

Oleate disrupts cAMP signaling, contributing to potent stimulation of pancreatic β -cell autophagy

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Autophagy is critical for maintaining cellular function via clearance of excess nutrients and damaged organelles. In pancreatic β -cells, it helps counter the endoplasmic reticulum (ER) stress that impairs insulin secretory capacity during Type 2 diabetes. Chronic exposure of β -cells to saturated fatty acids (FAs) such as palmitate stimulates ER stress and modulates autophagy, but the effects of unsaturated FAs such as oleate, which are also elevated during obesity, are less well understood. We therefore treated MIN6 cells and mouse islets for 8–48 h with either palmitate or oleate, and then monitored autophagic flux, signaling pathways, lysosomal biology, and phospholipid profiles. Compared with palmitate, oleate more effectively stimulated both autophagic flux and clearance of autophagosomes. The flux stimulation occurred independently of ER stress, nutrient-sensing (mTOR) and signaling pathways (protein kinases A, C, and D). Instead the mechanism involved the exchange factor directly activated by cAMP 2 (EPAC2). Oleate reduced cellular cAMP, and its effects on autophagic flux were reproduced or inhibited, respectively, by *Epac2* knockdown or activation. Oleate also increased lysosomal acidity and increased phospholipid saturation, consistent with improved autophagosomal fusion with lysosomes. We conclude that a potent stimulation of autophagy might help explain the known benefits of unsaturated FAs in countering the toxicity of saturated FAs in β -cells during obesity and lipid loading.

Macroautophagy (referred to as autophagy hereafter) is a critical degradative mechanism for maintaining cellular function and survival under both normal and stress conditions (1). It is characterized by formation of a double membrane structure, the autophagosome, which engulfs target proteins or organelles (2). Damaged organelles, aggregated protein, and excess nutrients are removed and metabolites recycled. Autophagy is classically activated by starvation, an effect mediated by inhibition

of the nutrient sensing protein kinases mammalian target of rapamycin C1 (mTORC1)⁴ or activation of AMPK (3–5). There is also growing evidence for noncanonical activation pathways for autophagy, which can occur in particular cell types or specialized situations (6).

Pancreatic β -cells play an essential role in maintaining glucose homeostasis by regulating the secretion of insulin, and impaired β -cell function is instrumental in the development of Type 2 diabetes (T2D). Recently, dysregulation of autophagy has emerged as a potential factor that might contribute to this disease setting (7–9). The evidence was based largely on mouse models employing β -cell-specific deletion of the essential autophagy gene, *Atg7*, which displayed impaired glucose tolerance, especially in the context of high-fat feeding or genetic predispositions to obesity and/or protein aggregation (10–14). Mechanistically, *Atg7* deletion inhibited both β -cell mass and insulin secretion, potentially explained by endoplasmic reticulum (ER) stress and mitochondrial defects (10, 11). Several studies have also pointed to an impairment of autophagic clearance in β -cells during T2D, based on alterations in morphology, and accumulation of the autophagosomal marker LC3II (15, 16). However, the latter is generated by the lipidation of the precursor molecule LC3I as part of the proximal signaling cascade, so its accumulation could represent either enhanced flux through the upstream pathway, as well as a reduction in distal clearance. To distinguish between these possibilities we (17), and others (18), have injected mice with chloroquine (CQ) to compromise lysosomal function and hence clearance. Under these conditions high-fat feeding causes an accumulation of LC3II puncta in β -cells. These results suggest that, regardless of what happens in frank diabetes, autophagic flux is activated

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⁴ The abbreviations used are: mTOR, mammalian target of rapamycin; 4EBP, eukaryotic initiation factor 4E-binding protein; 8-CPT-AM, 8-pCPT-2-O-Me-cAMP-AM; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside; ATF4, activating transcription factor 4; Atg7, autophagy-related 7; CHOP, C/EBP homologous protein; CQ, chloroquine; EPAC, exchange protein directly activated by cAMP; ER, endoplasmic reticulum; FA, fatty acids; GPR40, G-protein coupled receptor 40; Lamp-1, lysosomal-associated membrane protein 1; LC3, microtubule-associated protein 1A/1B light chain 3A; PBA, 4-phenylbutyrate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PI, phosphatidylinositol; PKA, protein kinase A; PKC, protein kinase C; PKD, protein kinase D; PS, phosphatidylserine; RFP, red fluorescent protein; T2D, type 2 diabetes; GFP, green fluorescent protein; SM, sphingomyelin; ANOVA, analysis of variance; DAPI, 4',6-diamidino-2-phenylindole; 8-Br-cAMP, cyclic 8-bromo-AMP; 6-Bnz-cAMP, 6-benzyl-cAMP.

