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Cell Culture Models of Fatty Acid Overload: Problems and Solutions

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Abstract

High plasma levels of fatty acids occur in a variety of metabolic diseases. Cellular effects of fatty acid overload resulting in negative cellular responses (lipotoxicity) are often studied *in vitro*, in an attempt to understand mechanisms involved in these diseases. Fatty acids are poorly soluble, and thus usually studied when complexed to albumins such as bovine serum albumin (BSA). The conjugation of fatty acids to albumin requires care pertaining to preparation of the solutions, effective free fatty acid concentrations, use of different fatty acid species, types of BSA, appropriate controls and ensuring cellular fatty acid uptake. This review discusses lipotoxicity models, the potential problems encountered when using these cellular models, as well as practical solutions for difficulties encountered.

Keywords

Lipotoxicity; lipids; fatty acid metabolism; fatty acids; cells; tissues

1. Introduction: *In vitro* lipotoxicity models

High circulating levels of free fatty acids (FAs) are associated with obesity and the development of Type 2 diabetes, among other diseases [1]. The term ‘lipotoxicity’, coined in 1994 [2], has been used for studies in which the negative effects of chronically elevated FAs

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on cellular function are monitored by adding supraphysiological concentrations of lipid. These *in vitro* studies enable researchers to investigate lipotoxicity in cell cultures, in a manner that is similar and complimentary to *in vivo* obesity models. This review covers biological effects of lipotoxicity, distinct lipotoxicity models and discusses characteristics of the FAs and BSA-conjugation systems used that should be taken into account when establishing these protocols. We begin by highlighting selected *in vitro* biological effects of FA overload in a few different tissues and cell types with which the authors of this review have experience.

1.1. Lipotoxicity in the liver

The liver plays a central role in lipid metabolism. Indeed, hepatic steatosis occurs when high concentrations of circulatory FAs reaching the liver and *de novo* lipogenesis are not counterbalanced by FA oxidation or lipid export as lipoproteins [3]. Lipid droplets (LD) are formed comprised of neutral lipid depots, surrounded by a monolayer of amphipathic lipids in a stable and controlled emulsion in the cytoplasm [4]. The neutral core in hepatic LDs contains mainly triacylglycerol [3; 5]. Interestingly, the accumulation of LDs in the liver could be a physiological and protective response. Overactivation of triacylglycerol synthesis favors steatosis but does not promote insulin resistance, while inhibition of triacylglycerol synthesis reduces steatosis and increases liver damage [6; 7]. Liver FA buffering is central not only for hepatic, but also whole body homeostatic maintenance [8]. In obesity, nearly 75% of hepatic FAs derive from white adipose tissue lipolysis and diet [9], and thus originate from circulating lipids.

Experimental strategies have been developed to understand how hepatocytes respond to lipid overload. Models in which hepatocyte lipid handling is challenged involve modulating FA concentration, acyl-chain lengths and unsaturation, and dissociated or not from other circulating signals such as hormones and cytokines. These *in vitro* assays induce FA accumulation in human hepatocyte lines 18–24 h after incubation in the presence of high lipid concentrations [10–18]. Some authors [13; 14] do not observe any differences in the accumulation induced by saturated or unsaturated FAs, while others [15–17] find that monounsaturated FAs promote higher lipid accumulation compared to saturated FAs when alone or as mixtures, in a dose-dependent manner. On the other hand, saturated FAs consistently promote lipotoxic cell death [8; 19]. In the HepG2 cell line, palmitate and stearate, but not monounsaturated palmitoleate or oleate, promote ER stress that promotes JNK pathway activation, leading to Bim-mediated Bax activation and apoptosis [14; 20]. These results were also observed using rodent cell lines and primary cultures [17; 21; 22]. Ceramide production is increased with saturated FA overload and could mediate FA-induced apoptosis. However, studies modulating ceramide production in hepatocytes yield conflicting results [23; 24]. Interestingly, palmitate is able to increase hepatocyte proinflammatory cytokine production. These cytokines may mediate cell death and contribute toward liver injury during steatosis progression [10; 12]. Kupffer cells, the liver resident macrophages, may also participate in liver lipotoxic effects by promoting local inflammation. Pardo and colleagues [25] elegantly used conditioned medium from macrophages pre-treated with palmitate on primary hepatocyte cultures, and found that it promoted ER stress, apoptosis, and insulin resistance. Oleate-conditioned medium did not

have the same effects [25]. These results associating lipotoxicity and inflammation are in agreement with *in vivo* data in which liver steatosis is accompanied by cell death and inflammation, markers for undesirable disease progression [5; 26].

Lipotoxicity in the liver may impair hepatic function, promoting metabolic disease. Saturated, but not monounsaturated, FAs induce insulin resistance in hepatocytes [11; 13; 27; 28], a possible consequence of lipid-induced post-translational modifications [29; 30] and/or palmitate-stimulated proteasomal degradation of insulin cascade proteins [13]. These metabolic effects are accompanied by mTORC1 activation [27], mitochondrial network fragmentation [18], and increased oxidative imbalance [11; 28; 31]. Egnatchik [32] demonstrated that palmitate promotes calcium release from the ER and its subsequent uptake by mitochondria; this disturbs the citric acid cycle and causes oxidative imbalance and cell death. Indeed, the toxic effects of palmitate were suppressed with a calcium chelator [32]. Overall, liver lipotoxicity studies have shown a link between steatosis, inflammation and insulin resistance, caused primarily by saturated FA. *In vivo*, these effects of high circulating lipids will interact with changes occurring in other key metabolic regulators.

