[Letter to the Editor] In vitro use of free fatty acids bound to albumin: A comparison of protocols

Long-chain free fatty acids (FFAs) are important metabolic substrates for energy production and lipid synthesis that are also involved in signaling processes (1,2). Two of the most common fatty acids in humans are the long-chain saturated FFA palmitate (C16:0) and the monounsaturated oleate (C18:1). Western diets rich in fatty acids are associated with increased levels of plasma cholesterol, hepatic steatosis, and a greater risk of cardiovascular disease (3,4). High levels of circulating FFAs, in particular of saturated FFAs, are implicated in insulin resistance and pancreatic β-cell dysfunction, and are predictive of diabetes development (5–8). In vitro exposure to high levels of FFAs leads to lipotoxicity, causing cellular dysfunction and death (5,9).

The low solubility of long-chain FFAs in aqueous solutions represents one of the major limitations for in vitro and in vivo studies. To overcome this problem, FFAs can be conjugated to albumin, allowing the preparation of solutions in the physiological concentration range. The fraction of unbound FFAs accessible for cellular uptake depends on the ratio of total FFAs to albumin (10). Thus, the biological effect of FFAs can be augmented by increasing the FFA concentration or by decreasing the bovine serum albumin (BSA) concentration. The unbound FFA concentration is also determined by the relative affinities of the FFAs for albumin (10). For example, when using a total FFA concentration of 0.5 mM in the presence of 1% w/v (151 µM) BSA, corresponding to an FFA/albumin molar

