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Effects of human serum albumin complexed with free fatty acids on cell viability and insulin secretion in the hamster pancreatic β -cell line HIT-T15

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ABSTRACT

Aims: The effects of human serum albumin (HSA) complexed with various free fatty acids (FFAs) on β -cells have not been studied in detail. In this study, we examined the effects of HSA and its mutants on FFA-induced cell viability changes and insulin secretion from the hamster pancreatic insulinoma cell line, HIT-T15.

Main methods: Cells were exposed to different FFAs in the presence of HSA or its mutants and/or bovine serum albumin (BSA) for 24 h. Cell viability, apoptosis, insulin secretion, and unbound FFA (FFA_u) levels were determined.

Key findings: In the presence of 0.1 mM HSA, palmitate and stearate induced significant cell death at 0.1 mM or higher, whereas myristate, palmitoleate, oleate, elaidate, linoleate, linoelaidate, and conjugated linoleate showed minimal changes on cell viability. Furthermore, oleate and linoleate were clearly cytoprotective against palmitate-induced cell death. The apoptosis inhibitors, cyclosporin A (csA) and the caspase inhibitor ZVAD-FMK, did not completely prevent FFA-induced cell death, although ZVAD-FMK blocked apoptosis with no differences in the presence of either HSA or BSA. In addition, insulin secretion from the cells was significantly reduced in the presence of HSA/oleate complexes. We also found differential effects of HSA mutants complexed with FFAs on cell viability.

Significance: In summary, our results showed that saturated FFAs induced more cell death than unsaturated FFAs. Furthermore, modified HSA/FFA interactions caused by mutations of key amino acids involved in the binding of FFA to HSA resulted in changes in cell viability, suggesting a possible role of HSA polymorphism on FFA-induced changes in cellular functions.

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Introduction

Previous studies have shown that elevated levels of plasma free fatty acids (FFAs) in obese subjects are associated with insulin resistance and pancreatic β -cell dysfunctions (Reaven and Chen, 1988; Shulman, 2000). Furthermore, there is compelling evidence that chronic exposure of β -cells to FFAs is associated with cytotoxicity (Morgan, 2009), particularly with long-chain saturated FFAs compared to monounsaturated FFAs. In fact, some studies have further documented that unsaturated FFAs can be cytoprotective against palmitate-induced β -cell death (Morgan and Dhayal, 2010). However, molecular mechanisms of how chronic FFA exposures induce β -cell death or the cytoprotective mechanisms by unsaturated FFAs against lipotoxicity are not yet clearly established. Recent studies suggested

that accumulation of long-chain acyl CoA in the cytosol activates cell-death signals by generation of nitric oxide (Shimabukuro et al., 1997) and ceramide (Paumen et al., 1997) as well as by mitochondrial perturbations (Newsholme et al., 2007). Lately, involvement of endoplasmic reticulum (ER) stress in induction of apoptosis on exposure of β -cells to palmitate has also attracted attention (Lai et al., 2008; Laybutt et al., 2007).

Therefore, transport of FFAs across the plasma membrane plays an important role in modulating FFA-induced cellular metabolism. In β -cells, concentration gradients across the membrane are believed to be a major contributing factor for this FFA transport (Hamilton et al., 1994). The free fraction of FFAs, unbound FFAs (FFA_u) in the extracellular space, which is available for this transmembrane transport, is greatly influenced by the extent of binding to their transport protein, human serum albumin (HSA). HSA is the principal carrier of FFAs in serum; it can bind with up to 11 FFA molecules, depending on the chain length with binding affinity ranging from 10^{-6} to 10^{-8} M (Carter and Ho, 1994; Curry et al., 1998; Peters, 1996). Under normal physiological conditions, an average of two FFA molecules are bound to HSA in circulation, but the molar ratio of HSA to FFA can rise up to 1:6 in disease states such as diabetes mellitus (Cistola and Small, 1991). Although the role of HSA as the principal FFA carrier in

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the blood has been clearly shown, its role in FFA effects on β -cells has not been studied in depth. Furthermore, previous studies used BSA to measure FFA effects on β -cells. Although HSA and BSA have a 76% sequence homology, they differ in their numbers of FFA-binding sites and binding affinities (Richieri et al., 1993; Spector et al., 1969) as well as in their three-dimensional structures (Huang et al., 2004). Therefore, use of HSA instead of BSA is relevant in studying the effects of FFA on β -cells.

In this study, we used a well-established insulinoma cell line, HIT-T15, to study the effects of various FFAs in the presence of HSA and/or BSA. We also studied the effects of FFAs in the presence of two typical apoptosis inhibitors, cyclosporin A (csA) and the general caspase inhibitor ZVAD-FMK, to determine the mechanism of FFA toxicity in β -cells. Furthermore, we studied the effects of mutations in HSA/FFA-binding sites on β -cell viability by treating β -cells with HSA mutants, which contained amino acid substitutions in FFA-binding sites (Simard et al., 2005) of subdomains 2A and 3A of HSA in the presence of FFAs. We also determined the FFA_u levels in the complexes of recombinant HSA and its mutants to FFAs using the fluorescent probe, acrylodan-labeled intestinal fatty acid-binding protein (ADIFAB).

Materials and methods

Materials

Hamster pancreatic β -cell line (HIT-T15), horse serum, fetal bovine serum (FBS), trypsin/EDTA, F12-K medium, and MTT (3,4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell proliferation assay kit were obtained from American Type Culture Collection (Manassas, VA). Tissue culture flasks and 96 well plates were purchased from BD Falcon (Franklin Lakes, NJ). Fatty acid free HSA, fatty acid free BSA, FFAs (palmitate, stearate, oleate, elaidate, myristate, linoleate, and palmitoleate), bicinchoninic acid assay kit (BCA), csA, and cibacron coupled to sepharose 6B were purchased from Sigma-Aldrich (St. Louis, MO). Conjugated linoleic acid (CLA) and linoelaidate were purchased from Cayman Chemical (Ann Arbor, MI). Antibiotic-antimycotic, phosphate buffered saline (PBS), plasmid vector, pHIL-D2, and the yeast strain *Pichia pastoris* were purchased from Invitrogen Life Technologies Inc. (Grand Island, NY). The non-esterified fatty acid kits (WAKO C and WAKO HR series) were supplied by the WAKO chemicals (Richmond, LA). Rat insulin ELISA kit was supplied by Crystal Chem Inc. (Downers Grove, IL). The general caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl) fluoromethylketone (ZVAD-FMK) was purchased from Promega Corporation (Madison, WI). Vent DNA polymerase was from New England Biolabs (Beverly, MA). Lipidex-1000 was from Packard Instruments (Meriden, CT). Staurosporine was purchased from EMD Chemicals (Gibbstown, NJ). Apoptotic DNA Laddering kit was purchased from Trevigen Inc. (Gaithersburg, MD). Acrylodan intestinal fatty acid-binding protein (ADIFAB) was purchased from Molecular Probes (Eugene, OR). All other chemicals used were of analytical grade.