1.2. Lipotoxicity in pancreatic beta cells

Sako et al. [33] first showed time-dependent effects of lipid infusion on isolated perfused pancreatic islet insulin secretion: it was enhanced after a short-term, 3 hour, lipid infusion, while 48 hours of lipid infusion blunted glucose-stimulated insulin secretion. An *in vitro* rat islet model of lipotoxicity [34] in which the effect of chronic exposure to palmitate, oleate, or octanoate for 48 hours was studied showed increased basal insulin secretion and inhibited secretion stimulated with high glucose levels. In a subsequent study, Bollheimer et al. [35] found similar changes. This study also found that although oleate potentiated preproinsulin mRNA production in islets, there was an inhibitory effect on proinsulin translation regulation by glucose. Grill and Qvigstad [36] discuss early work on effects of FAs on beta cell function and insulin secretion in numerous experimental *in vitro* and *in vivo* models. While the effects of FAs are similar on insulin secretion, effects on other parameters such as ER stress and apoptosis are disparate. As discussed above for liver, saturated FAs have been shown to be more detrimental when compared to unsaturated FAs in islets. Indeed, Maedler et al. [37] showed that unsaturated FAs oleate and palmitoleate display a protective role by restricting the apoptotic and anti-proliferative effects of saturated FA palmitate on human islets. Ceramide signaling mediated palmitate-induced apoptosis, as the use of fumosinin B1 abrogated the effects of palmitate. Palmitate downregulated the expression of Bcl2 and increased cytochrome c release, while addition of palmitoleate or oleate (which increased Bcl2 expression), or inhibition of ceramide synthesis, prevented the release of cytochrome c.

Palmitate has also been shown to promote to ER stress. Karaskov et al. [38] examined the consequences of chronic exposure of the insulin-secreting cell line INS1 to palmitate or oleate and determined that palmitate, but not oleate, stimulated ER stress, with increased phosphorylated PERK, nuclear ATF4 protein, XBP-1, and GADD153/CHOP. However, Grp78/94 and PDI ER chaperone protein levels were unchanged as compared to the BSA controls. Palmitate treatment resulted in marked morphological changes in ER, mitochondria and Golgi. This study, however, was performed in a serum free system, which would

increase sensitivity of the cells. Consistent with these findings are studies showing that unsaturated FAs and expression of desaturases (SCD1) are protective against palmitate-induced damage of beta cells [39; 40]. Nevertheless, chronic exposure to oleate and palmitate remains detrimental to beta cell function by inducing mitochondrial fragmentation and inhibition of autophagic flux [41; 42].

Oprescu et al. [43] showed the effects of oleate on insulin secretion *in vivo*, via 48 hour infusion by applying hyperglycemic clamps and *ex vivo*, in response to glucose at nonstimulatory, basal, and elevated glucose levels. In this study, oleate impairment of glucose-stimulated insulin secretion was abrogated by treatment with the antioxidants N-acetylcysteine, taurine and tempol, indicating a role of redox signalling in this process.

An *in vitro* model of glucolipotoxicity exposing primary mouse beta cells to palmitate and 20 mM glucose for 48 hours (glucolipotoxicity) found increased mitochondrial fragmentation associated with a reduction in mitochondrial fusion [41, 44]. Further characterization of the relative contributions of palmitate as compared to glucose in INS1 cells revealed that palmitate is primarily contributing toward mitochondrial fragmentation. Glucose displayed a synergistic amplifying effect. Conversely, treatment with glucose alone displayed a minimal effect [41].

Autophagy, which serves as a quality control mechanism for clearance of damaged cellular components, is also affected by lipotoxicity. Beta cell-specific ATG7 deficient mice exhibit impaired autophagy and glucose tolerance, as well as a more severe phenotype under high fat diets when compared to WT mice [45]. Furthermore, loss of ATG7 results in increased oxidative imbalance, mitochondrial dysfunction, reduced insulin content, and reduced beta cell mass [46; 47]. Given that lipotoxicity produces a similar phenotype, an *in vitro* model of lipotoxicity was used to investigate chronic effects of FAs on autophagic turnover [42]. Primary or clonal beta cells were exposed either to palmitate or oleate complexed to BSA and added in media containing 1% FBS. This resulted in inhibition of autophagic flux via impairment of lysosome acidification along with impaired bioenergetics and mitochondrial fragmentation. Lowering FBS concentrations is likely to stimulate autophagy in some cells, thus determination of changes of autophagosome formation and/or survivial upon reduction of FBS is suggested.

1.3. Lipotoxicity in the brain

Obesity correlates with saturated FA accumulation in the hypothalamus, the brain area controlling energy balance [48; 49] and hypothalamic lipid overload is accompanied by alterations in neuronal and glial cell function, leading to overfeeding and uncontrolled body weight gain [50]. *In vivo* studies suggest these changes are related to saturated FA-induced inflammatory and ER stress responses [51]. Indeed, the inhibition pathways leading to these responses alleviates several metabolic impairments caused by saturated FA accumulation [51]. *In vitro* studies using neuronal cell lines have shown that palmitate, the predominant saturated FA in fat-rich diets, rapidly induces ER stress followed by insulin resistance and loss of energy balance control [52–54]. These effects are prevented by chemical chaperones or oleate pre-treatment, indicating that palmitate-induced ER stress can directly modulate hypothalamic neuronal function [52; 54]. Notably, palmitate treatment is often accompanied