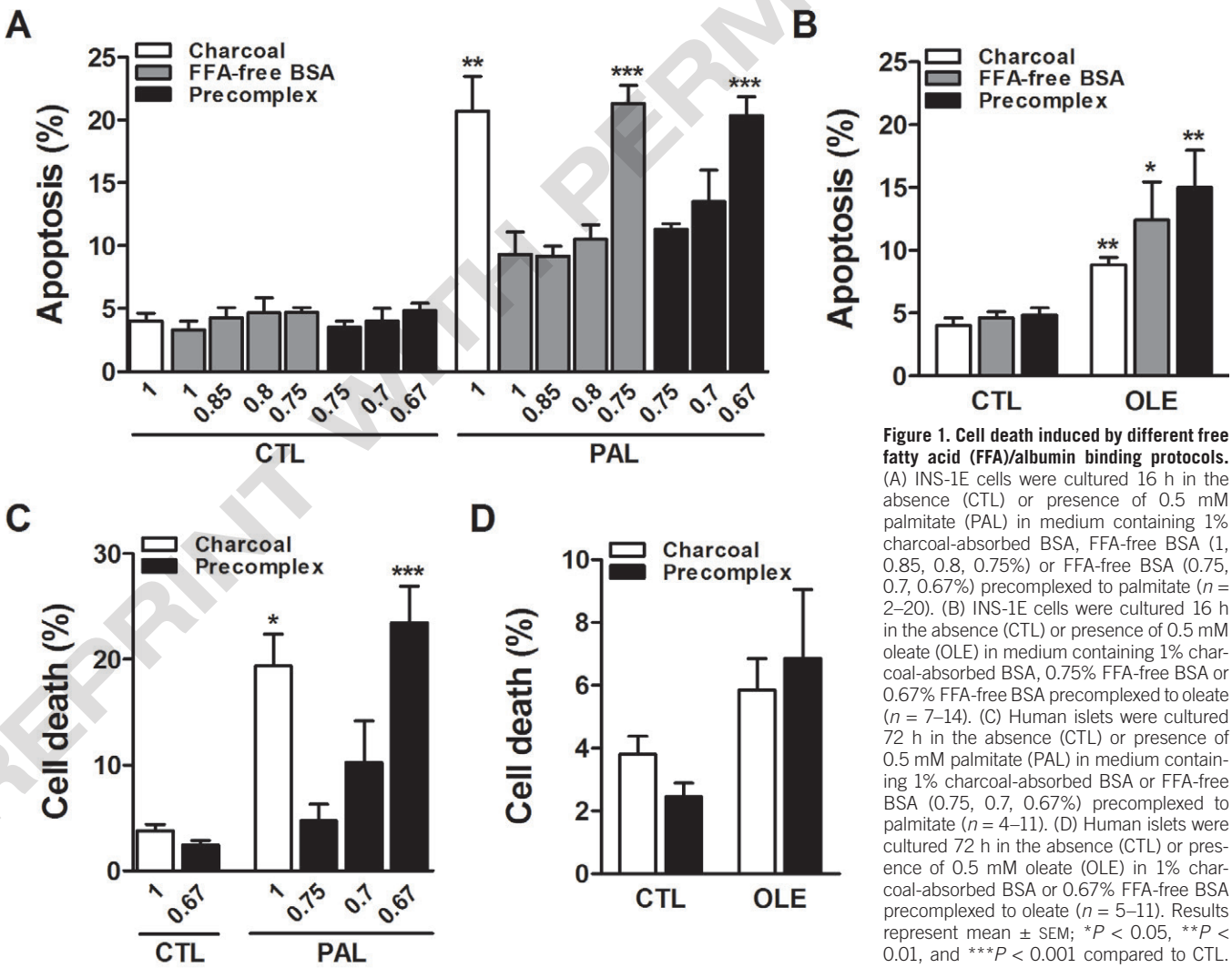

Figure 1. Cell death induced by different free fatty acid (FFA)/albumin binding protocols. (A) INS-1E cells were cultured 16 h in the absence (CTL) or presence of 0.5 mM palmitate (PAL) in medium containing 1% charcoal-absorbed BSA, FFA-free BSA (1, 0.85, 0.8, 0.75%) or FFA-free BSA (0.75, 0.7, 0.67%) precomplexed to palmitate (n = 2–20). (B) INS-1E cells were cultured 16 h in the absence (CTL) or presence of 0.5 mM oleate (OLE) in medium containing 1% charcoal-absorbed BSA, 0.75% FFA-free BSA or 0.67% FFA-free BSA precomplexed to oleate (n = 7–14). (C) Human islets were cultured 72 h in the absence (CTL) or presence of 0.5 mM palmitate (PAL) in medium containing 1% charcoal-absorbed BSA or FFA-free BSA (0.75, 0.7, 0.67%) precomplexed to palmitate (n = 4–11). (D) Human islets were cultured 72 h in the absence (CTL) or presence of 0.5 mM oleate (OLE) in 1% charcoal-absorbed BSA or 0.67% FFA-free BSA precomplexed to oleate (n = 5–11). Results represent mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to CTL.