Cell culture

The Syrian golden hamster pancreatic β -cell line HIT-T15 was cultured in 87.5% F12-K medium containing 7 mM glucose, 2 mM L-glutamine, 1500 mg/l sodium bicarbonate and supplemented with 10% horse serum (v/v), 2.5% FBS (v/v), 1% antimycotic-antibiotic (v/v), at 37 °C with 5% CO₂ and 95% air. Cells were grown and maintained in T75 cm² flasks and subcultured for individual experiments in collagen-coated 96-well culture plates until about 80% confluency.

Treatment of cells with FFAs, cyclosporin A (csA), ZVAD-FMK, and staurosporine

Stock solutions of FFAs were prepared by serial dilutions in 10% methanol in accordance with a previous study by Ha et al., 2006 (Ha et al., 2006). Stock solutions of palmitate and stearate were prepared at 70 °C,

myristate at 62 °C, elaidate at 50 °C, oleate at 37 °C and palmitoleate, CLA, linoleate, and linoelaidate at 25 °C. Proper amounts of stock solution of FFAs and HSA were dissolved in serum free-F12-K medium to give FFA working concentrations of 0.1 mM–0.8 mM and HSA concentration of 0.1 mM. In some instances, palmitate at 0.4 mM was mixed with either of the individual unsaturated FFAs at also the same concentration in the presence of 0.1 mM HSA. The mixtures were rotated overnight to ensure fatty acids were completely dissolved. Final concentration of methanol in working HSA/FFA solutions did not exceed 0.05% methanol. Confluent cells were treated with the working solutions and control cells received 0.1 mM HSA and 0.05% methanol. In experiments for testing apoptosis inhibitors, cells were treated with the general caspase inhibitor (ZVAD-FMK) and csA at final concentrations of 50 μ M and 5 μ M, respectively in serum free-F12-K medium in the presence of 0.4 mM palmitate and or stearate complexed to 0.1 mM HSA and/or BSA as described previously for 24 h (El-Assaad et al., 2003; Kong and Rabkin, 2000; Pritchard et al., 2000). csA and ZVAD-FMK were dissolved in 100% DMSO with final DMSO concentration not exceeding 0.05% and 0.25% in the working solutions, respectively. Another set of cells were also treated with the apoptotic inducer, staurosporine dissolved in serum free-medium containing HSA and/or BSA in the absence or presence of csA and ZVAD-FMK at a final concentration of 2 μ M. Untreated control cells received 0.1 mM HSA and/or BSA alone or in the presence of 5 μ M csA or 50 μ M ZVAD-FMK. For studies with HSA mutant proteins, the various mutant proteins at 0.1 mM were each complexed to either oleate or palmitate at 0.4 mM concentration in final working solutions and prepared as described earlier. Control cells were treated with the wild type recombinant HSA (rHSA) complexed to either oleate or palmitate.

Measurement of cell viability

HIT-T15 cells were treated with the FFAs, test compounds and control media as described earlier. After the 24-hour incubation, cell viability assay was determined using MTT cell proliferation assay kit according to the manufacturer's instruction with slight modification. Briefly, 20 μ l of MTT dye was added to the cells and the plates were incubated for 4 h at 37 °C. The medium was then aspirated and the cells were lysed with 100 μ l of the solubilization reagent. The absorbance was determined at 570 nm after overnight incubation in the dark at room temperature with gentle shaking. The MTT assay is a widely accepted way to examine cell proliferation based on the yellow tetrazolium MTT being reduced by viable cells to an intracellular formazan that can be solubilized and quantified colorimetrically. All spectrophotometric readings were made using a microplate reader (Bio-Rad Laboratories).

Assay of apoptotic DNA fragmentation

Detection of DNA fragmentation was assayed according to the manufacturer's apoptotic DNA laddering kit. Briefly, after treatment incubation, cells were lysed and DNA was isolated. Agarose gel electrophoresis was performed in 1.5% agarose gel to separate DNA fragments. The gel was then stained with ethidium bromide (0.5 μ g/ml) and the DNA bands visualized under UV illumination and photographed.

Determination of insulin secretion

Insulin secretion was determined by using an ELISA kit according to manufacturer's manual. After the 24-hour incubation in appropriate treatment media, cell culture media were collected for insulin ELISA assay. Cells were washed twice with 1 \times PBS and then lysed with 0.1 N NaOH and cell protein was quantified by BCA assay kit. Values of secreted insulin measured in ng was converted to ng per mg cell protein and expressed as percent of insulin secretion mediated by HSA alone (set as 100%).

Preparation of recombinant HSA and its mutant proteins

We synthesized recombinant HSA and the following single and double mutant proteins and the selections for these mutations were based on the specific FFA-binding data provided by X-ray crystallography studies which showed that up to seven fatty acids can bind to a single molecule of HSA (Carter and Ho, 1994; Curry et al., 1998; Sugio et al., 1999). Also, our recent studies using X-ray crystallography and NMR spectroscopy (Simard et al., 2005) showed that FFA-binding sites 4 and 5 within domain 3 represent two of the three high affinity FFA-binding sites on HSA (the third is on site 2 in domain 2). The mutants studied were, R410A/Y411A, W214L/Y411W, R410A, Y411A and W214L. Specific mutations were introduced into the HSA-coding region and the mutant proteins were expressed and purified as described previously (Ha et al., 2003).