by elevated levels of neuronal cell death, that must be considered when analyzing these data [52; 55; 56]. Studies have shown that palmitate can activate inflammatory markers in hypothalamic neurons in parallel with alterations in insulin signaling [53; 57–59]. Conversely, some authors suggest these cells are resistant to palmitate-induced inflammation and insulin resistance [60; 61]. In addition to differences between cell lines, these conflicting results may be a consequence of discrepancies in the lipotoxicity protocol. One relevant difference is the FA:BSA ratio used, that range from 3:1 to more than 6:1 [52; 53; 60]. Since FA:BSA ratios determine lipid availability and hence toxicity, neuronal inflammation could be a consequence of excessive cell death instead of direct activation of this pathway. While palmitate lipotoxicity assays have yielded conflicting results, *in vitro* experiments involving mono and polyunsaturated FA effects on neuronal inflammation have consistently shown that hypothalamic neurons treated with oleate and docosahexaenoic acid (DHA) present attenuated inflammatory cytokine expression and reduced cell death [53; 61].

Hypothalamic saturated FA accumulation also promotes inflammation by increasing the number of reactive microglia, the macrophages of the brain [51]. Microglial activation is triggered early on after rodent exposure to high fat diets and persists with the establishment of the obese phenotype [62]. *In vitro* studies have demonstrated that saturated FA treatment rapidly promotes an inflammatory response in these cells [51; 63–65]. Indeed, primary microglia and BV2 cells (a microglial cell line) incubated with palmitate and stearic acid increase inflammatory signaling and cytokine secretion [51; 63; 64]. In contrast, some studies describe palmitate-induced increases in anti-inflammatory marker expression in BV2 cells [66; 67]. This divergence could be a consequence of the use of a different lipotoxicity protocols, as there is a more than five fold difference in the FA:BSA ratio between these studies. To evaluate the contribution of different cells in lipotoxic hypothalamic inflammation, Valdearcos and colleagues [51] treated organotypic hypothalamic slice cultures with oleate and palmitate and found that only palmitate-treated slices presented increases in cytokine release and neuronal stress markers. These effects were absent in microglia-depleted slices, suggesting saturated FA-induced microglial activation triggers hypothalamic inflammation. Collectively, these data indicate that cell culture models are valuable to expand the understanding of pathways promoting hypothalamic dysfunction upon saturated FA overload. However, results in these systems must be compared carefully to *in vivo* models since saturated FA treatment is often accompanied by pronounced cell death. As such, it is advisible to use a combination of saturated and unsaturated FA to monitor physiologically relevant biological effects. Furthermore, the experimental definition of lipotoxicity remains loose when referring to protocols used to deliver lipids to cells. Different labs employ protocols that vary in characteristics such as FA complexation (usually to BSA), FA species used and concentrations, making comparisons difficult.

2. Fatty acid transport and uptake

Lipids are usually transported primarily by means of association with proteins in the blood forming hydrosoluble lipoprotein particles that allow for controlled recognition and delivery of lipids such as triglycerides and cholesterol to tissues [68]. Nonetheless, nonesterified FAs are transported mainly with serum albumin [69; 70], an abundant protein present in plasma and interstitial fluids able to bind FA and other lipophilic molecules [69; 70]. Albumin-FA

interactions maximize the amount of FA transported and cleared from the circulation [70; 71]. Studies conducted using primary hepatocyte cell cultures show that, in the presence of albumin, palmitate uptake by cells is more efficient [72], an effect that is not mediated by receptors [70; 73]. In *in vitro* systems, albumin-promoted FA delivery follows the "pseudofacilitation-model" [73; 74] in which FAs are delivered as function of their unbound concentration in the medium at a physiological concentration of albumin (~200 μM). At lower concentrations (< 150 μM), delivery will depend on albumin concentration.

FA uptake by cells is proposed to occur through membrane diffusion or facilitated transport by membrane proteins [75; 76]. Both types of transport require the FA movement from an aqueous solution to another, through a lipid bilayer, involving mechanisms that are still under debate [76–82]. Transport rates depend on FA size and number of unsaturations, membrane lipid composition, and protein composition [82; 83]. CD36 is the membrane receptor not consensually suggested to be a fatty acid translocator. The use of CD36 inhibitors AP5055 and AP5258 reduces triacylclygerol accumulation in cultured cells [84]. Some recent findings suggest that transport is probably not dependent on direct mediated FA translocation by CD36, but diffusion would be driven by increased cytosolic demand [81; 85].

Inside the cell, FAs will act as energy sources, signaling molecules, incorporated into membranes or as triacylglycerols [8]. Incorporation of lipids into the cell as well their intracellular fate under lipotoxic conditions may influence metabolic changes observed in different cellular systems - the balance between lipid oxidation and storage may be determinant toward lipotoxicity [86]. Interestingly, fatty acid oxidation may be decreased in cell cultures of muscular origin simply because of the lack of a physical workload in the *in vitro* system. Indeed, impaired fatty acid oxidation and consequent increment in lipid storage causes lipotoxicity in tissues with limited fat accumulation capacity such as skeletal muscle and the heart [86; 87]. Confirming this hypothesis, inhibition of FA oxidation in macrophages with etomoxir exacerbates palmitate-induced inflammation and ER stress [88]. Etomoxir is an irreversible inhibitor of acyl carnitine formation, preventing FA uptake by mitochondria, in which their oxidation occurs. On the other hand, increases in FA oxidation prevent inflammation and apoptosis in neuronal and muscle cells, respectively [58; 89; 90, 91]. Fatty acid binding proteins (FABPs) are intracellular lipid chaperones that bind to FAs and help redistribute them to exert their biological functions [92]. FABPs have important functions in metabolic control, and pathways sensitive to lipid signaling can be studied by inhibiting them [92; 93].