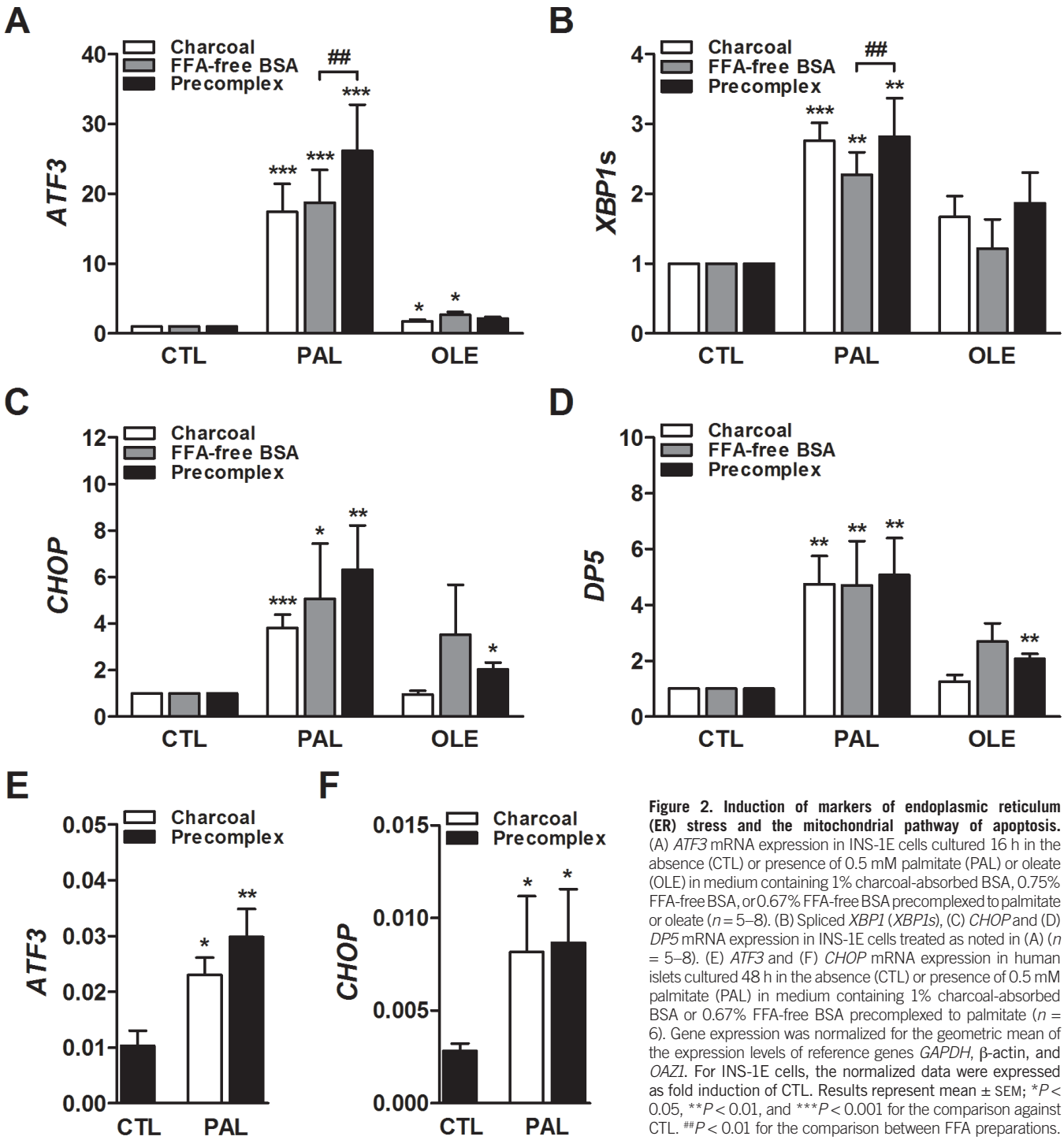

Figure 2. Induction of markers of endoplasmic reticulum (ER) stress and the mitochondrial pathway of apoptosis. (A) *ATF3* mRNA expression in INS-1E cells cultured 16 h in the absence (CTL) or presence of 0.5 mM palmitate (PAL) or oleate (OLE) in medium containing 1% charcoal-absorbed BSA, 0.75% FFA-free BSA, or 0.67% FFA-free BSA precomplexed to palmitate or oleate ($n = 5–8$). (B) Spliced *XBP1* (*XBP1s*), (C) *CHOP* and (D) *DP5* mRNA expression in INS-1E cells treated as noted in (A) ($n = 5–8$). (E) *ATF3* and (F) *CHOP* mRNA expression in human islets cultured 48 h in the absence (CTL) or presence of 0.5 mM palmitate (PAL) in medium containing 1% charcoal-absorbed BSA or 0.67% FFA-free BSA precomplexed to palmitate ($n = 6$). Gene expression was normalized for the geometric mean of the expression levels of reference genes *GAPDH*, β-actin, and *OAZ1*. For INS-1E cells, the normalized data were expressed as fold induction of CTL. Results represent mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for the comparison against CTL. $^{\#\#}P < 0.01$ for the comparison between FFA preparations.

ratio of 3.3, the theoretical unbound palmitate and oleate concentrations are 27 nM and 47 nM, respectively (11).