Determination of unbound FFA (FFA_u) in recombinant HSA/FFA complexes

200 μ g of ADIFAB powder was brought up in 130 μ l of storage buffer consisting of 50 mM TRIS, 1 mM EDTA and 0.05% sodium azide at pH 8.0, resulting to a stock of approximately 100 μ M. This low salt was used only for storage for not more than three months. The sodium salts of the FFAs, palmitate and oleate were used to prepare the aqueous solutions. Stock solutions were prepared at a FFA concentration of 30 mM in deionized water plus 4 mM NaOH and 25 μ M butylated hydroxytoluene (BHT). BHT was dissolved in ethanol at 50 mM and the final ethanol concentration in FFA-sodium salt solution did not exceed 0.05% of ethanol. 500 μ M dilutions of these FFA stocks were made in water plus 4 mM NaOH but no additional BHT. Total FFA was determined by WAKO NEFA C kit and aliquots stored in -20°C for no longer than 3 months. Upon excitation at 386 nm, ADIFAB fluorescence at 432 nm in the absence of fatty acid and at 505 nm in the presence of fatty acids (Richieri et al., 1999). Thus the intensity ratio (R) of 505 nm to 432 nm is indicative of the amount of FFA_u present. Measurements of R values were done with a photon technology international fluorometer using the photon counting mode. ADIFAB was used to determine FFA_u levels in HSA/palmitate or oleate complexes. rHSA and its mutants were all treated identically. For each complex, FFA_u levels were determined by measuring the fluorescence as described previously by Richieri et al., 1993 (Richieri et al., 1993) from three separate samples containing (1) HSA in buffer (blank), (2) HSA and ADIFAB in buffer (the R_0 value), and (3) FFA, HSA and ADIFAB in buffer (the R value). The measuring buffer was at pH 7.4 containing 20 mM HEPES, 140 mM NaCl, 5 mM KCl and 1 mM Na_2HPO_4 and same HSA (4 μ M) and ADIFAB (0.2 μ M) concentrations were used in each sample. Appropriate aliquots of FFA were added to the cuvette to give total FFA concentrations of between 0 and 24 μ M. Just prior to FFA addition, the concentrated stocks of FFA-sodium salts were warmed to temperatures above the FFA melting point (62°C for palmitate and 37°C for oleate). FFA was then added in small volumes to the third sample which was maintained at 37°C and immediately mixed by drawing the solution in and out of the pipette. Between each FFA addition the cuvette was allowed to incubate for 10 min at 37°C . After the 10 min incubation the 432 nm and 505 nm intensities were determined from the three samples all at 37°C . This procedure was repeated for total FFA to total albumin molar ratio values between 0 and 6 and in steps of 1.0. As a consequence, the FFA_u concentration was determined according to (Richieri et al., 1992) from the 505 nm and 432 nm fluorescence after each titration.

Analysis of data

Statistical differences between results were analyzed by two-tailed unpaired student's t -test or one way ANOVA and a P value of less than 0.05 was considered significant.

Results

Differential effects of FFAs bound to HSA on β -cell viability

HIT-T15 cells were treated with different types and amounts of FFAs mixed with HSA for 24 h and the cell viability was determined as described in Materials and methods. Two saturated FFAs, palmitate (C16:0) and stearate (C 18:0), showed significantly lowered cell viability with increasing FFA concentrations. Palmitate showed 12.2%, 26.4%, 40.9%, and 49.5% decreases in cell viability at HSA/FFA molar ratios of 1:1, 1:2, 1:4, and 1:8, respectively (Fig. 1) and stearate caused 17.5%, 30.0%, 43.7%, and 46.4% decreases at the same HSA/FFA molar ratios compared to the control group, which was treated with HSA only ($P < 0.05$), (Fig. 1). However, myristate (C14:0), a saturated FFA, showed no significant changes (less than 5%) in cell viability compared to the control at the same HSA/FFA molar ratios (Fig. 1).

Elaidate (C18:1), a trans-monounsaturated FFA, showed minimal effects on cell viability (1.9%, 7.7%, 5.0%, and 1.0% decreases at HSA/FFA molar ratios of 1:1, 1:2, 1:4, and 1:8 respectively), (Fig. 1), and palmitoleate (C16:1), a cis-monounsaturated FFA, also showed similar results as shown in Fig. 1 of 2.0%, 5.6%, 7.2%, and 5.1% decreases compared to the control, respectively (statistically not significant). Also, the effects of the monounsaturated fatty acid oleate on cell viability were considered as minimal at particularly HSA/FFA molar ratios of 1:4 and 1:8 (8.9% and 5.7% decreases, respectively, ($P < 0.05$ vs. control) when compared to its saturated counterpart, stearate at the same molar ratios (Fig. 1).

Effects of csA and ZVAD-FMK on FFA-induced β -cell death

We further examined whether the observed effects of palmitate and stearate complexed with HSA on the cell viability were due to apoptosis or not, by using two apoptosis inhibitors, csA and ZVAD-FMK. Additionally, we conducted a comparative study of these apoptosis inhibitors with FFAs in the presence of HSA and BSA. We used a FFA concentration of 0.4 mM mixed with 0.1 mM HSA and/or BSA as the treated control, in the presence of the apoptosis inhibitors as experimental variables. After the experiment was conducted, we assayed the cell viability. Treatments with FFAs and apoptosis inhibitors in the presence of HSA vs. BSA did not show any significant differences on cell viability changes (Fig. 2). As a positive control, cells were treated with the classical apoptotic inducer, staurosporine, which showed significant decreases in cell viability vs. untreated control (HSA only), by 40.1% and 36.2% decreases in the presence of HSA or BSA, respectively. This staurosporine-induced cell death was neither prevented by csA nor by the caspase inhibitor, ZVAD-FMK. Cells treated with the two FFAs and csA did not show significant changes on cell viability when compared to cells treated with only the two FFAs, palmitate and stearate (7.4% and 6.2% decreases in cell viability, respectively in the presence of HSA, and 3.1% and 4.6% decreases for BSA, respectively). In contrast, ZVAD-FMK co-treated with FFAs and HSA significantly enhanced cell viability when compared to the treated controls of palmitate or stearate with HSA, by 16.7% and 13.2% increases, respectively. For the case of BSA, ZVAD-FMK only led to significant 13.2% increase in cell viability in the presence of palmitate when compared to treated control while the 8.5% increase in the presence of stearate was not statistically significant.