Overall, BSA is a suitable method to deliver FA to cells since it is similar to the physiological system of nonesterified FA transport. However, care must be taken to adapt concentration of the molecules to avoid discrepancies in delivery kinetics. Since uptake *per se* cannot be effectively inhibited, modulation of FA fate inside the cell may be an interesting strategy to understand the signaling pathways sensitive to FA. Furthermore, precaution must be taken regarding the type of BSA used and the preparation of FA/BSA solutions, as will be discussed in detail below.

3. Types of BSA

The type of BSA used in cellular lipotoxicity models must be pondered carefully, since differences in isolation methods, purity and contaminants can interfere in the biological effects of lipid preparations. The choice of a specific BSA type and consistent maintenance of this type is thus necessary when planning experiments.

Albumin purity is the main feature to be considered since it directly alters lipid binding and availability. Indeed, different impurities in BSA used are able to modulate added FA binding to this protein [69; 94]. Contaminant bovine FAs, for example, are present in all less purified BSA samples, and should be avoided since they compete with added lipids for the albumin hydrophobic clefts. These endogenous FAs can induce biological effects themselves [69; 94; 95]. It is thus imperative to use FA-free BSA. FA removal is promoted through charcoal treatment or organic solvent precipitation [95]. Indeed, a wide variety of FA-free albumin is commercially available, with different purity levels. Commercial FA-free BSA usually binds lipids more efficiently than Charcoal-purified albumin, resulting in a smaller fraction of unbound FAs after conjugation [94]. This issue should be taken into consideration when comparing the biological effects of varying BSA concentrations.

In addition to endogenous bovine FA, BSA preparations can carry other contaminants depending on the isolation method employed. In fact, different ligands such as hormones, drugs and toxins can remain bound to albumin after isolation [71]. Moreover, globulins and other blood proteins are present in several types of commercial BSA [96]. The biological activity of these contaminants should be considered, since they can modulate the FA effects in different systems. Further albumin purification requires appropriate methods to remove residual contaminants. For example, globulins can be removed by heat treatment and glycoproteins by affinity chromatography [95; 96]. A wide diversity of albumin contaminant combinations can be found in commercially available BSA. The Sigma-Aldrich® catalog, for example, currently offers 48 types of BSA with different contaminant and purity grades, depending on the isolation/purification method applied.

4. Preparing FA/BSA solutions

Lipid and albumin conjugation and solubilization must be verified when preparing FA/BSA solutions. Indeed, differences in these formulations can generate disparate responses in the same system. For instance, microglial cells treated with palmitate at a FA:BSA ratio of 2:1 present an anti-inflammatory profile, while a 10:1 ratio induces a strong pro-inflammatory profile [9; 64; 66]. Therefore, FA/BSA protocols must be carefully controlled to ensure reliable results, and the ratios used must be described.

Long-chain FA solubilization may be difficult due to low solubility in aqueous solutions. The strategies employed to solve this issue usually include the use of organic solvents and heating. In fact, several groups have used ethanol to dissolve FA at concentrations ranging from 12.5 to 95% and heating to 50–70°C [64; 66; 97–100]. The final concentration of organic solvent has to be considered since it can affect cell viability [101]. Ethanol concentrations should not exceed 0.05% in the cell culture medium [102] and this solvent

should be avoided when studying FA effects on cells able to oxidize this alcohol [103]. In addition to ethanol, dimethyl sulfoxide (DMSO) has been employed to solubilize long-chain FA [41; 104], although the cytotoxicity of this solvent must also be considered, and concentrations over 1:200 (v:v) should be avoided. Heated aqueous solutions have also been used to dissolve FA [73; 105; 106]. In these protocols, the complete solubilization of the FA is achieved at 70°C, when the solution becomes clear [106]. The addition of NaOH can improve solubility when the acidic form of FA is used instead of the sodium salt [73; 105].

BSA is normally dissolved directly in water or cell culture media due to its high solubility in aqueous solutions [69]. However, concentrated BSA stock solutions can be hard to produce. Concentrated BSA solutions can be produced by heating the preparation to 37°C [106]. However, albumins undergo denaturation when heated to 50°C or above, and form water-insoluble aggregates [96; 107]. Thus, over-heating must be carefully avoided. It is worth highlighting that defatted or FA-free BSA is more sensitive to heat denaturation, whereas presence of FAs in BSA stabilizes BSA from heat denaturation [108].

Lipid conjugation to albumin represents a critical step in the preparation of FA/BSA solutions. Changes in temperature, time and FA:BSA ratios can produce solutions with different FA availability and, consequently, altered biological activity [94]. The temperatures used to complex lipids to albumin vary from 37°C to 70°C [66; 94; 105; 106]. Temperatures higher than 50°C, however, can lead to the formation of albumin aggregates, increasing FA availability in the solution [64; 107]. Lower temperatures, in turn, decrease FA solubility. This is particularly relevant when lipids are dissolved in aqueous or low ethanol solutions.