Under normal physiological conditions, an average of 2 FFA molecules are bound to each albumin molecule in the circulation, corresponding to concentrations of unbound FFAs less than 20 nM. The FFA/albumin molar ratio can rise to as high as 6 in pathological conditions (12,13). In experimental systems designed to evaluate mechanisms of lipotoxicity relevant to pathophysiological states, variable and sometimes high molar ratios are used (14–23). Ratios greater than 5 should be avoided as they may exceed FFA solubility, potentially inducing artifacts. In spite of the crucial role of albumin in determining biologically available FFA levels, the BSA concentration or FFA/albumin molar ratio are frequently not mentioned in scientific reports (24,25).

Albumin preparations may contain variable amounts of contaminants (26), which can affect FFA binding. Charcoal treatment of BSA allows the removal of

some contaminants, including endogenous bovine FFAs (26), and has been used by our group in previous publications (11,27–30). FFA-free albumins are commercially available and have a high degree of purity. FFAs can be added directly to albumin-containing culture medium or be precomplexed with albumin before being added to the medium.

Here we present a systematic comparison of the effects of different preparations of palmitate or oleate and BSA on lipotoxicity in pancreatic β-cells. Charcoal-absorbed BSA and FFA-free BSA were used to prepare FFA solutions, as described in the Materials and methods section in the Supplementary Material. Compared with charcoal-absorbed BSA, FFA-free BSA has the advantage of having less residual FFAs bound to it. Exposure of INS-1E cells for 16 h to 0.5 mM palmitate in 1% FFA-free BSA-containing medium (FFA/albumin ratio of 3.3) induced significantly less apoptosis compared with medium containing 1% charcoal-absorbed BSA (Figure 1A). We therefore asked whether the serial filtration steps that follow the charcoal incubation of BSA affect the final albumin concentration of the medium. The BSA concentration in the 1% charcoal-absorbed BSA preparation was 25% lower compared with 1% FFA-free BSA (n = 3–4, P = 0.03). In keeping with this result, a reduction from 1% to 0.75% FFA-free BSA (FFA/albumin ratio of 4.4) resulted in apoptosis levels comparable to the 1% charcoal-absorbed BSA preparation (Figure 1A). We next prepared FFA solutions by precomplexing palmitate with FFA-free BSA before diluting it into the medium (see Materials and methods section in the Supplementary Material). Compared with FFA stock solutions prepared in ethanol that are typically not kept for more than a month, the FFA-albumin complexes can be prepared in large amounts and stored for several years at -20°C. Precomplexed palmitate (0.5 mM, 0.75% FFA-free BSA) induced less apoptosis compared with the equivalent non-precomplexed preparation (Figure 1A). Similar apoptosis levels were only reached when the BSA concentration was lowered to 0.67% (FFA/albumin ratio of 5.0) (Figure 1A). We hypothesized that during the precomplexing of FFAs to BSA, some of the FFAs form aggregates resulting in lower FFA concentrations.

Oleate (0.5 mM) used in the presence of 1% charcoal-absorbed BSA or 0.75% FFA-free BSA, or precomplexed oleate used in the presence of 0.67% FFA-free BSA, induced comparable levels of apoptosis (Figure 1B), in keeping with the palmitate data. We also compared cell death induced in human islets by precomplexed FFA preparations against 1% charcoal-absorbed BSA, as used in previous reports (28–30). Because primary human islets are less susceptible to lipotoxicity than clonal rat INS-1E cells (29,31,32), islet cell death was assessed after 72 h of exposure. As in INS-1E cells, a reduction in the amount of BSA to 0.67% resulted in similar levels of palmitate- or oleate-induced cell death (Figure 1, C and D). These data illustrate that dose-response experiments are of value for determining suitable experimental conditions. Based on the cell death data, we chose 1% charcoal-absorbed BSA, 0.75% FFA-free BSA, or precomplexed FFAs in the presence of 0.67% FFA-free BSA for further signal transduction studies.

