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THE EFFECT OF SATURATED AND UNSATURATED FATTY ACIDS ON HEPG2 CELLS AND THE TREHALOSE PROTECTION OF HEG2 CELLS ON PALMITATE INDUCED TOXICITY

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YIFEI WU

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THE EFFECT OF SATURATED AND UNSATURATED FATTY ACIDS ON HEPG2 CELLS AND THE TREHALOSE PROTECTION OF HEPG2 CELLS ON PALMITATE INDUCED TOXICITY

By

Yifei Wu

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ABSTRACT

THE EFFECT OF SATURATED AND UNSATURATED FATTY ACIDS ON HEPG2 CELLS AND THE TREHALOSE PROTECTION OF HEPG2 CELLS ON PALMITATE INDUCED TOXICITY

By

Yifei Wu

Understanding the mechanism of saturated fatty acid-induced hepatocyte toxicity may provide insight into cures for diseases such as obesity-associated cirrhosis. Trehalose, a nonreducing disaccharide shown to protect proteins and cellular membranes from inactivation or denaturation caused by different stress conditions, also protects hepatocytes from palmitate-induced toxicity. Our results suggest that trehalose serves as a free radical scavenger and alleviates damage from hydrogen peroxide secreted by the compromised cells. We also observe that trehalose protects HepG2 cells by interacting with the plasma membrane to counteract the changes in membrane fluidity induced by palmitate. Unsaturated fatty acids such as oleate and linoleate are not toxic to HepG2 cells and do not induce significant biophysical changes to cell membrane. The experimental results are supported by molecular dynamics simulations of model cell membranes that closely reflect the experimental conditions.

Acknowledgment

My utmost gratitude goes to my advisor, Dr. Christina Chan, for her expertise, dedication, kindness, and most of all, for her patience. This thesis would not be possible without her guidance and support. I would also like to thank our collaborators Dr. Amadeu K. Sum and Sukit Leekumjorn from Virginia Polytechnic Institute and State University for their dedicated work and substantial contribution in making the publication possible. My thanks and appreciation goes to Dr. Patrick Walton and all members of Cellular & Biomolecular Lab, for their important inputs and helpful comments in this project. I am grateful to Dr. Neil Wright in the Department of Mechanical Engineering and Dr. John L. McCracken in the Department of Chemistry, for gracefully providing the DSC and EPR measurements.

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Chapter 1

Saturated fatty acid toxicity on HepG2 cells and the trehalose protection of toxicity

Introduction

Non-esterified long-chain free fatty acids (FFAs) are major sources of cellular energy (1) and essential components in triglycerides, cholesteryl esters, prostaglandins, and phospholipid syntheses (2, 3). There have been numerous reports on the toxic effects of fatty acids on model cells in vitro. Andrade et al. showed that both saturated and unsaturated fatty acids exert toxic effects on melanoma cells through the loss of membrane integrity or DNA fragmentation (4). Lima et al. evaluated the toxicity of various fatty acids on Jurkat (T-lymphocytes) and Raji (B-lymphocytes) cells (5) and found a positive correlation between the toxicity and the chain length and number of double bonds in the fatty acids. Their experiments identified palmitate among the most toxic of the fatty acids. FFAs, especially saturated fatty acids, can cause cell death in many types of cells, including pancreatic β-cell (6, 7), cardiomyocytes (8, 9), and hepatocytes (10-12).

Most of the research up until now on the mechanism of cell death focused on the production of potential or toxic intermediates, such as stearoyl-CoA desaturase 1 (13-15), acids from omega oxidation (16,17), reactive oxygen species (ROS), ceramide (18,19), reduced mitochondrial potential (8), and reduction of mitochondrial Bcl-2/Bax ratio (11). Recent studies in our lab suggest that palmitate can cause lipotoxicity in liver cells through increased production of hydrogen

peroxide (H_2O_2) and hydroxyl (*OH) radicals (12). Measurements indicated that the cytotoxicity was not completely prevented upon treatment with mitochondrial complex inhibitors or free radical scavengers. This suggests that mechanisms other than ROS production in the mitochondria may be contributing to the toxicity of palmitate. Therefore, we investigated the possibility that palmitate-induced toxicity may be due to hydrophobic effects on the cellular membrane. Fatty acids are known to have toxic and fusogenic effects on cells (20, 21). The mechanism by which fatty acids exert cytotoxicity has been identified as the detergent-effect (22). According to this hypothesis, ionized fatty acid micelles solubilize membrane lipids or proteins and disrupt the physical and functional integrity of cell membranes (20, 21).

Identifying chemical agents to prevent or reverse the effect of fatty acid induced cellular toxicity has been a major focus of research in the past decades. Studies have suggested that saturated and unsaturated fatty acids have different effects on toxicity. For example, there has been evidence indicating that dietary oleic acid can protect endothelial cells against hydrogen peroxide-induced oxidative stress and reduce the susceptibility of LDLs to oxidative modifications (23-25). Similarly, we found that oleic acid does not induce the same level of cytotoxicity as palmitic acid in HepG2 cells at similar concentrations (12) and the addition of oleic acid reduces the cytotoxicity induced by palmitate (26). In another related study, Kinter et al. investigated the protective role of unsaturated FFA in oxygen-induced toxicity of hamster fibroblasts and found that monounsaturated FFA increased cell survival as compared to saturated and polyunsaturated FFAs (27). Furthermore, it has been

shown that unsaturated FFAs rescued palmitate-induced apoptosis by converting palmitate into triglycerides (13). Recently, Natali et al. investigated the effects of various types of FFAs in glial cells and found that oleic acid was a potent inhibitor of fatty acid and cholesterol synthesis (28). In recent years, it has been established experimentally that trehalose has a stabilizing effect on biological membranes (29) by protecting cells from dehydration, heat, and cold (30-32). Moreover, evidence is mounting suggesting that trehalose acts as an antioxidant, possibly serves as a free radical scavenger (33-35), and inhibits the peroxidation of unsaturated fatty acids by heat or oxygen radicals (35,36). In addition, trehalose has been found to protect yeast cells and cellular proteins from damage by oxygen radicals during oxidative stress (37). In light of the role trehalose plays in the stabilization of cells, we investigated whether trehalose is protective in the presence of palmitate, and if so, we aimed at understanding how trehalose protects against palmitate-induced toxicity.

Therefore, to gain insight into how trehalose interacts with liver cells (human hepatocellular carcinoma cell line, HepG2 cells) in the presence of palmitate, we performed a series of experimental and computational measurements. The experimental measurements focused on determining the influence of palmitate and trehalose on the fate of HepG2 cells, and the computational part aimed at interpreting and understanding the experimental results, shedding light into the role of palmitate and trehalose in the toxicity of HepG2 cells. Insight into these mechanisms will add to our understanding of processes (i.e., metabolic, signaling, and biophysical) that are

altered by palmitate. We found that palmitate decreased the cellular membrane fluidity of HepG2 cells. The addition of trehalose to palmitate cultures prevented this lowering in membrane fluidity. Thus, we found that trehalose protects also against palmitate-induced toxicity in liver cells. This study represents the first attempt to obtain a comprehensive understanding of the biochemical and biophysical processes leading to and resulting from the toxicity of palmitate on cells.

Materials and methods

Cell culture

Human hepatocellular carcinoma cell line, HepG2 (American Type Culture Collection, Manassas, VA), was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (American Type Culture Collection) and 2% penicillin-streptomycin (Invitrogen). They were seeded in six-well plates and incubated at 37°C in humidified atmosphere containing 10% CO_2. After cells reached confluence, the media were replaced with 2 ml control medium (4% fatty-acid free bovine serum albumin, or BSA) or fatty acid (0.7 mM with 4% BSA) and changed every 24 h. The BSA level used was close to physiological conditions (38). A quantity of 0.7 mM FFAs was employed in this study because the plasma FFA levels in the obese and type 2 diabetic patients have been reported to be approximately this level (39-42). Experiments were conducted after 48 h of treatment. To determine the optimal amount of trehalose to add, we performed a dose-response in HepG2 cells with trehalose ranging from 0 to 0.2 mM.