In respect to conjugation time, FAs can be added to BSA immediately before cell treatment or lipids can be pre-complexed to albumin and stored prior to use [94; 97; 106]. Pre-conjugation is frequently performed under continuous stirring with serial additions of FA to BSA solutions, a process that can last from minutes to hours [94; 106]. Of note, pre-complexed solutions can result in a lower content of unbound FA, allowing the use of reduced albumin concentrations to induce the same effects compared to freshly conjugated preparations [94].

5. FA:BSA ratios versus free FA levels

During lipid/albumin conjugation, FA:BSA ratios should be considered since they determine FA availability. In healthy humans, serum FA:BSA ratios range from 1:1 to 3:1. These ratios can be higher than 5:1 in disease states [109]. Accordingly, the use of high FA:BSA ratios in experiments enhances the biological effects of lipids [8; 9]. A variable to keep in mind under these conditions is cell death, since decreased viability can introduce several experimental biases.

BSA has six (or possibly seven) high affinity binding sites for FAs and is thus an efficient carrier serving to substantially increase the solubility of FAs in aqueous solutions [110]. Because of this characteristic, it is important to report the FA to BSA molar ratio or, ideally, to report the fraction of bound to unbound FA, which may vary in different preparations and create difficulties in reproducibility of biological effects. For example, the amount of

unbound FA has been shown to be the main contributor to changes in pancreatic beta cell insulin secretion [111]. While it is possible to estimate the fraction of unbound FA by using previously developed algorithms [69; 112], tools that enable the direct experimental measurement of unbound FA are now available. Measurements of FA fractions can be achieved by several means such as the use of colorimetric Nonesterified Fatty Acid (NEFA) kits consisting of an enzymatically-coupled assay with a detection sensitivity of 15 μM (Cell Biolabs, [113]) to determine the total free FA concentration. This measures non-esterified fatty acids, both bound and unbound to BSA. This colorimetric probe measures total free fatty acids since it uses a reaction that consumes the unbound FA, and so more will be released due to changes in the equilibrium. An alternative is the use of FA fluorescent probes such as Acrylodan-Labeled Intestinal Fatty Acid Binding Protein, which has a detection sensitivity of 1 nM for unbound, non-estereified FA [114; 115]. It will only detect unbound FAs, since it has a lower affinity does not modify these FAs.

The use of FA-free BSA to deliver FA to the cell makes the selection of proper controls challenging. Cell culture sera typically consist of 2% albumin, to which FAs obviously also bind, thus necessitating a reduction in the serum concentration of the media to avoid a change in the FA:BSA ratio. Addition of FA-free BSA to 1% serum has been deemed sufficient to maintain the endogenous BSA of the media low, while still supporting the necessary cellular function that the fetal bovine serum provides [42; 116]. These experiments require controls, such as the presence of BSA without added FA, which may not accurately reflect real biological systems. For example, while studying the effects of lipotoxicity on beta-cell secretory function, we have found that overnight incubation with BSA promotes decreased insulin secretion, in a dose dependent manner (Shirihai et al., unpublished observations). On the other hand, Straub et al. [117] have shown that rat islet incubation in BSA significantly augments insulin secretion. The differences from our findings may be due to time-dependent changes. The second study system is more reflective of acute changes, since islets are incubated in BSA for 4 hours [117], whereas the first study incubates the islets with the FA/BSA solution overnight.

Haber et al. [118] looked into the possibility that FA-free BSA may serve as a sink for cellular lipids. This study measured FA efflux from the beta-cells, leading to the depletion of lipid content and impairment of insulin secretory function. The addition of FA-free BSA in the control media traps and absorbs free FAs, thereby altering secretory function [118]. An experimental example of this effect is shown in Figure 1, in which cellular fatty acid content in fetal bovine serum is clearly higher than in BSA devoid of added FA. It is valuable to mention that BSA has also been shown to have antioxidant properties, which may affect beta-cell function, since oxidants have been implicated as a signal for beta-cell insulin secretion [119; 120].

Thus, it seems necessary to reevaluate the use of FA-free BSA as a control in cellular lipotoxicity experiments. Titrating lower concentrations of FA to the BSA in control experiments, avoiding endogenous lipid quenching, may be an adequate control. An alternative solution is to dissolve the FA of interest and add directly to media containing FBS as in [34] or to complex the FA directly to the BSA in FBS, as used by Erion et al.

[121]. The advantage of this method is that it minimizes the undesirable effects of FA-free BSA while maintaining the serum concentration at 10% during culture conditions.

Incorporation of lipids into the cell under conditions of lipotoxicity may influence metabolic changes observed in different cellular systems. A relatively straightforward way to examine accumulation of lipid is the use of fluorescence-based methods such as FA uptake kits with dyes dyes such as Nile Red for measurement of lipid droplets or BODIPY® lipid probes to assay different length FAs. Alternatively, the use of radiolabeled ^{14}C FA may also be employed to measure uptake of the FA of interest. Dubikovskaya et al. [91] overview various methods to measure FA uptake.

6. Mixtures of different types of FA

FAs have a gross structural similarity, but have distinct physical and biological features depending on the number of carbons and unsaturations in the acyl chain [122]. The capacity of a FA to bind to BSA relates to hydrophobic and electrostatic interactions between the FA and one of the 6–7 possible binding sites within the protein [110; 13]. Therefore, saturated FA and unsaturated FA will bind to BSA with different affinities at the same sites, and this will lead to competition between these FA [73; 123; 124].