FFAs cause β-cell apoptosis via the induction of endoplasmic reticulum (ER) stress and activation of the mitochondrial pathway of cell death through up-regulation of pro-apoptotic BCL-2 proteins (29). We compared the effect of different palmitate and oleate preparations on the induction of transcription factors that mediate the ER stress response, namely activating transcription factor 3 (ATF3), spliced

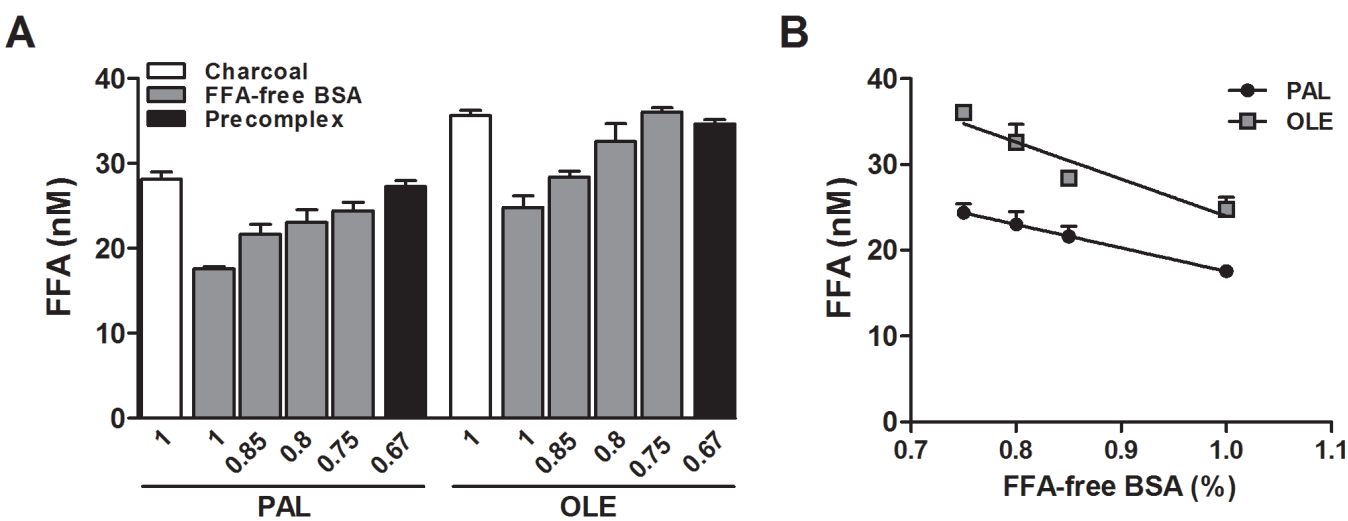

Figure 3. Unbound free fatty acid (FFA) concentrations in different FFA/albumin preparations. (A) Unbound FFA concentrations were measured using the ADIFAB2 probe in 0.5 mM (total concentration) palmitate (PAL)- or oleate (OLE)-containing phenol red–free RPMI medium containing 1% charcoal-absorbed BSA, the indicated concentration of FFA-free BSA, or 0.67% FFA-free BSA precomplexed to palmitate or oleate. (B) Relationship between unbound palmitate or oleate concentrations and the percentage of FFA-free BSA in the solution. Linear regression is shown for palmitate (R^2 = 0.99) and oleate (R^2 = 0.91). Results represent mean ± SEM of 3–9 independent experiments.

X-box binding protein 1 (XBP1s) and C/EBP homologous protein (CHOP), as well as the proapoptotic BCL-2 family member death protein 5 (DP5; also known as harakiri). Exposure to palmitate using the different BSA preparations induced similar levels of *ATF3*, *XBP1s*, *CHOP,* and *DP5* mRNA expression (Figure 2, A–D), with somewhat greater induction for precomplexed palmitate. The activation of the PERK and IRE1 branches of the ER stress response and the induction of *DP5* mRNA by oleate is milder compared with palmitate (28,29). Overall, gene expression was modified by oleate in a comparable manner across the different oleate/BSA preparations (Figure 2). Human islets exposed to palmitate with 1% charcoal-absorbed or 0.67% FFA-free BSA showed a similar induction of *ATF3* and *CHOP* mRNA expression (Figure 2, E and F). Taken together, the gene expression data are in keeping with the apoptosis findings.