Cytotoxicity assay

Cell viability was assessed by lactate dehydrogenase (LDH) leakage through the membrane into the medium. The cell culture supernatant from control and palmitate-treated cultures were tested after 48 h of incubation for the presence of LDH [*LDH(medium)*] using an LDH assay kit (Roche Applied Science, Indianapolis, IN). Cells were washed with phosphate-buffered saline (PBS) and lysed with 1% triton-X 100 for 12 h at 37°C. The cell lysate was then centrifuged at 5000 *g* for 10 min and tested for LDH activity [*LDH(trtoin)*]. The LDH released was normalized to the total LDH, given by

$$\%LDH = \frac{LDH(medium)}{LDH(medium) + LDH(triton)} \times 100$$

Membrane fluidity

Two different stearic-acid derivatives were used to detect changes in the membrane fluidity, 5-*n*-doxylstearic acid (5-*n*-SASL) and 16-*n*-doxylstearic acid (16-*n*-SASL) (Invitrogen, Carlsbad, CA). The 5-*n*-SASL probe monitors the portion of the membrane closest to the lipid headgroups, while the 16-*n*-SASL reflects changes in the middle/end of the lipid hydrocarbon chains (43).

A stock solution of the spin labeled stearic acids at 10^{-3} M was prepared in dimethyl-sulfoxide and the aliquots stored at –20°C. Immediately before use, the stock solution was thawed and diluted 50 times with PBS. Preliminary experiments

were conducted to confirm that the spin-label solution did not affect the cell viability. Cell suspensions collected after Trypsin-EDTA (GIBCO, Billings, MT) exposure were centrifuged and the pellets were resuspended in spin label solution and kept on ice. The labeled cell suspensions were then transferred to a flat cell and placed in the cavity of the electron paramagnetic resonance (EPR) spectrometer (model No. ESP-300E X-band; Bruker AXS, Madison, WI). The microwave power was set at 15.8 mW, the modulation frequency at 100 kHz, and the modulation amplitude at 2.53 G. For indexes of membrane fluidity, we evaluated the values of the outer and inner hyperfine splitting ($2T_{\perp}$ and $2T_{\parallel}$ in Gauss, respectively) in the EPR spectra for 5-*n*-SASL. The order parameter was calculated from $2T_{\perp}$ and $2T_{\parallel}$ by

$$S = \frac{T_{\parallel} - (T_{\perp} + C)}{T_{\parallel} + (2T_{\perp} + C)} \times 1.66 ,$$

where $C = 1.4 - 0.053(T_{\parallel} - T_{\perp})$. In the EPR spectra for 16-*n*-SASL, we used the peak height ratio ($h_o/h - 1$) for an index of the membrane fluidity (44,45), where h_o and $h - 1$ are the heights of the central and high-field peaks, respectively. The greater the values of the order parameter and peak height ratio, the lower the freedom of motion of the spin labels in the membrane bilayers, indicating lower membrane fluidity (46).

Results

Palmitate cytotoxicity and trehalose protective role

We previously found that palmitate-induced toxicity led to ROS production in HepG2 cells (12), but its effect was not prevented upon treating with ROS scavengers. During oxidative stress, yeast cells produce trehalose to protect themselves from damage by oxygen radicals (37). Therefore, we evaluated the effect of trehalose on palmitate-induced toxicity in HepG2 cells. The level of cytotoxicity was measured by the relative amount of LDH released in the medium. The control consisted of HepG2 cells exposed to DMEM with 4% BSA. From Fig 1, our measurements indicate that palmitate significantly increased the amount of LDH released, relative to the control. As the concentration of trehalose increased, the LDH released reduced significantly. This protective effect reached a maximum at a trehalose concentration of 0.13 mM, whereupon further increase in the trehalose concentration was detrimental to the HepG2 cells. Although the mechanism of trehalose-induced toxicity is not a focus of this study, we infer from previous studies, including our own, that trehalose preferentially binds to the membrane and possibly causes surface modifications that may affect cell activity (47-52). As it will be demonstrated from our computational studies, we found that trehalose can induce local hydrophobic/hydrophilic domains along the bilayer interface, a membrane reorganization process which may have potentially detrimental effects. Based on these findings, a trehalose concentration of 0.13 mM was optimum for alleviating the palmitate-induced toxicity in HepG2 cells and it was used in all subsequent

experiments.

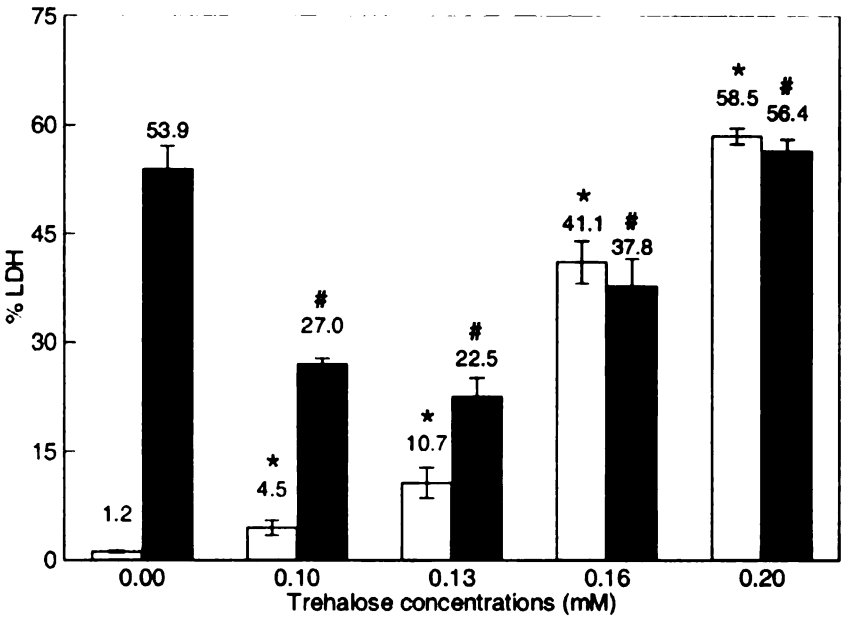


FIGURE 1 Effect of trehalose on the HepG2 cells cytotoxicity in response to palmitate. Confluent HepG2 cells in bovine serum albumin (BSA) medium were exposed to 0.7 mM palmitate in the presence of different concentrations of trehalose. The LDH released was measured after 48 h. Open and shaded bars represented the effect of trehalose alone or the mixtures of trehalose/palmitate, respectively. Note that the first shaded bar shows the effect of palmitate alone. Error bars are standard deviation of three independent experiments. The symbols * and # indicate statistical difference from control and palmitate, respectively ($p < 0.05$).

Trehalose on H_2O_2 release

We previously identified H_2O_2 as one of the ROS species involved in the palmitate-induced toxicity of hepatoma cells. To determine whether trehalose protected HepG2 cells by scavenging H_2O_2, the measured H_2O_2 released into the medium was normalized to total cellular protein. As shown in Fig 2, 48 h of palmitate exposure enhanced H_2O_2 release into the medium, while trehalose significantly reduced the H_2O_2 release in the presence of palmitate. The results suggest that trehalose protects the cells in part by reducing H_2O_2 release.

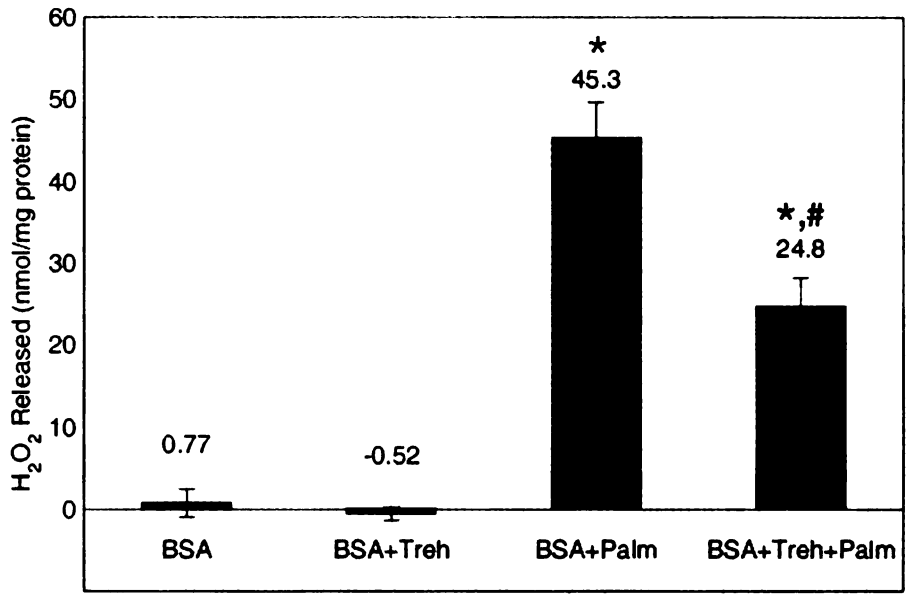


FIGURE 2 Effects of trehalose and palmitate on H_2O_2 release. Confluent HepG2 cells in BSA medium were treated with 0.7 mM palmitate (*Palm*) with or without 0.13 mM trehalose (*Treh*) for 48 h. The H_2O_2 released into the medium was measured and normalized to total cellular protein. Error bars are standard deviation of three independent experiments. The symbols * and # indicate statistical difference from control and palmitate, respectively ($p < 0.05$).

