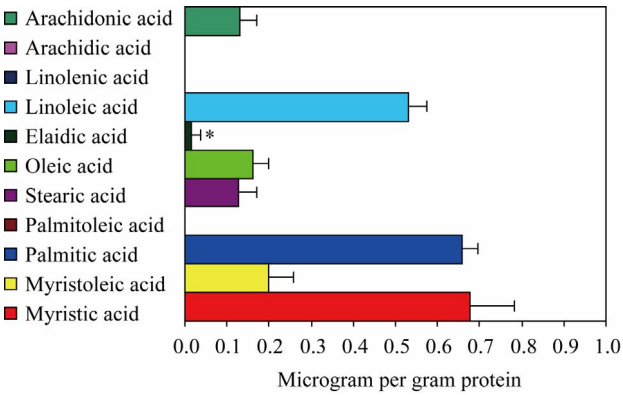

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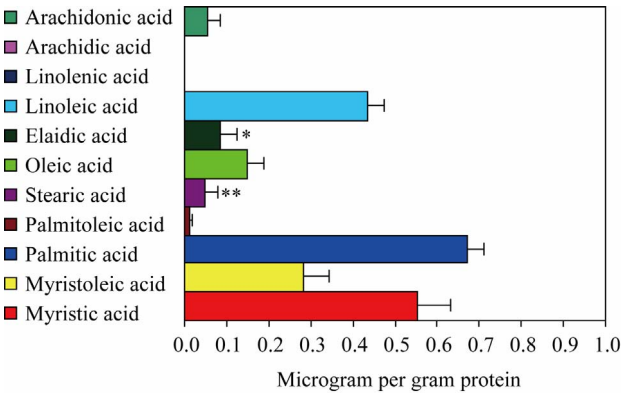

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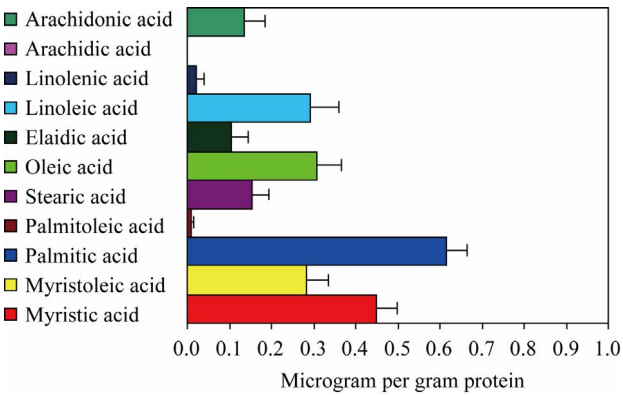

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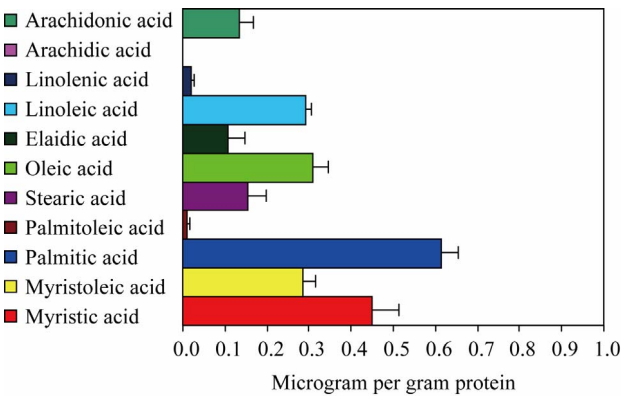

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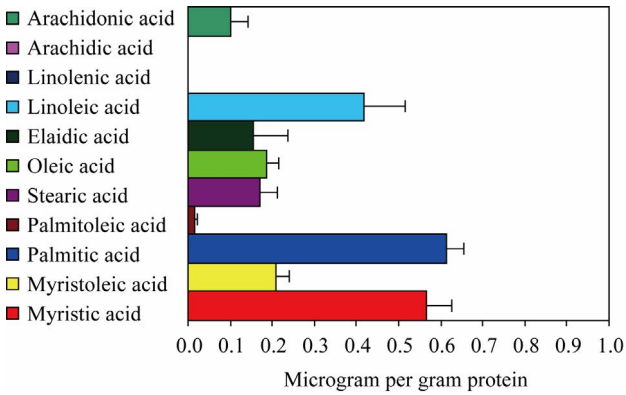