The results obtained with different FFA and BSA preparations might be explained by changes in the unbound FFA concentrations. These concentrations can be theoretically calculated using the multiple stepwise equilibrium model (10,11), but more recently it has become possible to directly measure them using the fluorescent probe ADIFAB2, which is an acrylodan-derivatized intestinal fatty acid binding protein (33). Increasing the concentration of FFA-free BSA in the medium resulted in decreased unbound concentrations of FFAs (Figure 3A) in a linear manner across the range of concentrations examined (Figure 3B). In keeping with the comparable cellular responses to FFAs (Figures 1 and 2), FFAs used in the presence of 1% charcoal-absorbed BSA, 0.75% FFA-free BSA, or precomplexed FFAs used in the presence of 0.67% FFA-free BSA resulted in similar unbound FFA concentrations, ~26 nM for palmitate and ~35 nM for oleate (Figure 3A). These data confirm that the FFA-BSA precomplexing process probably results in some loss of FFAs due to aggregation.

The measurement of unbound FFA concentrations allowed us to compare apoptosis induced by similar unbound concentrations of oleate (24.8 ± 1.4 nM) and palmitate (24.4 ± 1.0 nM) (n = 3–6, P = 0.9). As expected, the saturated FFA palmitate induced significantly more cell apoptosis (17 ± 3%) than unsaturated oleate (7 ± 1%) (n = 4, $P < 0.001$).

Because of the importance of the unbound FFA concentrations, the use of fetal bovine serum (FBS) in cell culture also deserves consideration. FBS contains albumin and other FFA binding proteins, which will lower unbound FFA concentrations. The albumin concentration of FBS is ~2.5 g/dl. The use of 10% FBS in medium will increase the albumin concentration by 0.25%, which may significantly alter unbound FFA concentrations and cellular effects (Figure 1A and Figure 3A). The mixture of bovine FFAs present in FBS may also affect experimental results. The addition of unsaturated fatty acids is protective against palmitate, at least in part through modulation of the ER stress response (28).

An equimolar combination of palmitate and oleate (total concentration 0.5 mM) in medium containing 0.75% FFA-free BSA resulted in an unbound FFA concentration of 34 nM, a value close to that observed for oleate.

In conclusion, when using FFAs for in vitro experiments, the concentrations and preparation of FFAs and BSA should be carefully considered. Our results demonstrate that the source of albumin and the method of FFA conjugation affect the unbound FFA concentrations and, consequently, cellular outcomes. In publications using FFAs, the FFA and BSA concentrations and the FFA/albumin molar ratio need to be provided. In addition, we recommend measuring unbound FFA concentrations when using FFAs for in vitro experiments.

Author contributions

D.A.C. and M.C. contributed to the experimental design of the study. A.F.O., D.A.C., L.L., and M.I.E. carried out experiments and data analysis. M.B. and P.M. contributed materials. A.F.O., D.A.C., and M.C. wrote the manuscript.

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Competing interests

The authors declare no competing interests.