Membrane fluidity for HepG2 cells

Since palmitate is hydrophobic, there may be nonspecific cytotoxic effects due to its hydrophobicity. It has been established experimentally that trehalose has a stabilizing effect on biological membranes (53), therefore we investigated the changes in cellular membrane structure upon exposure to palmitate in the presence and absence of trehalose. The cellular membrane fluidity of HepG2 cells in the presence of palmitate or trehalose was measured by EPR. The EPR spectra of the spin-label agents were used to detect changes in the freedom of motion of the lipids in the cell membrane, thus providing a measure of the membrane fluidity. The membrane fluidity of HepG2 cells were measured after 48 h of exposure to palmitate,

trehalose, and combinations thereof. The control was HepG2 cells exposed to DMEM with 4% BSA. Using 16-*n*-SASL as a probe to monitor the ordering of the lipid tails near the center of the bilayer core, we observed a greater peak height ratio for the palmitate-treated cells as compared to the control, which correlated with reduced freedom of motion of the spin labels in the membrane. This indicates a decrease in membrane fluidity of the cells treated with palmitate for 48 h, as shown in Fig. 3a. The exposure of HepG2 cells to trehalose had an insignificant effect on the bilayer core region since trehalose is excluded from the bilayer. The interaction of trehalose with the membrane is only at the surface of the bilayer. Treating HepG2 cells with trehalose and palmitate increased the peak height ratio. This suggests that a complex interaction exists between the cellular membrane, palmitate, and trehalose. Similarly, using 5-*n*-SASL as a probe to monitor the lipid carbons near the lipid headgroups, we observed that the presence of trehalose in the palmitate-treated cells increased the fluidity near the surface of the membrane (see Fig. 3b).

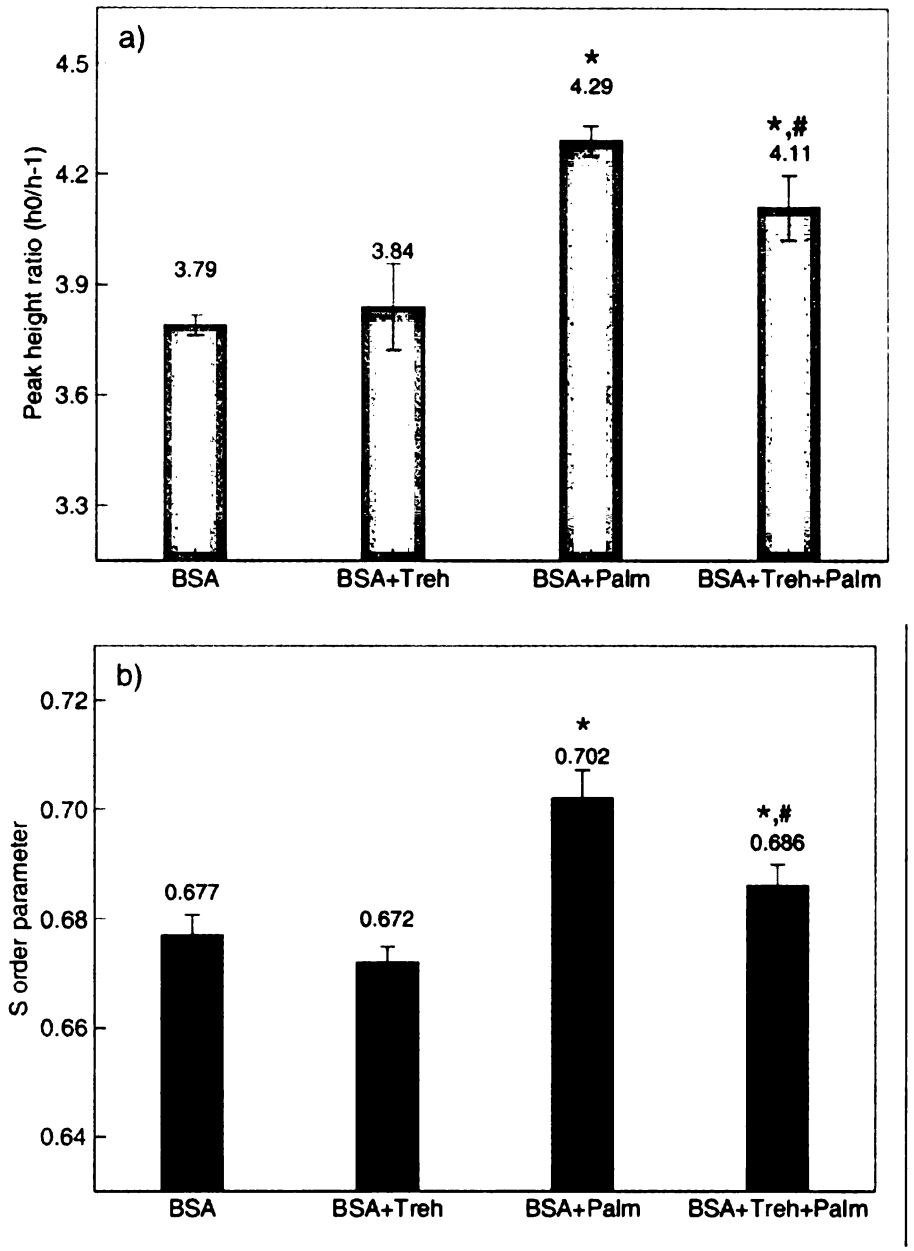


FIGURE 3 Effect of palmitate exposure on cellular membrane fluidity. Cells were treated with 0.7 mM palmitate in the presence or absence of 0.13 mM trehalose for 48 h. The cellular membrane fluidity was measured using EPR. (*a*) Values are peak height ratio for 16-*n*-SASL-labeled HepG2 cells. (*b*) Values are order parameter for 5-*n*-SASL-labeled HepG2 cells. Error bars are standard deviation of four independent experiments. The symbols * and # indicate statistical difference from control and palmitate, respectively ($p < 0.05$).

Discussion:

FFAs are known to play important roles in the development of many hepatic disorders. A number of studies have shown that elevated levels of fatty acids are important mediators of lipotoxicity and can impair cellular functions and/or cause cell death (54). Others have found that the negative effect of FFA-induced toxicity may be reduced or alleviated by the addition of unsaturated fatty acids, antioxidants, or, as of more recently, disaccharides. To evaluate whether palmitate-induced toxicity can be reduced by adding trehalose, we exposed HepG2 cells to palmitate alone or a combination of trehalose and palmitate. As demonstrated from our experiment (Fig 1 and 2), cells exposed to palmitate resulted in a significant amount of LDH and H_2O_2 released into the medium, indicating cell death or compromised cellular membrane. With increasing trehalose concentration, reduced amount of LDH released is observed in the presence of palmitate, up to ~13 mM, an optimal concentration for HepG2 cells. Furthermore, a significant reduction in H_2O_2 released is observed for these cells. To provide insight into the biochemical and biophysical processes altered by the presence of palmitate in HepG2 cells and to interpret these results from a molecular level, we collaborated with Dr. Amadeu K. Sum and Sukit Leekumjorn from Virginia Polytechnic Institute and State University, and they studied the effect of palmitate and trehalose on model cell membranes (lipid bilayers) using molecular dynamic simulations. The simulations results reveal the early stages of how palmitate induces biophysical changes to the cellular membrane and the role of trehalose in protecting the membrane structure. (Chapter 3)

Chapter 2

Unsaturated Fatty Acid Effect on HepG2 cells

Introduction

Unbound free fatty acids (FFAs), derived from dietary triglycerides (TGs) and phospholipids, are aliphatic monocarboxylic acids and among important energy sources for cells and tissues (55, 56). Typically containing a lipid chain of 4 to 28 carbons, they are classified according to the degree of unsaturation: saturated, monounsaturated, and polyunsaturated (57-60). The optimal FFA concentrations in the plasma stream are regulated by plasma protein albumin that leave about less than 0.01% unbounded (61, 62). Recently, in vitro studies confirm that saturated FFAs induced significant toxic effects on various cells types, however, unsaturated FFAs have been shown to induce less toxic effects or reduce and prevent the toxic effects by saturated FFAs (63-66).