Apoptosis is involved in FFA-induced β -cell death

To further confirm the possible role of apoptosis in FFA-induced β -cell death, we carried out apoptotic DNA laddering experiments. DNA laddering is indicative of a hallmark of apoptosis. A comparison was also made between the presence of HSA vs. BSA in the absence or presence of the various apoptotic inhibitors. We observed no detectable DNA

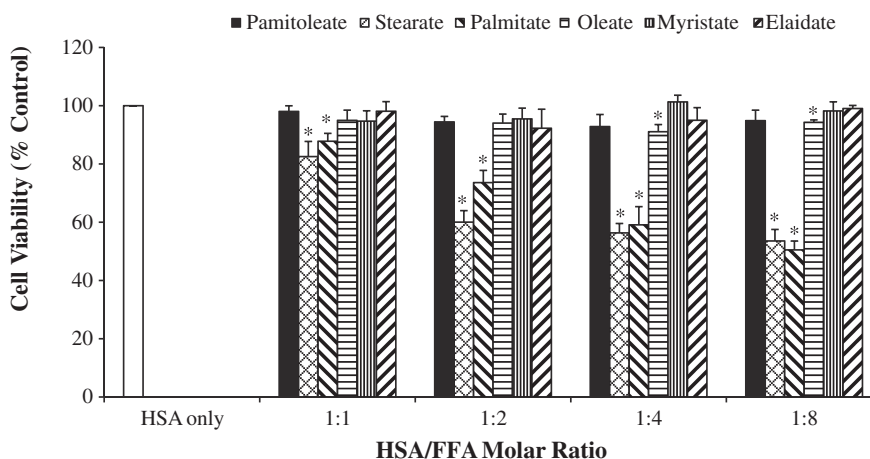


Fig. 1. Effects of HSA/free fatty acid (FFA) complexes on HIT-T15 cell viability. Cells were treated with 0.1 mM HSA alone or with the various HSA/FFA complexes at various molar ratios (1:1, 1:2, 1:4, and 1:8) for 24 h. Cell viability was determined by MTT assay as described in [Materials and methods](#). Results are expressed as percentage of HSA only (control) treatment set at 100%. The values represent the mean \pm S.D. of 3 independent experiments performed in duplicate. * $P < 0.05$ vs. control.

laddering in control cells (Fig. 3; lanes C1–C6) that contained either HSA or BSA only (C1 and C2, respectively), or in the presence of csA (C3 for csA and HSA, and C4 for csA and BSA), and ZVAD-FMK (C5 for ZVAD-FMK and HSA, and C6 for ZVAD-FMK and BSA). As a positive control, the classical apoptotic inducer, staurosporine showed a smear indicative of extensive DNA degradation in the presence of HSA or BSA (C7 for staurosporine and HSA and C8 for staurosporine and BSA, respectively). There was faint DNA band smear in the presence of HSA/palmitate (P1) while BSA/palmitate (P2), HSA/palmitate + csA (P3) and, BSA/palmitate plus csA (P4) had observable DNA laddering. A similar result was seen in stearate treatments (S1–S4), except that HSA/stearate (S1) had elaborate DNA laddering in comparison to HSA/palmitate (P1). In the presence of ZVAD-FMK in both the palmitate (P5 and P6) and stearate (S5 and S6) treated cells, there was no observable DNA laddering which was a similar result as to that exhibited by the control cells treated with either HSA or BSA in the presence of ZVAD-FMK only (C5 and C6, respectively).

Oleate and linoleate are cytoprotective against palmitate-induced cell death

In an attempt to simulate physiological conditions where FFAs usually exist as mixtures, we examined the effects of palmitate alone or

in the presence of individual unsaturated FFAs (palmitoleate, oleate, linoleate, linoelaidate, or CLA) on β -cell viability. The fatty acid concentrations used was 0.4 mM since at this concentration, palmitate had a notable cell-death inducing effect and thus all other fatty acids were also tested at the same concentration and all mixed with 0.1 mM HSA. As shown in Fig. 4, palmitate caused significant reduction on cell viability by 27.1% when compared to HSA-only treated control cells ($P < 0.05$). However, palmitoleate, elaidate, linoelaidate, and CLA had minimal changes while oleate and linoleate, when compared to the control, caused increases on cell viability by 21.6% and 24.9%, respectively. The cell death induced by palmitate was completely negated by the presence of oleate or linoleate when compared to the HSA/palmitate treated control cells. Interestingly, HSA/palmitoleate or elaidate mixtures when either was in combination with palmitate caused further reductions on cell viability from the HSA/palmitate treated control cells (8.4% and 15.2% decreases, respectively).

24-hour exposure of cells to oleate decreases insulin secretion

Long-term (24 h or longer) exposure of β -cells to fatty acids has been shown to cause reduced insulin secretion (Yaney and Corkey, 2003). However, most studies used mainly BSA/FFA complexes, so we