(i)

Figure 8. (a) The effect of DON on the fatty acids of the liver 3 hours after dosing. Values are means ± SEM of 8 animals; (b) Effect of DON on fatty acids of the liver 24 hours after dosing. Values are means ± SEM of 8 animals. *Palmitic acid significantly decreased from 3 after dosing ($p < 0.02$); (c) Effect of DON on fatty acids of the liver 72 hours after dosing. Values are means ± SEM of 7 animals. *Palmitic acid significantly increased from 24 hours after dosing ($p < 0.01$); (d) Effect of LPS on fatty acids of the liver 3 hours after dosing. Values are means ± SEM of 8 animals; (e) Effect of LPS on fatty acid of the liver 24 hours after dosing. Values are means ± SEM of 8 animals. *Elaidic acid significantly decreased from 3 hours after dosing ($p < 0.01$); (f) Effect of LPS on fatty acids of the liver 72 hours after dosing. Values are means ± SEM of 7 animals. *Elaidic acid significantly decreased from 3 hours after dosing ($p < 0.01$). **Stearic acid significantly decreased from 3 hours after dosing ($p < 0.03$); (g) Effect of LPS and DON concurrent administration on fatty acids of the liver 3 hours after dosing. Values are means SEM of 8 animals; (h) Effect of LPS and DON concurrent administration on fatty acids of the liver 24 hours after dosing. Values are means SEM of 7 animals; (i) Effect of LPS and DON concurrent administration on fatty acids of the liver 72 hours after dosing. Values are means SEM of 8 animals.

and LPS. For example, the LPS binding protein CD14 recognizes LPS and aids in the loading of LPS onto the LPS receptor complex [48]. It has been observed by Wache *et al.*, 2009 [49] that DON decreased the cell surface expression of CD14 in a dose-dependent manner. Also, macrophages stimulated by LPS produce large amounts of NO [50] and DON has been shown to suppressed LPS-induced NO production by the mouse macrophage cell line, RAW264, in a concentration-dependent manner. Significant inhibitory effect was also produced at a concentration as low as 500 ng/mL. DON also suppressed LPS-induced iNOS promoter activity and the expression of iNOS protein in a similar concentration range to that of the inhibition effect of NO production [41]. NO production by iNOS contributes to host defence and pathophysiological changes in inflammation, including sepsis [51]. Low concentrations of NO produced by iNOS are beneficial for the antimicrobial activ-

ity of macrophages against pathogens [52], and excessive production of NO and its derivatives can provoke pathogenesis by septic shock and autoimmune disorders [53]. Palmitoylation of iNOS is necessary for the intracellular transit towards subcellular domains where NO synthesis is required [36]. NO production by palmitoyl-specific radioprotective domain (P-SRD) has been shown to inhibit the inflammatory responses caused by endotoxin [40] and the inhibition was probably due to the competition of the P-SRD for LPS binding site on the macrophage cells. Since iNOS is S-acylated with palmitic acid, the inhibition of LPS-induced NO production by DON has consequentially diminished the utilization of palmitic acid that is required for palmitoylation in NO synthesis.

6. Conclusion

This study provides a model to explore the involvement of the palmitoylation process in the interaction of LPS with environmental toxins, such as DON, that can adversely affect the body. The data provide insights on the role of fatty acids in bacterial pathogenesis and some understanding of the diversity of bacterial survival strategies and the important role that NO plays for host protection against invading bacteria.

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Abbreviations

FFAs, free fatty acids; LPS, lipopolysaccharide; DON, deoxynivalenol; ROS, reactive oxygen species; $NaAsO_2$, sodium arsenite; AsH_2O, $NaAsO_2$ in deionized water; AsHH, $NaAsO_2$ in half and half dairy cream; NO, nitric oxide; iNOs, inducible NO synthase; CAMP, cationic antimicrobial peptides; TLR4, Toll-like receptor 4.