It was suggested that saturated FFAs-induced toxicity may be due to hydrophobic effects on the cellular membrane rather than ROS, mainly because the cytotoxicity was not completely prevented upon treatment with mitochondrial complex inhibitors or free radical scavengers (66). Currently, there is very limited knowledge on how unsaturated FFAs play a role in preventing or reducing the toxicity.

There have been numerous studies that examined the effects of unsaturated FAs on lipid bilayers or liposomes at various conditions. For example, Sunamoto et al. investigated the autoxidation of phosphatidylcholine (PC) liposome containing

arachidonic and linoleic acids (67). Using 1,1- diphenyl-2-picrylhydrazyl as radical, they found that the oxidation rate of unsaturated FAs or lipids became faster in liposomes compared to the reaction in solution. Their results suggested that the reactions are preferential within the bilayer core regions. In another related studies, Lee et al. exposed liposomes containing different amounts of oleic, linoleic, and arachidonic acid to oxidizing medium and found that liposomes containing linoleic and arachidonic acid were less susceptible to oxidation than oleic acid (68). After exposing the fragments of lipid peroxidation to endothelial cells and found that the amount of monocyte chemotaxis and monocyte adhesion were significantly increased, they concluded that oxidation products of linoleic and arachidonic acid can triggered cellular immune response. Furthermore, Samuni et al. investigated the oxidative damage of egg phosphatidylcholine (EPC) liposome containing arachidonic acid (C20:4), cis- 7,10,13,16,19-docosapentaenoic acid (C22:5), and cis-4,7,10,13,16,19-docosahexaenoic acid (C22:6) in the presences of vitamin E, antioxidant (Tempo), and cholesterol (69). Their results showed that all polyunsaturated FFAs are highly sensitive to oxidation and hydrolytic degradation. Based on the residual fragment of FFAs collected overtime, cholesterol demonstrated some protective effect and Tempo were better antioxidant than vitamin E. Furthermore, Hyv¨onen et al. investigated the membrane properties at the final stage of the phospholipases A2 enzyme process where phospholipids within the bilayer are hydrolyzed to fatty acids (70). Using PLPC, lyso-PC molecules (a PC headgroup, glycerol backbone, and palmitic acid chain), and linoleate/linoleic acid in their

models, they found that the bilayers become unstable, as a result of water penetrating into the bilayer core region. Recently, Watabe et al. examined the decomposition rate of unsaturated FAs in DPPC liposome containing photoporphyrin IX (PpIX) from light irradiations and determined the oxidation rate from fast to slow: arachidonic acid < oleic acid < linoleic acid (71). Although the oleic acid contains less number of double-bond than linoleic derivatives, they have a greater oxidation rate because the locations of double-bonds are in close range of PpIX molecules embedded within the bilayer. In summary, experimental studies mentioned here are based on the resulting products of lipid oxidation, however, none have addressed the interactions between lipid constituent and FA at the initial stage of this process.

In Chap1, We found that trehalose, a non-reducing disaccharide widely used as a stabilizer and preservant, has a protective role in palmitate-induced toxicity. Unlike trehalose, which is impermeable to membranes (72), unsaturated FFAs can be transported through the membrane into cells through passive and active transport. Once inside the cells, FA can modify the membrane lipid compositions by altering the membrane fluidity and in turn affect cellular function (73,74). Based on this fact that saturated FA decreased membrane fluidity in Chap1, we speculated that the presence of unsaturated FA may help maintain or restore membrane fluidity to its normal state. To compare the effect of saturated and unsaturated FAs on cell membranes, we conducted the cytotoxicity experiments using HepG2 cells exposure to palmitate, oleate, and linoleate. Computational investigations through MD simulations were used to confirm the experimental results and determine the role of palmitate, oleate, and

linoleate on model DOPE bilayers. To relate experimental and computational results, the phase transition study of DOPC liposome containing various concentrations of saturated and unsaturated FAs was used. We try to interpret and understand the interactions of FAs embedded inside the lipid bilayers and identify the role of unsaturated FAs in preventing the changes in membrane fluidity. Insight into these mechanism will add to our understanding of processes (i.e., metabolic, signaling, and biophysical) that are induced by FFAs.

Experimental Materials and Methods

Cell culture

Human hepatocellular carcinoma cell line HepG2 was cultured in DMEM containing 10% fetal bovine serum and 2% penicillin-streptomycin. They were seeded in six-well plates and incubated at 37 °C in humidified atmosphere containing 10% CO2. After cells reached confluence, the media were replaced with 2 ml control medium (4% fatty-acid free bovine serum albumin, or BSA) or fatty acids (0.7 mM palmitate or oleate or linoleate with 4% BSA) and changed every 24 h. Experiments were conducted after 48 h of treatment.

Cytotoxicity assay

Same as in Chap 1.

Membrane fluidity

Same as in Chap 1.

Liposome preparation and DSC measurement

To correlate the fluidity measurements to our computational studies, a simpler model cell membrane also was used. Liposomes (multilamellar vesicles) were prepared by the thin film method according to the protocol from Avanti Polar Lipids. The lipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids, Alabaster, AL) and fatty acid (palmitic acid, oleric acid or linolenic acid , Sigma-Aldrich, St. Louis, MO), obtained in chloroform stock solutions, were mixed in appropriate amounts in a glass tube. After vortexing, the solvent was dried under nitrogen. This formed a thin lipid film on the inside wall of the glass tube. The film was dried in a freeze-dryer to ensure complete evaporation of chloroform. Deionized water was added into the tube before it was placed in a bath sonicator for 10 min. Differential scanning calorimetry (DSC) analysis were performed on the liposomes samples at a scan rate of 1°C/min. Samples containing 20 mg/ml of lipid and 10 μL of liposome suspensions were used. DOPC was used in these experiments because its phase transition temperature (~–19°C) allowed us to obtain clean and clear DSC scans, whereas POPC (one of the lipids used in the simulations studies) has a phase transition temperature near the normal melting point of water (–2°C for POPC), which causes severe interference in obtaining reliable data.

Results

The experimental results are divided into four sections: cytotoxicity, peroxide (H2O2), membrane fluidity, DSC measurements. Cytotoxicity and peroxide measurements are used to determine HepG2 cells viability after exposure to palmitate, oleate, or linoleate. Membrane fluidity is measured by EPR using stearic acid probes, 5-n-SASL

and 16-n-SASL embedded inside HepG2 cells. Lastly, phase transition study of DOPC liposome containing various concentrations of palmitate, oleate, and linoleate are measured by DSC.

Cytotoxicity Measurements

The level of cytotoxicity was measured by the relative amount of LDH released in the medium after exposure of HepG2 cells with palmitate, oleate, or linoleate. The control consisted of HepG2 cells exposed to DMEM with 4% BSA alone. From Fig. 4, our measurements indicate that palmitate significantly increased the amount of LDH released, relative to the control. Oleate and linoleate did not induce toxic effect on HepG2 cells.