References

1. **Amri, E.Z., G. Ailhaud, and P.A. Grimaldi.** 1994. Fatty acids as signal transducing molecules: involvement in the differentiation of preadipose to adipose cells. J. Lipid Res. *35*:930-937.
2. **Hara, T., D. Kashihara, A. Ichimura, I. Kimura, G. Tsujimoto, and A. Hirasawa.** 2014. Role of free fatty acid receptors in the regulation of energy metabolism. Biochim. Biophys. Acta *1841*:1292-1300.
3. **Siri-Tarino, P.W., Q. Sun, F.B. Hu, and R.M. Krauss.** 2010. Saturated fat, carbohydrate, and cardiovascular disease. Am. J. Clin. Nutr. *91*:502-509.
4. **Roden, M.** 2006. Mechanisms of Disease: hepatic steatosis in type 2 diabetes--pathogenesis and clinical relevance. Nat. Clin. Pract. Endocrinol. Metab. *2*:335-348.
5. **Cnop, M.** 2008. Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes. Biochem. Soc. Trans. *36*:348-352.
6. **Paolisso, G., P.A. Tataranni, J.E. Foley, C. Bogardus, B.V. Howard, and E. Ravussin.** 1995. A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM. Diabetologia *38*:1213-1217.
7. **Blaak, E.E.** 2003. Fatty acid metabolism in obesity and type 2 diabetes mellitus. Proc. Nutr. Soc. *62*:753-760.
8. **Giacca, A., C. Xiao, A.I. Oprescu, A.C. Carpentier, and G.F. Lewis.** 2011. Lipid-induced pancreatic β-cell dysfunction: focus on in vivo studies. Am. J. Physiol. Endocrinol. Metab. *300*:E255-E262.
9. **Schaffer, J.E.** 2003. Lipotoxicity: when tissues overeat. Curr. Opin. Lipidol. *14*:281-287.
10. **Spector, A.A.** 1975. Fatty acid binding to plasma albumin. J. Lipid Res. *16*:165-179.
11. **Cnop, M., J.C. Hannaert, A. Hoorens, D.L. Eizirik, and D.G. Pipeleers.** 2001. Inverse relationship between cytotoxicity of

free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. Diabetes *50*:1771-1777.