It is important to keep in mind that the biologically active fraction of the FA/BSA preparation is the unbound free FA. Free FA levels depend on binding constants of the FA for each binding site, FA:BSA molar ratios, and FA interactions with other molecules, including other FAs and BSA itself [125; 126]. Figure 2 shows how free FA quantities vary distinctly with different FAs when presented with BSA. Despite the well-known interaction of BSA with FAs, drugs, and some other endogenous ligands such as bilirubin [71], little information is available about the kinetics of *in vitro* FA binding in the presence of two or more types of FA and how they influence each other´s binding. This scarcity of knowledge is possibly due to the difficulty in developing specific and sensitive methods to deal with the chemical similarities among different FAs in the nanomolar concentration range.

Kleinfeld's group has described methods to calculate free FA fractions in mixtures using fluorescent probes [126–128]. They demonstrated that human serum is composed of at least 40 different kinds of free FAs. Markedly, the unbound fraction does not represent the total fraction of a given FFA [126]. A similar observation was made when comparing saturated with unsaturated FAs in an *in vitro* mix. Huber and co-workers [127] found that in a mixture containing arachidonate, linoleate, oleate, palmitate, and stearate, with the total molar fractions of, 10, 15, 24, 21, and 29%, the free FA fractions observed were 23, 21, 20, 2 and 15%, respectively. This outcome is expected, since the solubility and albumin affinity for each type of FA is different. Thus, to obtain the same unbound fraction, more stearate is needed than arachidonate, since the first has higher affinity for albumin than the latter [127].

These discrepancies observed in total versus free FA fractions are important to highlight questions about *in vitro* assays that use BSA as a vehicle. If the experiment is constructed to keep the initial FA:BSA ratios constant and compare palmitate versus oleate or mixtures containing both, for example, free FA concentrations will be different among these

preparations. If the aim is to test the same free FA fraction for each type of FA, however, the total FA fractions will be different and measurements should be conducted to establish these conditions. For both objectives, the “vacancy” of some binding sites in albumin can directly interfere with the cell. As seen in Figure 1, these sites can serve as interaction spots for cellular lipids and decrease their bioavailability. To maintain results produced by different labs comparable, it is critical to acknowledge the exact composition of FA and BSA in the methodology and, preferentially, measure and indicate the initial free FA concentrations, when possible.

7. Immune responses to FA/BSA preparations

Various studies have suggested that high fat diets are direct inducers of obesity-related low grade inflammation [8; 129]. This has led to an increase in the number of studies aiming to uncover mechanisms by which FAs can activate immune cells. FA/BSA preparations have thus been widely employed in assays testing the inflammatory effects of lipids. Nonetheless, some other effects of these protocols should be considered to ensure correct result interpretations.

Lipotoxicity is one of the main side effects which should be analyzed, since cell viability is compromised at elevated FA:BSA ratios [8; 94]. Indeed, cell damage and death are classical inducers of immune cell activation to a pro-inflammatory profile [128–130]. In this sense, the inflammatory response verified in the presence of high FA:BSA proportions can be promoted by cell death instead of a direct activation of inflammatory responses by the FA. Therefore, an accurate control for cell viability is necessary to prevent data misinterpretation when working with FA/BSA solutions and inflammation.

An additional side effect that is usually neglected is the intrinsic immune reactivity of BSA. Evidence suggests that conformational and linear epitopes of BSA are potent inducers of allergic reactions [131]. In fact, BSA epitopes are fully recognized by sensitized IgE from sensitized subjects, and are able to induce T cell proliferation [131–133]. This could be an undesired side-effect when the immunological properties of lipids are tested in *in vivo* infusion studies. Furthermore, a specific BSA epitope has been related to autoimmune reactions against pancreatic islet cells [134]. However, most of these studies were conducted in human models, and the immune reactivity of BSA in rodents remains to be elucidated.

Contaminants carried by albumin, such as globulins and endotoxins, are also able to activate immune cells and should be avoided to prevent biases in data interpretation [135]. Indeed, we find that BSA itself induces the expression of pro-inflammatory cytokines by innate immune cells (Kowaltowski et al., unpublished observations). Corroborating this, BSA-treated human adipocytes present an increase in the expression of IL-6, IL-8 and TNF-α [136]. Interestingly, methyl-β-cyclodextrin, which can be used to replace BSA as a FA carrier, also promotes increases in the expression of pro-inflammatory cytokines by adipocytes [137]. This suggests that lipid homeostasis plays a central role in the control of inflammatory marker expression.

Altogether, these data highlight the necessity of controls when working with FA/BSA solutions. Within this perspective, BSA must always be present in the control groups. Some of these effects can be prevented by reducing albumin content in the preparations, such as when using pre-complexed FA/BSA solutions [94].

8. Conclusions

When conducting lipotoxicity studies, many factors should be considered, such as those compiled in Table 1, which lists technical issues to be considered in FA:BSA lipotoxicity models, as well as solutions that have been adopted for these problems. We find it important that the effect of BSA be controlled by including an experimental group with identical concentrations of BSA and organic solvent used to dissolve the FA. However, as we have discussed throughout this manuscript, albumin has biological effects itself [136; 138; 139], including lipid quenching, that should be considered specifically for each model. If endogenous lipid quenching occurs, determining an appropriate concentration of serum or titrating low quantities of FA to inhibit lipid quenching by FA-free BSA are possible solutions to prevent this undesired effect. Furthermore, we believe FA and BSA concentrations, as well FA:BSA molar ratios, must be described in the methodology. Since the biologically-active fraction will be free FA [127], this will create awareness of differences between laboratory protocols. It is equally important to consider the amount of albumin inherently present in the serum, if the cells require serum supplementation concomitantly to FA treatment [105]. Finally, FAs can be cytotoxic [8] in a manner dependent on the type of cell, type of FA, FA and BSA concentrations, and exposure time [140–142]. The treatment effect must therefore be separated from the cytotoxic effect by measuring cell viability.