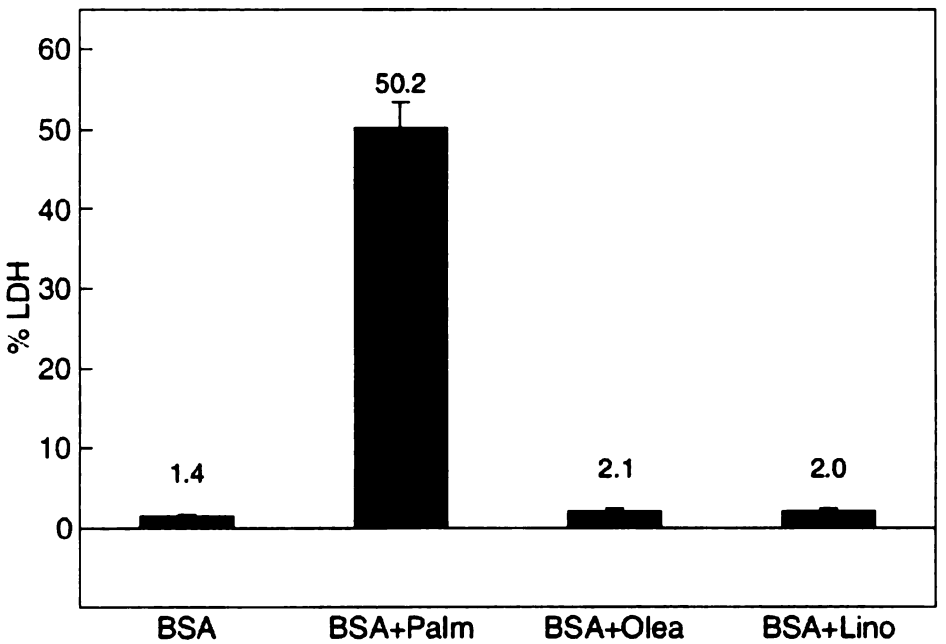


FIGURE 4. HepG2 cells cytotoxicity in response to FFAs. Confluent HepG2 cells in Bovine serum albumin (BSA) medium were exposed to 0.7 mM palmitate, oleate, or linoleate. The LDH released was measured after 48 hrs. Error bars are standard deviation of three independent experiments.

Peroxide Measurements

Relative to the amount of LDH released, we found a direct correlation between the amounts of H2O2 release into the medium. The amount H2O2 release after 48 hrs exposure of HepG2 to palmitate, oleate, or linoleate are shown in Fig. 5. The results suggested palmitate induced cell death by triggering cellular immune response.

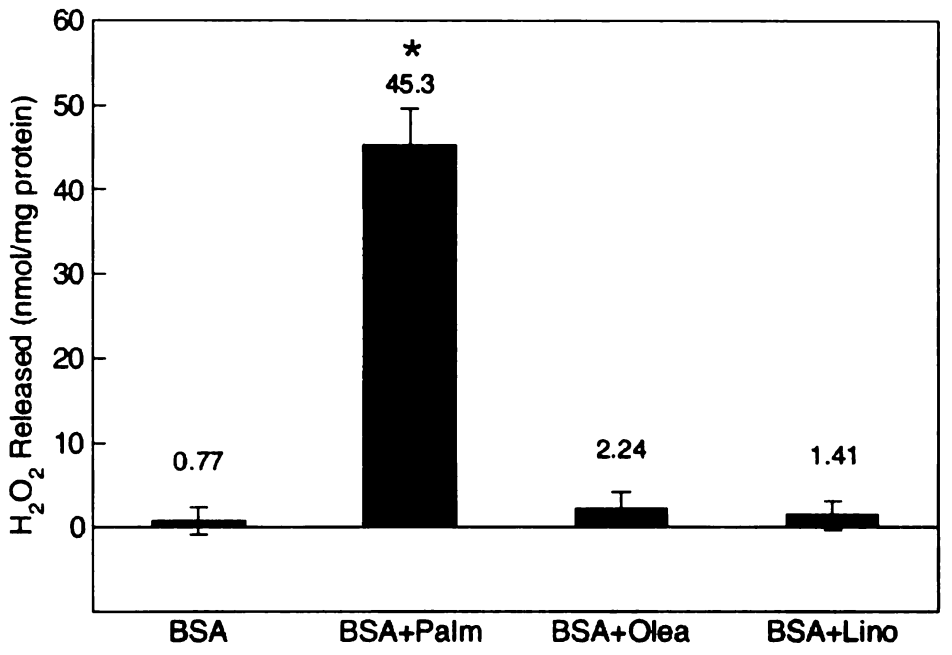
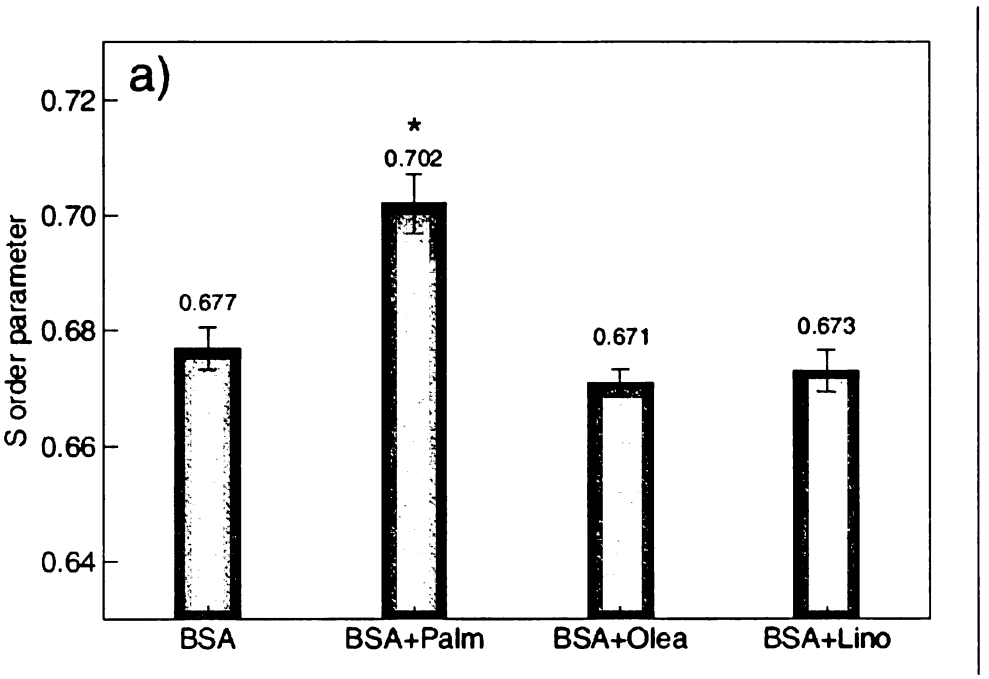


FIGURE 5. Effects of FFAs on H2O2 release. Confluent HepG2 cells in Bovine serum albumin (BSA) medium were treated with 0.7 mM palmitate (Palm), oleate (Olea), or linoleate (Lino) for 48 hrs. The H2O2 released into the medium was measured and normalized to total cellular protein. Error bars are standard deviation of three independent experiments. The symbols * indicates statistical difference from control ($p < 0.01$).

Membrane Fluidity Measurements

Since typical FFAs are hydrophobic in nature, we investigated the non-specific cytotoxic effects due to their hydrophobicity. For this study, we investigated the changes in cellular membrane structure upon exposure to saturated and unsaturated FAs using EPR. The membrane fluidity of HepG2 cells were measured after 48 hrs of exposure to palmitate, oleate, or linoleate. The control was HepG2 cells exposed to

DMEM with 4% BSA. Using both 5-n-SASL and 16-n-SASL as probes to monitor the ordering of the lipid tails near the lipid headgroups and the center of the bilayer core, we observed an increase in the S order parameter and peak height ratio for HepG2 cells exposed to palmitate, as shown in Fig. 6. No significant changes are observed for HepG2 cells exposed to either oleate or linoleate, in comparison to the control. The results suggested a grater reduction of membrane fluidity due to the hydrophobic effect of saturated FA than unsaturated FAs.