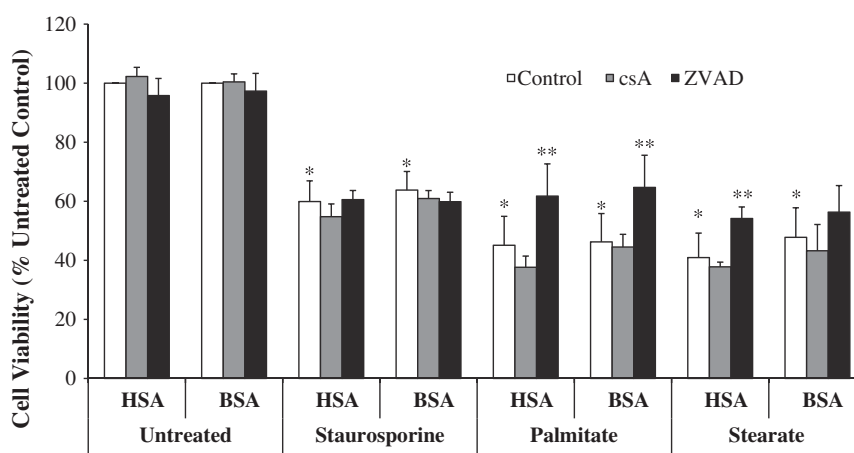


Fig. 2. Effects of cyclosporin A (csA) and caspase inhibitor (ZVAD-FMK) on palmitate and stearate-induced cell death on HIT-T15 cells. Cells were treated with palmitate or stearate at 1:4 molar ratios of HSA/FFA and/or BSA/FFA for 24 h in the absence and presence of 5 μ M csA or 50 μ M ZVAD-FMK. Also, cells were treated in the absence or presence of 2 μ M staurosporine in the presence of 0.1 mM of HSA or BSA. Untreated cells received 0.1 mM HSA and/or BSA in the absence and presence of csA or ZVAD-FMK. Cell viability was determined by MTT assay as described in methods. Results are expressed as percentage of untreated control (HSA and/or BSA only) set at 100%. The values represent the mean \pm S.D. of 3 independent experiments performed in duplicate. * $P < 0.05$ vs. untreated control, ** $P < 0.05$ vs. treated control.

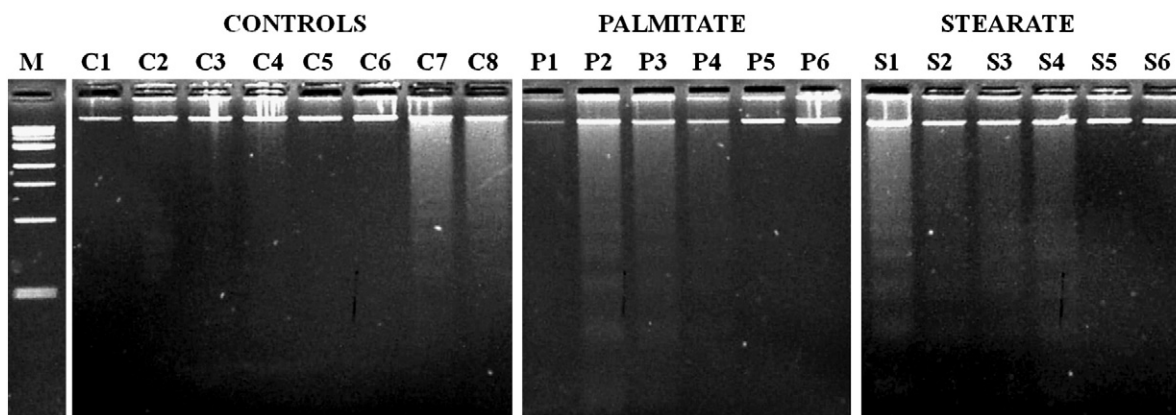


Fig. 3. Apoptotic DNA laddering analysis in ethidium-bromide stained agarose gel after fatty acid treatments. HIT-T15 cells were treated for 24 h in the presence of 0.1 mM HSA only (C1), 0.1 mM BSA only (C2) or in the presence of 5 μ M cyclosporin A (C3 for HSA, and C4 for BSA) or with the caspase inhibitor, ZVAD-FMK at 50 μ M (C5 for HSA, and C6 for BSA). Lanes C7 and C8 represent cells treated with 2 μ M of staurosporine in the presence of 0.1 mM of HSA and/or BSA, respectively. The middle and last panels represent palmitate (0.4 mM) and stearate (0.4 mM) treated cells, respectively in the absence or presence of various test compounds (P1, S1 HSA/FFA only; P2, S2 BSA/FFA only; P3, S3 HSA/FFA + csA; P4, S4 BSA/FFA + csA; P5, S5 HSA/FFA + ZVAD-FMK; P6, S6 BSA/FFA + ZVAD-FMK). DNA was extracted after treatment incubations and 2 μ g DNA was resolved in a 1.5% agarose gel. Lane M represents 1 kb DNA marker.

sought to determine HSA's effect on HIT-T15 cells, using 0.1 mM HSA co-treated with different concentrations of oleate (1:1, 1:2, 1:4, and 1:8 HSA/FFA molar ratios). We chose oleate because it is one of the most abundant FFA in human plasma (Richieri and Kleinfeld, 1995) and also shows minimal cytotoxicity as observed in our cell viability assays. A significant reduction in insulin secretion was observed (Fig. 5) with increasing molar ratios of HSA/FFA (8.8%, 45.1%, 39.0%, 37.7% decreases compared to the control treated with HSA only, $P < 0.05$).

Various HSA mutants show differential effects on FFA-induced β -cell death

Having shown the differential effects of fatty acids in the presence of HSA on cell viability, we attempted to further characterize the effects of modified interactions between HSA and FFA on cell viability. We hypothesized that the changes in fatty acid-binding sites of HSA might change the concentration of FFA_u, which is available to cells and thus responsible for different effects on cell viability. We used various HSA mutant proteins complexed to the FFAs, oleate and palmitate, at a concentration of 0.4 mM (1:4 ratio of HSA/FFA) and assayed cell

viability after 24-h incubation. Fig. 6 shows the results on assessment of the cell viability using the MTT assay kit by which reduction in absorbance at 570 nm is indicative of loss of viability and vice versa and the results shown are in percentages relative to the control (rHSA and FFA co-treatment). We found that among the 5 HSA mutant proteins tested, the double mutant R410A/Y411A showed significant increase on cell viability when treated with either oleate (28.4% increase) or palmitate (24.8% increase) compared to the control group treated with rHSA/FFA complexes ($P < 0.05$). Treatment of mutant proteins, R410A, W214L, and W214L/Y411W complexed with oleate showed significant reductions on cell viability (28.8%, 37.0%, and 14.2% decreases, respectively) compared to the control treated with rHSA/oleate complexes ($P < 0.05$). Also, the mutants, W214L and W214L/Y411W complexed with palmitate showed significant reductions on cell viability when compared to control group treated with rHSA/palmitate complexes (35.3%, and 32.9% decreases, respectively, $P < 0.05$). However, R410A complexed to palmitate did not show similar significant reductions on cell viability as shown with oleate treatment (14.7% decreases). Furthermore, the HSA mutant Y411A showed no significant effect whether complexed with oleate or palmitate (3.1% increase, and 6.2% decrease, respectively).