12. **Kleinfeld, A.M., D. Prothro, D.L. Brown, R.C. Davis, G.V. Richieri, and A. DeMaria.** 1996. Increases in serum unbound free fatty acid levels following coronary angioplasty. Am. J. Cardiol. *78*:1350-1354.
13. **Nehra, V., P. Angulo, A.L. Buchman, and K.D. Lindor.** 2001. Nutritional and metabolic considerations in the etiology of nonalcoholic steatohepatitis. Dig. Dis. Sci. *46*:2347-2352.
14. **Hatanaka, M., B. Maier, E.K. Sims, A.T. Templin, R.N. Kulkarni, C. Evans-Molina, and R.G. Mirmira.** 2014. Palmitate induces mRNA translation and increases ER protein load in islet β-cells via activation of the mammalian target of rapamycin pathway. Diabetes *63*:3404-3415.
15. **Lee, S.H., S. Athavankar, T. Cohen, R. Piran, A. Kiselyuk, and F. Levine.** 2013. Identification of alverine and benfluorex as HNF4α activators. ACS Chem. Biol. *8*:1730-1736.
16. **Listenberger, L.L., X. Han, S.E. Lewis, S. Cases, R.V. Farese, Jr., D.S. Ory, and J.E. Schaffer.** 2003. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc. Natl. Acad. Sci. USA *100*:3077-3082.
17. **Chu, K.Y., Y. Lin, A. Hendel, J.E. Kulpa, R.W. Brownsey, and J.D. Johnson.** 2010. ATP-citrate lyase reduction mediates palmitate-induced apoptosis in pancreatic β cells. J. Biol. Chem. *285*:32606-32615.
18. **Ariyama, H., N. Kono, S. Matsuda, T. Inoue, and H. Arai.** 2010. Decrease in membrane phospholipid unsaturation induces unfolded protein response. J. Biol. Chem. *285*:22027-22035.
19. **Martinez, S.C., K. Tanabe, C. Cras-Meneur, N.A. Abumrad, E. Bernal-Mizrachi, and M.A. Permutt.** 2008. Inhibition of Foxo1 protects pancreatic islet β-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis. Diabetes *57*:846-859.
20. **Listenberger, L.L., D.S. Ory, and J.E. Schaffer.** 2001. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J. Biol. Chem. *276*:14890-14895.
21. **Hall, E., P. Volkov, T. Dayeh, K. Bacos, T. Ronn, M.D. Nitert, and C. Ling.** 2014. Effects of palmitate on genome-wide mRNA expression and DNA methylation patterns in human pancreatic islets. BMC Med. *12*:103.
22. **Lai, E., G. Bikopoulos, M.B. Wheeler, M. Rozakis-Adcock, and A. Volchuk.** 2008. Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic β-cells. Am. J. Physiol. Endocrinol. Metab. *294*:E540-E550.
23. **Hatanaka, E., A. Dermargos, A.E. Hirata, M.A. Vinolo, A.R. Carpinelli, P. Newsholme, H.A. Armelin, and R. Curi.** 2013. Oleic, linoleic and linolenic acids increase ros production by fibroblasts via NADPH oxidase activation. PLoS ONE *8*:e58626.
24. **Inoguchi, T., P. Li, F. Umeda, H.Y. Yu, M. Kakimoto, M. Imamura, T. Aoki, T. Etoh, et al.** 2000. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. Diabetes *49*:1939-1945.
25. **Koshkin, V., X. Wang, P.E. Scherer, C.B. Chan, and M.B. Wheeler.** 2003. Mitochondrial functional state in clonal pancreatic β-cells exposed to free fatty acids. J. Biol. Chem. *278*:19709-19715.
26. **Chen, R.F.** 1967. Removal of fatty acids from serum albumin by charcoal treatment. J. Biol. Chem. *242*:173-181.
27. **Cnop, M., J.C. Hannaert, and D.G. Pipeleers.** 2002. Troglitazone does not protect rat pancreatic β cells against free fatty acid-induced cytotoxicity. Biochem. Pharmacol. *63*:1281-1285.
28. **Cunha, D.A., P. Hekerman, L. Ladrière, A. Bazarra-Castro, F. Ortis, M.C. Wakeham, F. Moore, J. Rasschaert, et al.** 2008. Initiation and execution of lipotoxic ER stress in pancreatic β-cells. J. Cell Sci. *121*:2308-2318.
29. **Cunha, D.A., M. Igoillo-Esteve, E.N. Gurzov, C.M. Germano, N. Naamane, I. Marhfour, M. Fukaya, J.M. Vanderwinden, et al.** 2012. Death protein 5 and p53-upregulated modulator of apoptosis mediate the endoplasmic reticulum stress-mitochondrial dialog triggering lipotoxic rodent and human β-cell apoptosis. Diabetes *61*:2763-2775.
30. **Cnop, M., B. Abdulkarim, G. Bottu, D.A. Cunha, M. Igoillo-Esteve, M. Masini, J.V. Turatsinze, T. Griebel, et al.** 2014. RNA sequencing identifies dysregulation of the human pancreatic islet transcriptome by the saturated fatty acid palmitate. Diabetes *63*:1978-1993.
31. **Cnop, M., L. Ladrière, P. Hekerman, F. Ortis, A.K. Cardozo, Z. Dogusan, D. Flamez, M. Boyce, et al.** 2007. Selective inhibition of eukaryotic translation initiation factor 2α dephosphorylation potentiates fatty acid-induced endoplasmic reticulum stress and causes pancreatic β-cell dysfunction and apoptosis. J. Biol. Chem. *282*:3989-3997.
32. **Ladrière, L., M. Igoillo-Esteve, D.A. Cunha, J.P. Brion, M. Bugliani, P. Marchetti, D.L. Eizirik, and M. Cnop.** 2010. Enhanced signaling downstream of ribonucleic acid-activated protein kinase-like endoplasmic reticulum kinase potentiates lipotoxic endoplasmic reticulum stress in human islets. J. Clin. Endocrinol. Metab. *95*:1442-1449.
33. **Richieri, G.V., R.T. Ogata, and A.M. Kleinfeld.** 1996. Kinetics of fatty acid interactions with fatty acid binding proteins from adipocyte, heart, and intestine. J. Biol. Chem. *271*:11291-11300.

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