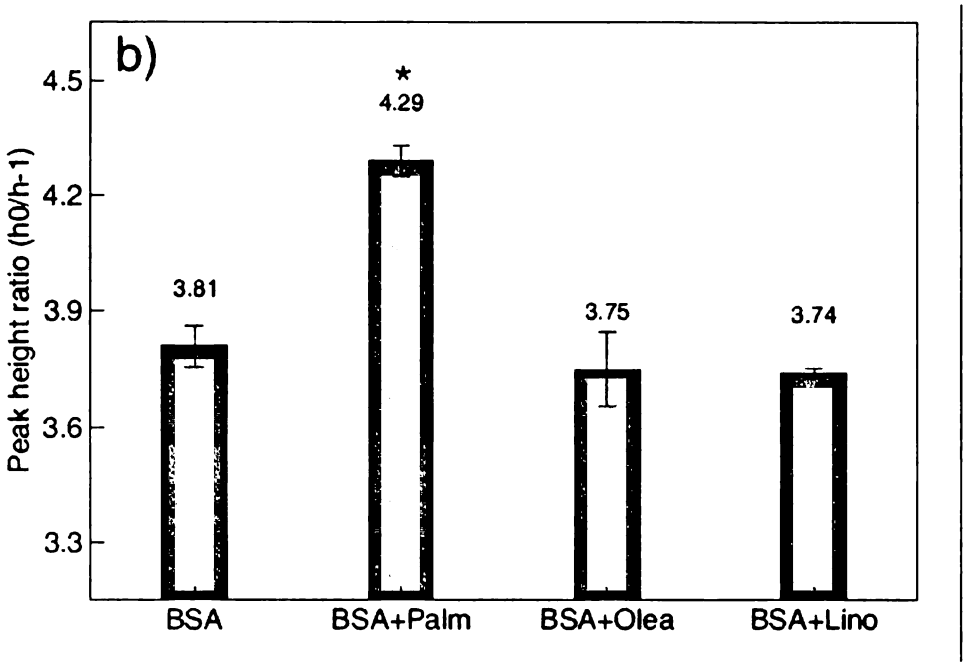
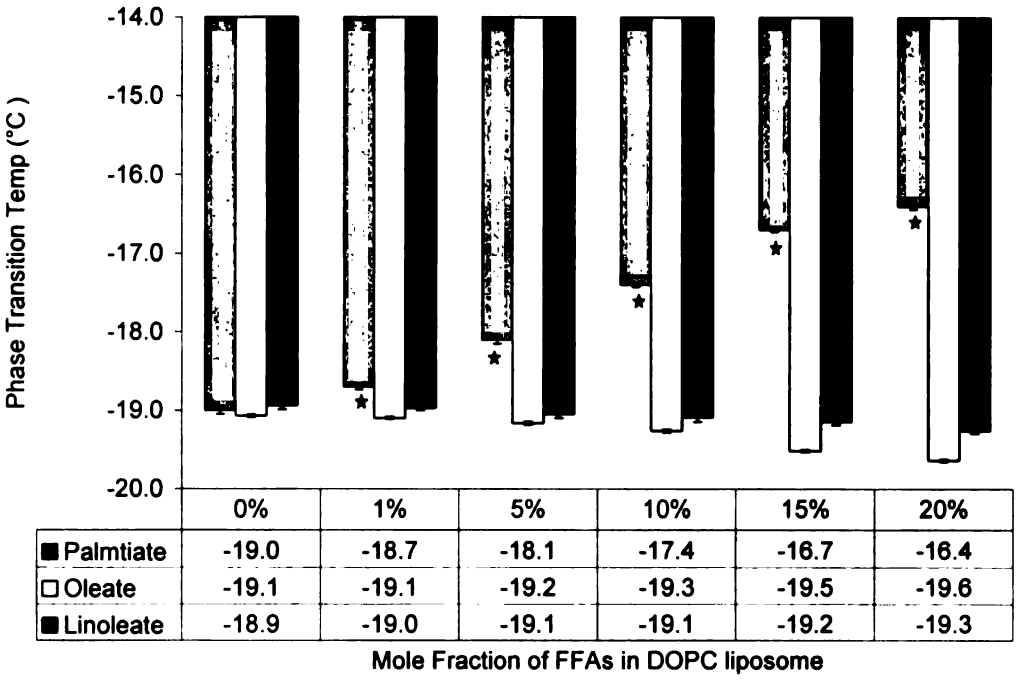


FIGURE 6. Effect of FFAs exposure on cellular membrane fluidity. Cells were treated with 0.7 mM palmitate (Palm), oleate (Olea), or linoleate (Lino) for 48 hrs. The cellular membrane fluidity was measured using EPR. a) Values are order parameter for 5-n-SASL labeled HepG2 cells. b) Values are peak height ratio for 16-n-SASL labeled HepG2 cells. Error bars are standard deviation of three independent experiments. The symbols * indicates statistical difference from control ($p < 0.05$)

DSC Measurements

To corroborate the membrane fluidity results, we measured the phase transition temperature of DOPC liposomes by DSC measurements. The DSC thermographs for DOPC liposomes with varying mole fractions of palmitate, oleate, or linoleate are shown in Fig.7. The figure demonstrated a significant increase in the phase transition temperature of the DOPC liposomes with increasing concentration of palmitate. However, slight changes are observed for DOPC liposome containing the same concentration of oleate and linoleate. This suggests that only palmitate increase the ordering of the phospholipids in the liposomes, which correlates with the decrease in membrane fluidity measured by EPR.



	0%	1%	5%	10%	15%	20%
■ Palmtiate	-19.0	-18.7	-18.1	-17.4	-16.7	-16.4
□ Oleate	-19.1	-19.1	-19.2	-19.3	-19.5	-19.6
■ Linoleate	-18.9	-19.0	-19.1	-19.1	-19.2	-19.3

Mole Fraction of FFAs in DOPC liposome

FIGURE 7 Effect of FFAs on phase transition temperature of DOPC liposome. Phase transition temperature of DOPC liposomes containing various concentrations of palmitate, oleate and linoleate(grey, white and black bar). The phase transition was measured with DSC. Error bars are standard deviation of four independent experiments. The symbols "*" indicates statistical difference from control ($p < 0.01$)

Discussion

Unlike most FFAs, palmitate has been shown to be very toxic to HepG2 cells at 0.7 mM. Unsaturated FFAs, on the other hand, have been shown to have both positive and negative effects among various cell types. We found from the cytotoxicity and peroxide measurements that oleate and linoleate are not toxic to HepG2 cells at same concentration considered for palmitate. Our EPR measurements indicated that there is a significant change in membrane fluidity in the presence of palmitate, compared to oleate and linoleate systems. This change is mainly associated with hydrophobic effect of saturated FA which resulted in reducing membrane fluidity. To compare the

biochemical and biophysical processes associate with the change in membrane fluidity in the presence of palmitate, oleate, or linoleate at a molecular level, we collaborate with Dr. Amadeu K. Sum and Sukit Leekumjorn from Virginia Polytechnic Institute and State University, and they studied the effect of these FFAs on model cell membranes (DOPC lipid bilayers) using molecular dynamic simulations. (Chapter 3)

Chapter 3

Molecular dynamics Simulation

(Dr. Amadeu K. Sum and Sukit Leekumjorn from Virginia Polytechnic Institute and State University performed all the simulations discussed in this chapter)

To provide insight into the biochemical and biophysical processes altered by the presence of palmitate, oleate, or linoleate in HepG2 cells and to interpret these results from a molecular level, we collaborated with Dr. Amadeu K. Sum and Sukit Leekumjorn from Virginia Polytechnic Institute and State University, and they studied the effect of FFAs and trehalose on model cell membranes (lipid bilayers) using molecular dynamic simulations.

The lipid bilayers used here are equimolar 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPC/POPE) bilayers with a total of 288 lipid molecules evenly distributed in each leaflet. This mixed bilayer was chosen for these studies because it represents the main phospholipid constituents of HepG2 cells (75). All simulations were performed at 310 K, by Dr. Amadeu K. Sum and Sukit Leekumjorn with the GROMACS 3.3.1 software package in parallel using Virginia Tech's System X (dual 2.3 GHz Apple Xserve G5). Images in this chapter are presented in color.

The initial stage of cell exposure to palmitate is modeled by introducing a single palmitate molecule in the aqueous phase of previously equilibrated bilayers with and without trehalose. Snapshots of the two systems at the start of the simulation are shown in Fig 8. Fig 8 b and d, show representative trajectories of palmitate along the simulation (trajectory is traced by the position of the central carbon atom in the palmitate tail). As shown in Fig 8, palmitate can penetrate the bilayer within the simulation time considered. Eight of the ten simulations resulted with palmitate in the bilayer and palmitate remained in the aqueous phase for the duration of the

simulations. For the simulations with trehalose, similar results were obtained. The observed penetration of palmitate in the bilayer is consistent with experimental studies that demonstrated that palmitate can be readily incorporate into the hydrophobic region of the bilayer (76,77).