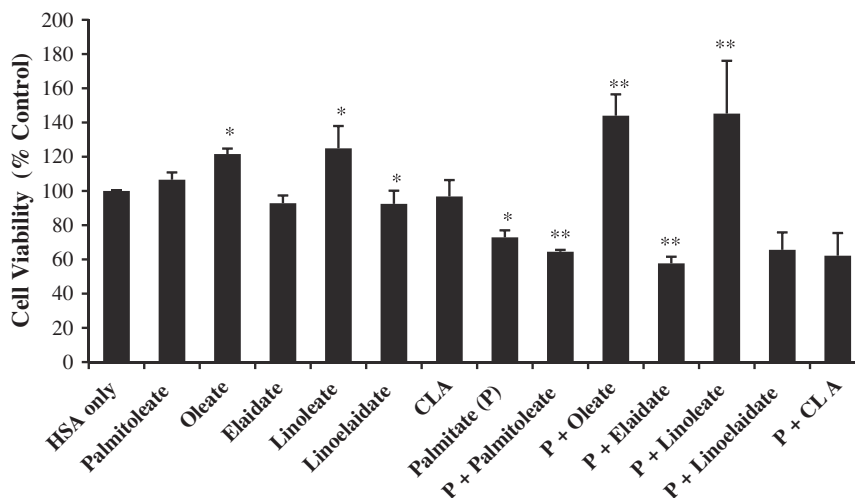


Fig. 4. Effects of unsaturated FFAs on palmitate-induced β -cell death in the presence of HSA. Cells were treated with 0.1 mM HSA alone or with the various individual unsaturated FFAs at concentrations of 0.4 mM with or without 0.4 mM palmitate for 24 h. Cell viability was determined by MTT assay as described in Materials and methods. Results are expressed as percentage of HSA only (control) treatment set at 100%. The values represent the mean \pm S.D. of 3 independent experiments performed in duplicate. * $P < 0.05$ vs. control, ** $P < 0.05$ vs. HSA/palmitate.

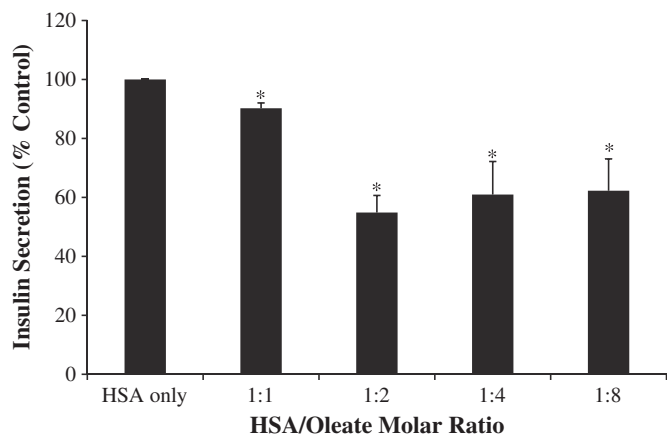


Fig. 5. Effects of different HSA/oleate molar ratios on insulin secretion from HIT-T15 cells. Cells were treated with 0.1 mM HSA alone or the various molar ratios of HSA/Oleate complexes (1:1, 1:2, 1:4, and 1:8 molar ratios) for 24 h. Insulin secretion was quantified by ELISA method and values of insulin secretion were normalized in ng per mg cell protein. Results are expressed as percentage of insulin secreted from cells treated with HSA only (control) set as 100%. The values represent the mean \pm S.D. of 3 independent experiments performed in duplicate. * $P < 0.05$ vs. control.

Unbound FFA (FFA_u) levels in recombinant HSA/FFA complexes

We also determined the FFA_u levels in complexes of each of the two double mutants, R410A/Y411A and W214L/Y411W as well as the wild type rHSA with either palmitate or oleate using the fluorescent probe ADIFAB in HSA to FFA molar ratios of 1:1–1:6. As shown in Fig. 7, there was a gradual rise in FFA_u levels with increasing total FFA concentrations without changing HSA concentrations and with sharp increases beyond HSA to FFA molar ratios of 1:2. In correlation with the HSA to FFA molar concentrations of 1:4 that were used in cell viability assays, the FFA_u levels in rHSA/oleate, R410A/Y411A/oleate, and W214L/Y411W/oleate complexes were determined to be 71.6 nM, 59.0 nM, and 36.9 nM, respectively. On the other hand the FFA_u levels in rHSA/palmitate, R410A/Y411A/palmitate, and W214L/Y411W/palmitate complexes were 62.6 nM, 38.2 nM, and 44.0 nM, respectively.

Discussion

Our study investigated the effects of FFAs and HSA complexes on HIT-T15 cell viability by incubating the cells with various amounts of

FFAs at concentrations ranges of 0.1–0.8 mM in the presence of a fixed concentration of HSA (0.1 mM). The two principal saturated FFAs of human serum, palmitate (C16:0) and stearate (C18:0), induced cell death under all concentrations tested in a dose-dependent manner, while their cis monounsaturated counterparts, palmitoleate (C16:1) and oleate (C18:1), showed minimal changes on cell viability. Myristate (C14:0), and elaidate (C18:1, a trans unsaturated counterpart of stearate), had no significant effects on cell viability. These results are in good agreement with earlier studies that showed that long-term exposure to β -cells of saturated FFAs of 16 carbon chain length or greater induce β -cell death while unsaturated FFAs have minimal changes on the cell viability in the presence of BSA (Azevedo-Martins et al., 2006; Diakogiannaki et al., 2007; Eitel et al., 2002; El-Asaad et al., 2003; Furstova et al., 2008; Lupi et al., 2002). Furthermore, cytoprotection by the cis unsaturated FFAs against palmitate-induced toxicity was recently shown to be more potent than that by trans unsaturated FFAs (Dhaval et al., 2008). In this respect, we treated cells with mixtures of palmitate and individual unsaturated FFAs in the presence of HSA and found that the cis FFA, oleate and the polyunsaturated fatty acid, linoleate were cytoprotective against palmitate-induced cell death (potentially increasing cell proliferation) while their trans counterparts, elaidate and linoelaidate, were not. In fact, linoelaidate caused further slight reduction in cell viability of palmitate treated cells; palmitoleate, a cis FFA elicited similar results. Our results might be suggestive of a cell line-specific response, as also observed by Lai et al., 2008 (Lai et al., 2008), and the possible deleterious effects might be due to high levels of FFAs. Recent studies have suggested that the toxic effects of palmitate are due to its lower esterification rates and higher conversion rates to ceramide than those of oleate (Cnop, 2008; Cnop et al., 2001).