during obesity or pre-diabetes, and this might potentially help in delaying β -cell failure.

Palmitate and oleate are, respectively, the most prevalent saturated and unsaturated fatty acids (FAs) in the circulation, and both are augmented by high-fat diets. Chronic or excessive exposure to saturated FAs is often considered detrimental to multiple organs and cell types including pancreatic β -cells, whereas unsaturated ones are viewed as less toxic, and potentially beneficial (19). *In vitro* studies on β -cell autophagy have thus focused predominately on palmitate and have generally (10, 20–25) shown an enhancement of autophagy when conducted in conjunction with lysosomal inhibitors to allow quantification of flux. Mechanistically, this increase is thought to be secondary to the stimulation of ER stress (20, 25) that occurs in response to chronic palmitate treatment of β -cells (26). But there are also indications of an impairment of fusion between autophagosomes and lysosomes potentially explained by changes in luminal pH or lipid remodelling (22–25). More limited evidence suggests that oleate might enhance LC3II accumulation under steady-state conditions (10, 21, 22). But there have been no detailed studies specifically addressing the effect of oleate on autophagic flux in β -cells. This contrasts with the situation in other cell types, where unsaturated FAs have been shown to promote proximal autophagy via a variety of mechanisms (27–30). Therefore, the aim of the current study was to compare the effects of palmitate and oleate on both proximal and distal autophagy in β -cells, and to address underlying mechanisms.

Results

Oleate and palmitate stimulate autophagic flux in mouse pancreatic β -cells

We sought to establish a threshold for FA-stimulated autophagy in MIN6 cells, as monitored by accumulation of LC3II (Fig. 1A). The steady-state levels of this autophagic marker were unaltered by 48 h pretreatment with either the saturated FA, palmitate, or the unsaturated FA, oleate, at 0.2 or 0.4 mM palmitate alone, or in combination at 0.2 mM. As expected under these conditions, the ER stress marker CHOP was increased by palmitate, with a threshold at 0.4 mM (Fig. S1). Oleate was without effect, even in combination with palmitate (Fig. S1A). As measured in the presence of CQ, however, autophagic flux was selectively enhanced by 0.4 mM oleate (Fig. 1A). In terms of time dependences, neither FA was active at 8 h (Fig. 1B), whereas oleate promoted a transient increase in steady-state LC3II levels at 24 h, and augmented flux by 24 h with a maximal effect at 48 h (Fig. 1, C and D). In many further experiments conducted over several years, with multiple batches and passages of cells, we established that palmitate was more variable in its responses than oleate, such that the unsaturated FA was confirmed as the more effective stimulator of autophagic flux overall (summarized Fig. 1E). Similar results were obtained using isolated mouse islets maintained for 48 h with the different FAs (Fig. 1F).

Oleate enhances lysosomal size and acidification

Accumulation of LC3II is a function of both its generation via upstream signaling and degradation following fusion of