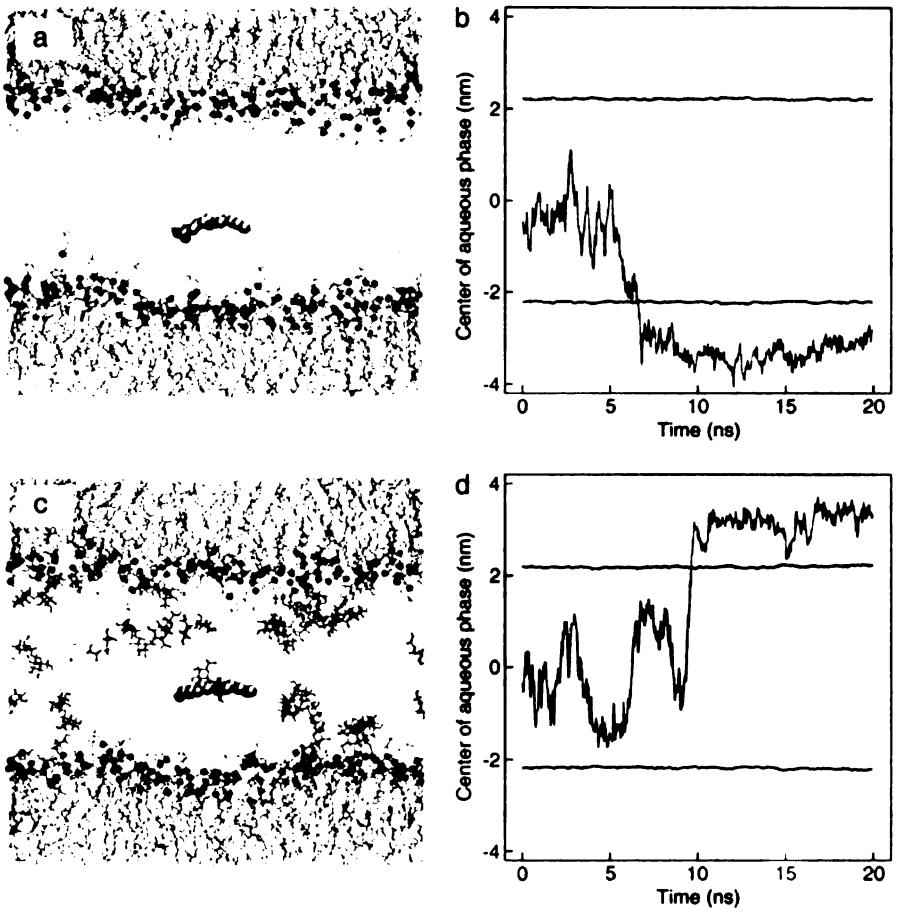


FIGURE 8 Starting structure of POPC/POPE bilayer with one palmitate inserted into the aqueous phase. Bilayers are modeled (a) without and (c) with trehalose. Colored molecules are POPC/POPE headgroup (blue), lipid tails (red), water (gray), trehalose (green), and palmitate (cyan). The dynamics of palmitate, represented by the position of central carbon atom in its tail, are shown in panels b and d. Blue horizontal lines correspond to the average position of the phosphorus atoms of POPC and POPE along the interface and are used to identify the interface. Gray area denotes the aqueous regions. The position z = 0 corresponds to middle of the aqueous phase.

To investigate the protective role of trehalose on palmitate-induced toxicity, an extensive hydrogen-bond analysis was carried out to investigate the interactions

between lipids, trehalose, and palmitate. [See previous publication (78) for details] The observations led us to conclude that palmitate, a hydrophobic molecule, prefers to penetrate the bilayer through hydrophobic regions. And trehalose has the ability to modify the bilayer surface (79, 80), creating large hydrophobic regions exposed to the aqueous phase. The results also demonstrates the dual role of trehalose: on the bilayer surface, trehalose can alter the H-bond distribution, thus inducing hydrophobic regions for palmitate to penetrate the bilayer, while in the aqueous phase, trehalose can interact with palmitate and prevent it from approaching the bilayer surface. These two competing processes help us to understand why the experimental measurements have shown that trehalose at high concentrations (>0.13 mM) is detrimental to HepG2 cells. At high trehalose concentrations, the bilayer surface is significantly modified by trehalose such that hydrophobic regions are more accessible for palmitate to penetrate the bilayer.

From the simulation, we found single palmitate can penetrate the bilayer. However, attempts to model multiple palmitate molecule dynamics was not successful. Palmitate molecules aggregate, as shown in Fig 9, due to the hydrophobic/hydrophilic interactions. The aggregation reduces the driving force for palmitate to penetrate the bilayer. To investigate effects of palmitate on bilayer, model bilayers with palmitate embedded in them were created.

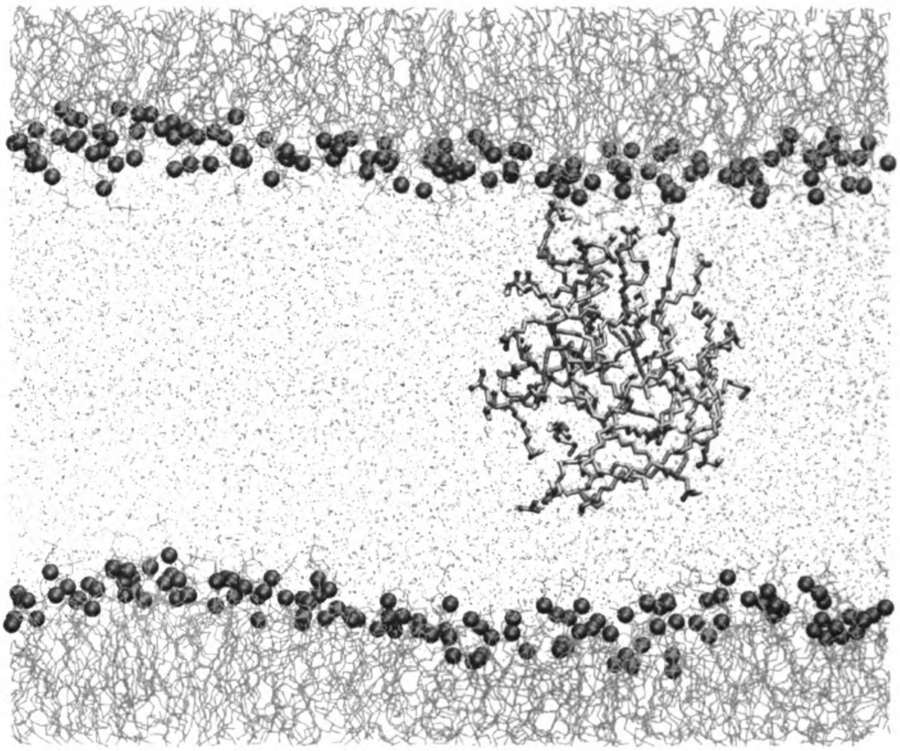


Figure 9: Snapshot of palmitate aggregation in the aqueous phase. The colored groups correspond to the DPPC headgroups (blue), lipid tails (light gray), palmitate (red/dark gray), and water (pink).

Fig 10 shows the changes to the bilayer structure caused by the addition of palmitate for the cases where the bilayer freely expands and remains constrained as palmitate is embedded in the bilayer. To mimic the local effect of palmitate embedded in the bilayer, the lateral expansion of the bilayer was constrained. As shown in Fig 10c, straight lipid tails with tilted arrangements are observed at higher palmitate concentrations. This is related to the ordered lipid phase, which has been shown to be detrimental to cells by limiting their transport activities (81, 82), binding sites for pathogens and toxins (83-85), and possibly the cause of palmitate-induced toxicity. As the palmitate concentration decreases, the arrangement of the lipid tails become more random as observed in Fig 10a, thus restoring the bilayer to its normal structure.

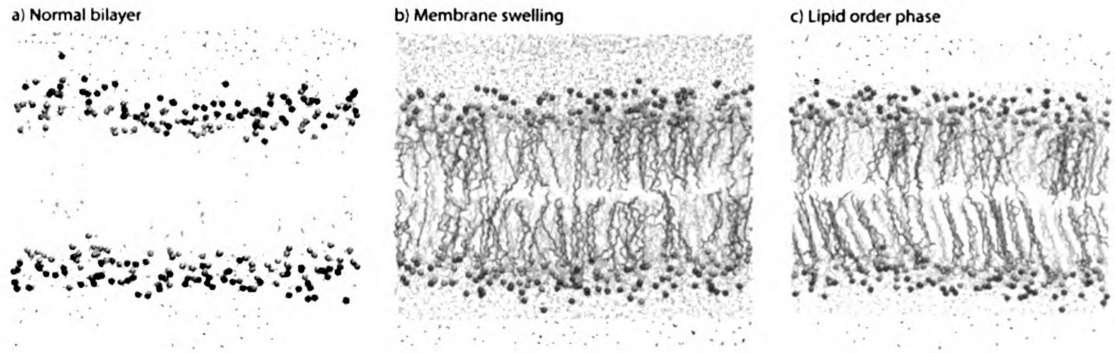


FIGURE 10 Snapshots of bilayer structures with (44 mol %) and without palmitate. (a) No palmitate is embedded in the bilayer. (b) The bilayer is allowed to expand as more palmitate molecules are embedded in the bilayer. (c) The bilayer is constraint in the lateral directions as more palmitate molecules are embedded in the bilayer. Colored molecules are POPC headgroup (blue), POPE headgroup (green), lipid tails (silver), water (red), and palmitate (brown).