Of note, apoptosis induced by FFAs has been previously described in β -cells (El-Asaad et al., 2003; Kharroubi et al., 2004; Lupi et al., 2002; Maestre et al., 2003; Shimabukuro et al., 1998) but the mechanistic pathways leading to apoptosis are not well established. Since most of these studies utilized BSA in the presence of FFAs, our studies provide comparative effects of cell viability changes and apoptosis by FFAs in the presence of BSA and HSA. Mitochondrial perturbations are believed to cause plasma membrane disruption and/or apoptosis that cause overall cell death (Kroemer et al., 1998). In the event of apoptosis, the mitochondrial permeability transition pore (MPTP) is known to form in the contact site between inner and outer mitochondrial membranes, which facilitates the efflux of pro-apoptotic proteins such as cytochrome c (Halestrap et al., 2002; Kroemer et al., 1998). Some studies have also indicated that elevated FFAs, particularly palmitate, induced MPTP formation in β -cells and cytochrome c release (Koshkin et al., 2008;

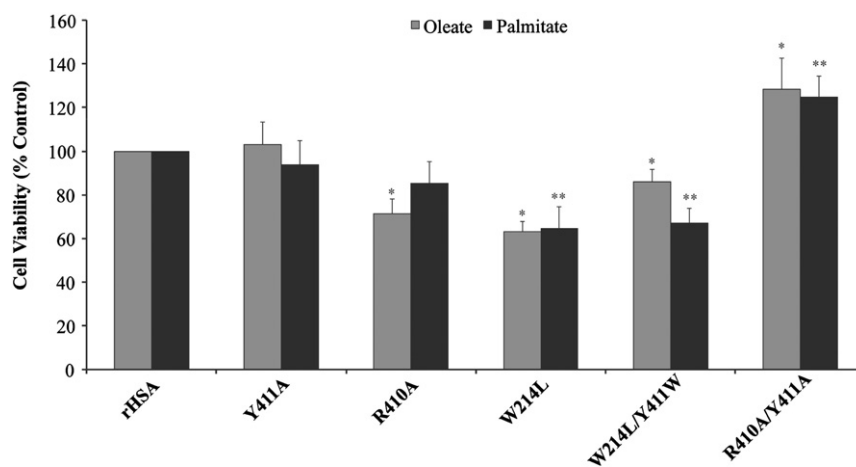


Fig. 6. Effects of various HSA mutants complexed to either oleate or palmitate on cell viability. Cells were treated with 0.1 mM HSA mutants complexed to either oleate or palmitate at 0.4 mM for 24 h. Cell viability was determined by MTT assay as described in Materials and methods. The results are expressed as percentage of cell viability of cells treated with rHSA/FFA complexes set as 100%. The values represent the mean \pm S.D. of 3 independent experiments performed in duplicate. * $P < 0.05$ vs. rHSA/oleate treated cells, ** $P < 0.05$ vs. rHSA/palmitate treated cells.

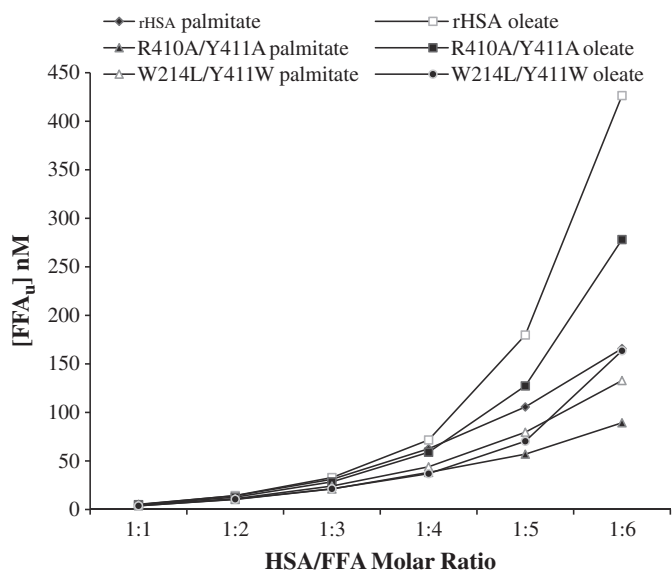


Fig. 7. Unbound free fatty acid (FFA_u) concentrations in complexes of recombinant HSA (rHSA) and its variants bound to palmitate or oleate. FFA_u concentrations were determined as described in methods using the fluorescent probe ADIFAB (0.2 μM) in a fixed albumin concentration of 4 μM and FFA concentrations between 4 and 24 μM. For clarity, results are shown as averages from six determinations excluding ± S.D.

Maedler et al., 2003; Maedler et al., 2001; Maestre et al., 2003). Release of cytochrome c into the cytosol activates caspase-induced apoptosis. The immunosuppressive drug csA inhibits MPTP formation thus preventing the release of pro-apoptotic signals (Kong and Rabkin, 2000; Piot et al., 2008; Pritchard et al., 2000). Our results showed that treatment of HIT-T15 cells with two saturated FFAs, palmitate and stearate bound to HSA or BSA in the presence of csA, did not significantly lower the cell death induced by the FFAs. Cell death induced by the classical apoptotic inducer, staurosporine was also not prevented by the presence of csA. However, we found increases in cell viability in the presence of the caspase inhibitor ZVAD-FMK co-incubated with the FFAs, although it did not completely block cell death. We went ahead further to assay for apoptotic DNA fragmentation, and we observed that csA did not block DNA fragmentation but ZVAD-FMK prevented the DNA fragmentation, thus indicating that other non-caspase-dependent pathways might be responsible for FFA-induced cell death, accounting for the overall cell death. Some earlier systems have also indicated a switch from apoptosis to necrosis by ZVAD-FMK (El-Assaad et al., 2003) in FFA-treated cells, while other studies are now examining the possible roles of autophagy (Choi et al., 2009; Fujitani et al., 2010) in β-cell death or survival. Furthermore, the involvement of ER stress on exposure of β-cells to FFA and its induction of apoptosis (Cunha et al., 2008; Diakogiannaki et al., 2008; Karaskov et al., 2006; Lai et al., 2008; Laybutt et al., 2007) have now been revealed as possible pathways that might be parallel to those stemming from mitochondrial dysfunctions.