Altogether, working with FA and BSA does require some special attention to control all the particularities of the system, as outlined in Table 1, but also provides unique mechanistic insights into the toxic effects of lipids. Our objective in compiling these particularities and discussing solutions, is to contribute toward better interpretation and reproducibility of lipotoxicity findings in the future.

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Highlights

- Lipid-treated cells are used as models to study mechanisms of metabolic disease
- Lipotoxicity has been monitored in distinct tissues and cells, with varying protocols
- Fatty acids are poorly soluble and must be adequately prepared and conjugated
- The use and choice of conjugation systems (such as BSA) must be carefully considered

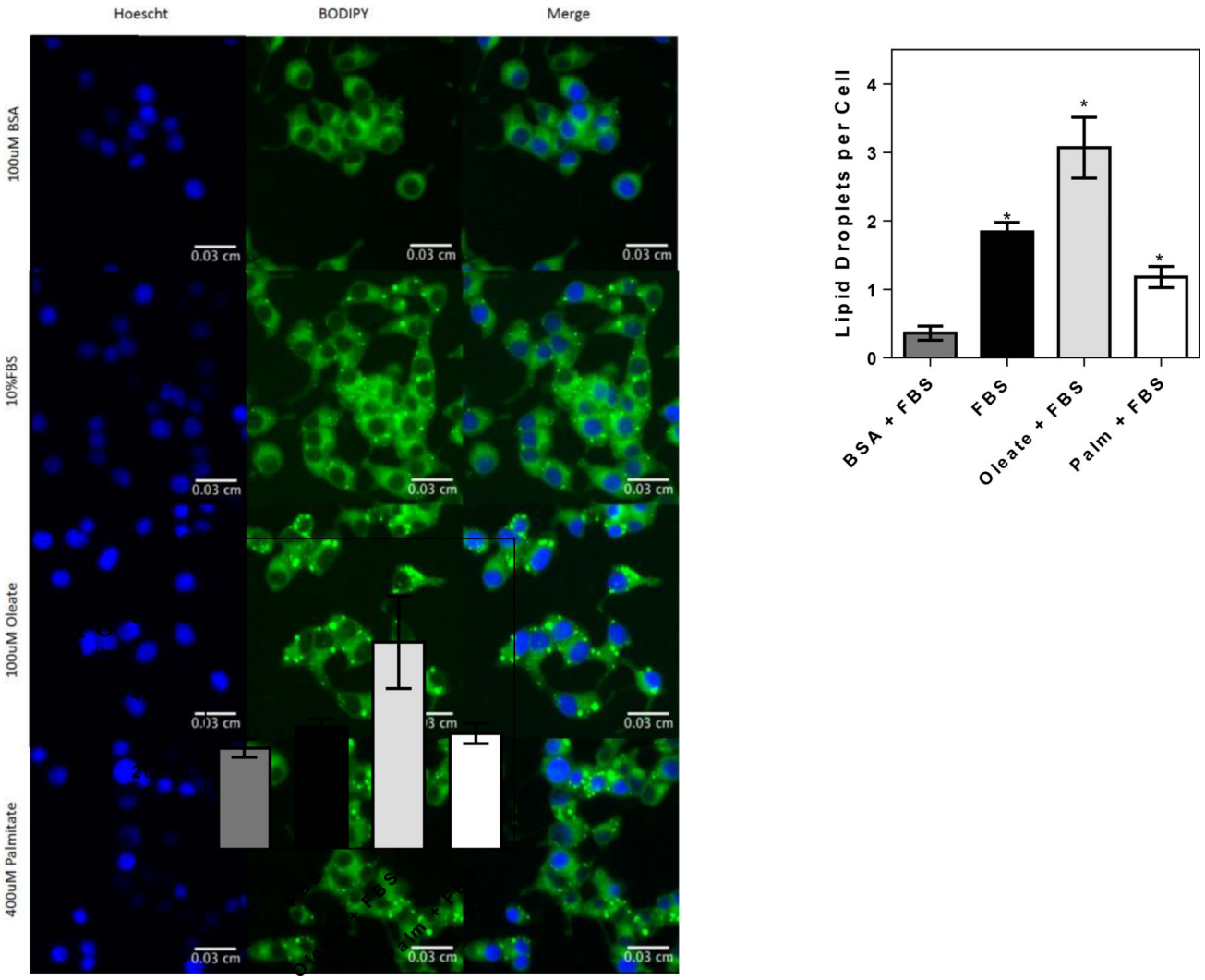

Figure 1. BSA decreases cellular lipid content

INS1 cells were grown to 70% confluence in 10% FBS RPMI culturing media, after which they were treated with 100 μM BSA plus 1% FBS or 400 μM palmitate conjugated to BSA/FBS in a 4:1 ratio for 16 h. Oleate (100 μM) was conjugated directly to 10% FBS. Cells were then fixed with 4% PFA for 15 min and stained with Hoechst and BODIPY493 for 15 min. Cells were washed twice with PBS and imaged using the Operetta imager, after which the lipid droplets per cell were quantified. Typical images shown left, quantifications of the number of lipid dropets and lipid droplet area, right. Note the decrease in lipid content with BSA. * $p < 0.05$ versus BSA plus 1% FBS.