As seen from Fig 10, the increase in the ordering of the lipid tails is related to the mixing of lipid and fatty acid components, where straight chain fatty acid exhibits higher order parameters. Since the ordering of the lipid tails is directly related to the phase transition, the simulation results agree well with the DSC measurements for DOPC liposomes containing palmitate, EPR measurements of HepG2 cells exposed to palmitate (Chap 1&2), all of which showed an increase in the phase-transition temperature with increasing palmitate concentration.

Palmitate has been shown to be very toxic to HepG2 cells. Unsaturated FAs, on the other hand, are not toxic. To obtain a better understanding on how unsaturated FA interacts with the lipid bilayers in comparison to the effect induced by saturated FA, hydrogen bonding was analyzed to characterize the effect of unsaturated FAs (oleate and linoleate) on the properties of DOPC bilayers. The goal was to investigate the effect of lipid hydration with increasing FA concentrations. Using the hydrogen bond analysis previously described by Brady and Schmidt (86) with the first hydration

cut-off from RDFs, Fig. 11 shows the average number of hydrogen bonds per lipid between lipid oxygen atoms and water for all systems considered. As seen in the figure, the average number of hydrogen bonds reduces significantly with increasing palmitate concentrations. This is because the increase in lipid packing resulted in the removal of potential binding sites for water. On the other hand, the number of hydrogen bonds for oleate systems remains relatively the same regardless of the oleate or linoleate concentrations. This is related to fact that oleate and linoleate can reduce the packing between lipids, thus maintaining the suitable area per lipid and the level of hydration. From this analysis, we confirm that the role of saturated and unsaturated fatty acid are very different in that unsaturated FA induced less change in the bilayer structure and help maintain the level of hydration.

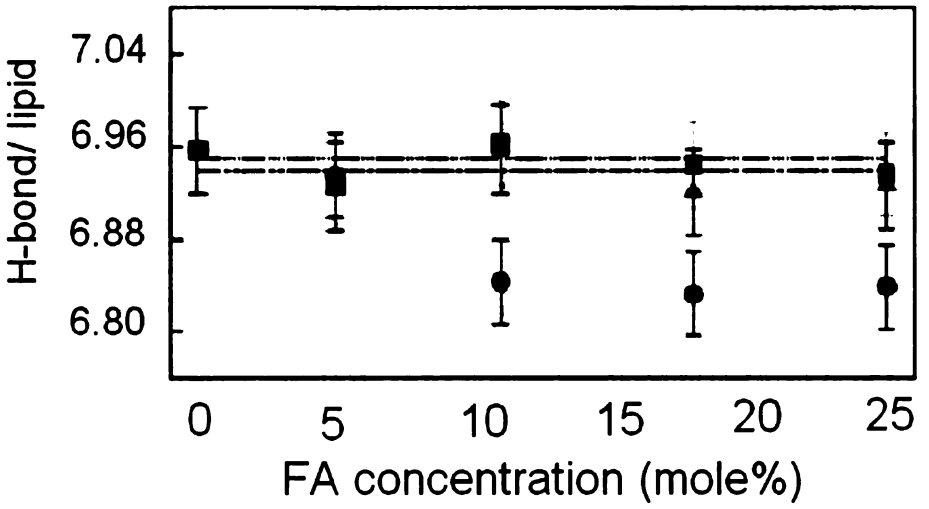


Figure 11. Average number of hydrogen bonds per lipid for bilayers with palmitate (circles), oleate (square) and linoleate (triangle); water as H-donors. Solid, dotted, and dash lines are drawn to guide the eye, respectively. Error bars represent standard deviations.

Discussion

Modification of the membrane lipid composition may alter the membrane fluidity and in turn affect cellular function (87, 88). As palmitate molecules are embedded within the membrane, it is observed from the EPR measurements that the membrane fluidity is significantly decreased. This phenomenon can be explained by many factors. First, palmitate is hydrophobic in nature and by exposing it to cell membranes, palmitate is most likely dissolving into the membrane, thus reducing the membrane fluidity. Second, as palmitate can be metabolized into phospholipid components of cell membranes, these additional components can cause an increase in the packing between the lipids, consequently decreasing the membrane fluidity (87,88). Last, since H_2O_2 and *OH are present in cultured cells exposed to palmitate, it has been reported that unsaturated phospholipids are oxidized into fragment of saturated hydrocarbons with various headgroup functionalities, some of which are highly toxic to cells (89-92). Although, the oxidation of unsaturated lipids generally results in an increase in fluidity and permeability of the membrane (93-95), the remaining fragments inside the membrane, which are hydrophobic in nature, can reduce the membrane fluidity. In this study, we found that palmitate decreased the cellular membrane fluidity of HepG2 cells. This was expected, since others have shown that fatty acids incorporated into the membrane disrupted the bilayer structure and changed the lipid phase-transition temperature (76,77). We have also observed increasing the concentration of palmitate increases the phase-transition temperature of DOPC.

Conclusions and Future work

We performed a series of experimental and computational measurements to gain insight into how trehalose interacts with HepG2 cells in the presence of palmitate, and the different effects of saturated and unsaturated fatty acids on HpeG2 cells. Experimentally, we found that healthy HepG2 cells exposed to palmitate resulted in a significant amount of LDH and H2O2 released into the medium, indicating cell death or compromised cellular membrane. However, cells exposed to oleate and linoleate did not show cell damage. Furthermore, it is observed from EPR measurements that the membrane fluidity is significantly decreased in the presence of palmitate, while fluidity is not significantly changed in the presence of oleate and linoleate. The leading hypotheses for the observed results are:

1. Palmitate dissolves into the membrane, thus reducing the membrane fluidity.
2. Palmitate metabolizes into phospholipid components of cell membranes, thus increasing the packing between the lipids.
3. The remaining fragments of oxidized lipids inside the membrane (oxidized by H2O2 and *OH), which are hydrophobic in nature, reduce the membrane fluidity.

The simulation is aimed at interpreting and understanding the experimental results, providing knowledge at the molecular level into the role of fatty acids and trehalose in the toxicity of HepG2 cells. As illustrated by the results, the computation analyses confirmed that palmitate can dissolve into the bilayers within a short time. As the

palmitate concentration in the bilayer increased, it forces the surrounding lipid molecules to become highly packed, resulting in a more ordered structure. The local effect of palmitate embedded in the bilayer was also considered. The simulation results yielded a highly order bilayer structure with the lipid tails in a tilted arrangement. These results agreed well with DSC measurements for DOPC liposomes containing palmitate and EPR measurements of HepG2 cells exposed to palmitate. Furthermore, we verified that the direct interactions of trehalose and palmitate in the medium through hydrogen bonding potentially hinder palmitate from dissolving into the bilayer. The binding of palmitate to trehalose seems beneficial to cell membranes; however, we have also discovered that trehalose can potentially modify the bilayer surface by altering the surface hydrogen-bond distribution, thus inducing hydrophobic regions for palmitate to penetrate the bilayer. We confirmed that the role of saturated and unsaturated fatty acid are very different in that unsaturated FA induced less change in the bilayer structure and help maintain the level of hydration.

We have demonstrated a potential mechanism by which palmitate incorporates into the bilayer. We further hypothesize that palmitate can aggregate in lipid bilayer to form small domains, and possibly pores. However, due to the limited time scale of simulation, we were not able to observe significant diffusion of palmitate in bilayer. To get evidence for aggregation, we can use fluorescence resonate energy transfer (FRET). Fatty acids and phospholipids would be tagged covalently with optical donor and acceptor chromophores. A time correlated single photon counting system can be used to measure the fluorescence lifetime of donor and acceptor incorporated in the

liposome. Aggregation would be seen as a deviation from the predictions of the Forster model for a random distribution of the chromophores. Understanding the mechanism of saturated fatty acid-induced hepatocyte toxicity may provide insight into cures for diseases such as obesity-associated cirrhosis.

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