On the other hand, as previously stated in the results section that oleate proved to be less cytotoxic in comparison to palmitate, and since oleate is one of the most abundant FFAs in human serum, it was pivotal to show the effects of oleate on insulin secretion from β-cells under the HSA/FFA molar ratios used in the viability assays. Our results showed that HSA/oleate complexes in the presence of a 7 mM glucose concentration caused a significant reduction in insulin secretion. Previous studies have shown that long-term exposure to FFAs inhibited glucose-stimulated insulin secretion by lowering the rate of insulin gene expression (Dubois et al., 2004; Jacqueminet et al., 2000; Moffitt et al., 2005; Sako and Grill, 1990).

We also postulated that changes in HSA affinity for FFAs might affect the fraction of FFA_u in plasma which in turn induces different cellular effects. We examined this aspect in β-cell viability using rHSA proteins

with specific mutations of key amino acid residues in FFA-binding sites of subdomains 2A and 3A of HSA. Our results showed that among the five HSA mutant proteins used, the double mutant R410A/Y411A caused a significant reduction in cell death induced by palmitate and oleate compared to the control comprising rHSA/FFA complexes. This might indicate that this mutant protein strongly binds to FFAs and thus decreases the fraction of FFA_u available to the β-cells. This was in agreement with an earlier study in that this mutant complexed with oleate showed a significantly reduced rate of oleate transport into HepG2 cells and thereby lowered apoB secretion by 70.4% (Ha et al., 2006). In particular, these results suggest that double substitution involving changes of the polar amino acids arginine and tyrosine at positions 410 and 411 to alanines enhanced binding, thus implying that hydrophobicity might be the major ligand-binding force for binding of FFA to HSA. This was an interesting result since X-ray crystallographic data has shown that R410 and Y411 are the key amino acids in the binding pocket of subdomain IIIA of HSA (Simard et al., 2005). In contrast, the single mutant Y411A did not produce significant changes in cell viability, indicating that the single substitution of small hydrophobic alanine alone did not cause significant changes in the FFA-binding pocket. The single mutant R410A complexed to palmitate also had no significant effect, but when it was complexed to oleate, it had no improvement on the viability. This suggests that the R410A HSA mutant might more strongly bind to palmitate than to oleate. Interestingly, the W214L HSA mutant showed a significant reduction in cell viability indicating that these changes were unfavorable for FFA binding. Therefore, lowered affinity to FFAs consequently increased the FFA_u pool to the β-cells which resulted in deleterious effects on the cell viability. Furthermore, our FFA_u level determination in rHSA/palmitate or oleate complexes showed a pattern of higher FFA_u concentration profiles when compared to R401A/Y411A complexed to either of the two FFAs. These FFA_u concentrations were all less than 100 nM in complexes of molar ratios below 1:4 of HSA to FFA and approximately in the range of previous determinations (Richieri et al., 1993; Richieri and Kleinfeld, 1995). These results are consistent with other results which further support that the enhanced effects on cell viability of the double mutant R410A/Y411A when complexed to FFAs are due to the reductions in cytotoxic FFA availability to β-cells, thus increasing cell viability. However, it is noteworthy that rHSA or its mutants/palmitate complexes were more cytotoxic than those of oleate, and yet their FFA_u profiles were not drastically different. Also, the FFA_u profiles of the other tested representative double mutant, W214L/Y411W to FFA complexes were lower than the rHSA/FFA complexes, yet it elicited reduced cell viability from the rHSA/FFA treated cells. Detailed analyses of the effects of this mutant HSA on cell viability is beyond the scope of this study and therefore further studies are required to clarify these results. Previous studies have shown that FFA_u levels are stronger determinants of insulin secretion (Warnotte et al., 1999) and degree of β-cell cytotoxicity (Cnop et al., 2001) than total FFA concentrations. Thus, the differential effects of the rHSA mutants on cell viability in the presence of FFAs suggest that HSA variants might play a role in modulating the FFA_u fractions that is more relevant under higher levels of circulating FFAs such as in obese subjects. There have been very few studies that have attempted to study FFA binding to genetic variants of HSA. Although a few noted increased FFA binding (Minchiotti et al., 2008), majority of the mutations had none or less effect, especially those on the surface of the protein (Otagiri and Chuang, 2009). Therefore, while the existence of other genetic HSA variants in the populace remains to be screened or unknown, our study presents a possible mechanism on what alterations on HSA has on FFA binding since we carried out amino acid substitutions in key FFA-binding pockets of HSA.

Conclusion

In conclusion, our studies showed that various FFAs mixed with HSA have differential effects on HIT-T15 cell viability, which depends

on the degree of saturation and chain length of FFAs. Furthermore, we showed that palmitate and stearate had adverse effects on the cell viability that casA and caspase inhibition could not reverse, irrespective of the presence of HSA or BSA. However, caspase inhibition blocked apoptotic DNA fragmentation, thus indicating that FFA-induced β -cell death is not due to any single mechanism of cell death. Insulin secretion was also reduced after 24-hour exposure of β -cells to HSA/oleate complexes. Also, we showed that changes in key amino acids residues in FFA-binding sites of HSA resulted in altered cell viability and that these changes might be due to modified HSA binding with FFA, as observed in the FFA_u profiles. Our study suggests a possible role of HSA polymorphism in FFA-induced changes in β -cell metabolism.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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