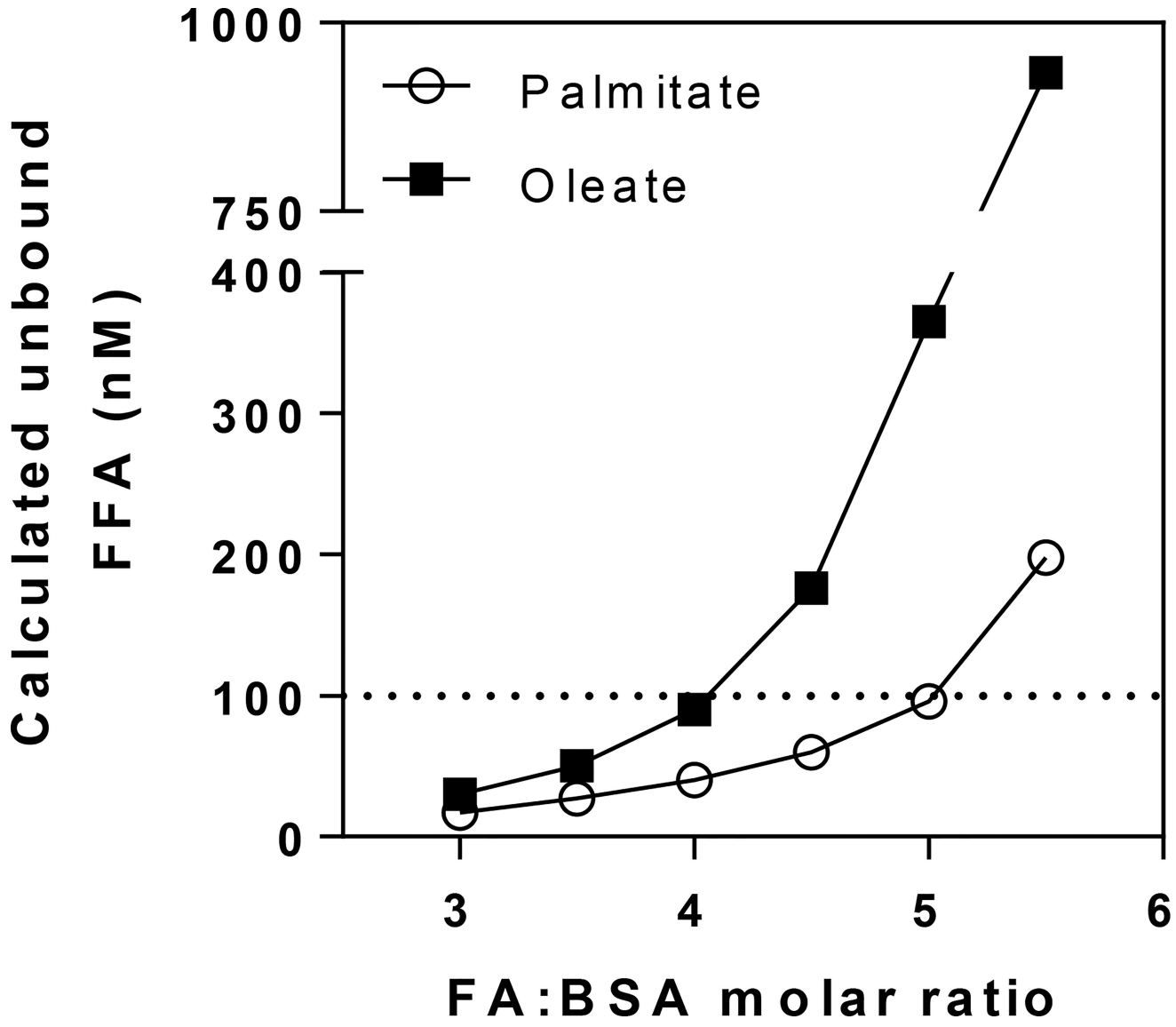

Figure 2. The unbound FFA fraction in solution increases with FA:BSA molar ratios

The unbound fraction was calculated for BSA and palmitate or oleate by multiple stepwise equilibrium analysis as described in [123].

Table 1

BSA/Lipotoxicity Trouble Shooting Checklist

Issue	Solution	References
BSA Purity	Check purification method and source, as well as contaminants described in the product sheet. Consistently use the same BSA type.	[71; 94; 96]
FA solubility	Determine most appropriate solvent and solubility method. Check cell viability.	[94; 102; 106]
Temperature	Determine and maintain appropriate temperature for FA solution preparations, under 50°C for FA/BSA.	[96; 106; 107]
Conjugation time	Maintain consistent time points for serial FA additions.	[94; 106]
FA:BSA Ratios	Use realistic healthy *vs* disease state FA:BSA molar ratios. Check cell viability.	[126; 142]
Unbound FA fraction	Measure FFAs.	[114; 123; 126]
BSA effects in the control groups	Check BSA purity, inflammatory markers and endogenous lipid quenching. Titration of lower FA concentrations to avoid quenching.	[91; 134; 136]
Ensuring that added FAs change intracellular lipid levels	Measure changes in lipid uptake and accumulation.	[15; 91]
Choice of FAs (saturated/ unsaturated)	Check cell viability, differences in FFAs. Ascertain cells can metabolize FA used and the FA choice matches the experimental goal.	[15; 141]