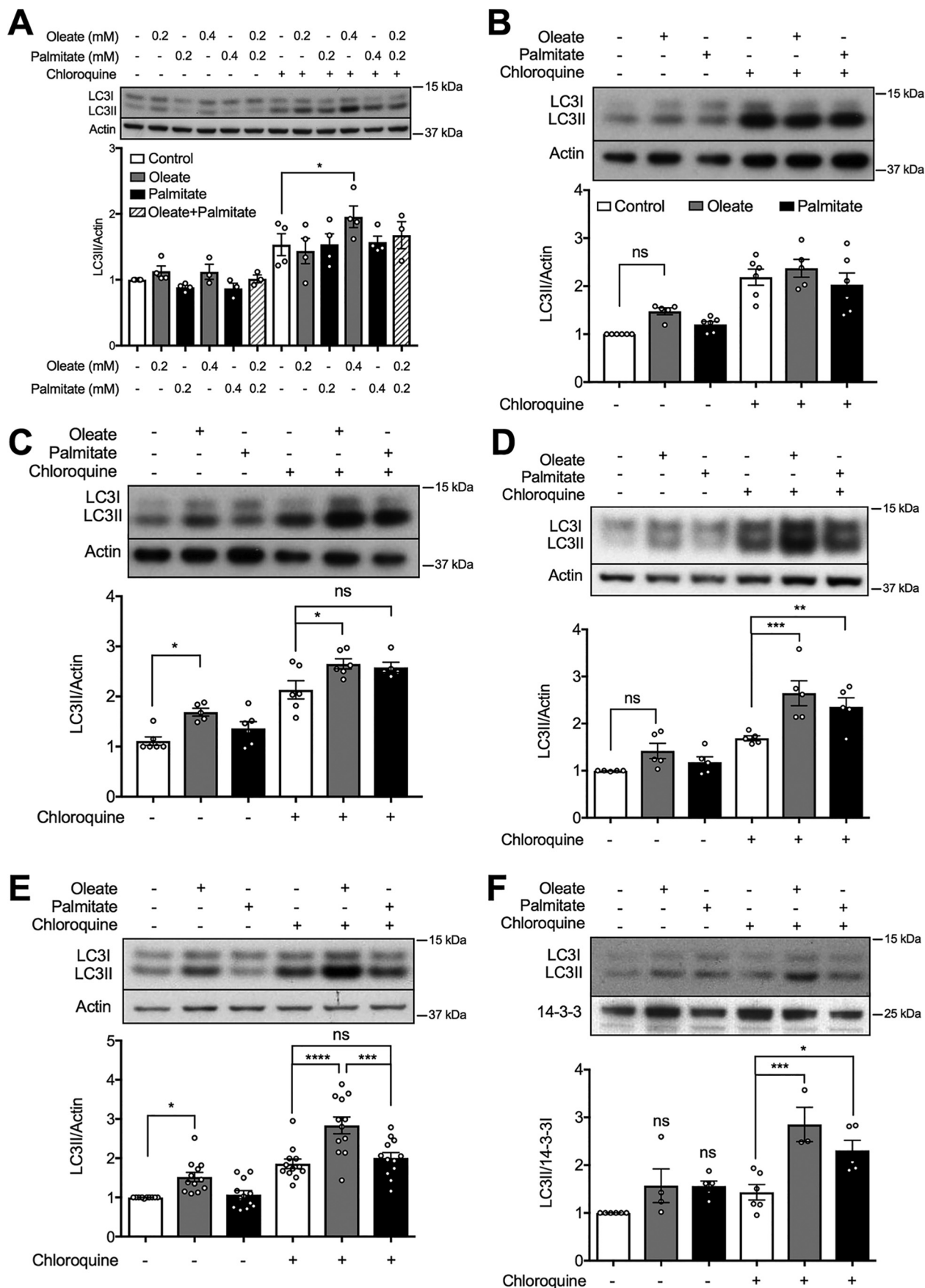
autophagosomes with lysosomes. We first determined whether FAs alter the size of the lysosomal compartment, as assessed using both the dye LysoTracker (Fig. 2A), and staining for the lysosome-associated membrane protein 1 (Lamp-1) (Fig. S2). Indeed lysosomes were markedly expanded but with little distinction between FAs or treatment times, suggesting that changes in the numbers of lysosomes do not account for the differential effects of oleate and palmitate on autophagic flux. To address functional alterations we employed LysoSensor, which exhibits a yellow to blue shift with increases in acidity, which is independent of the size of the uptake compartment. In this manner we determined that both FAs slightly decreased lysosomal pH at 8 h, with more modest effects at 48 h (Fig. 2B). This would suggest improved functionality, because many lysosomal enzymes require low pH for optimal activity.

Oleate augments autophagosome–lysosome fusion and phospholipid saturation

We next monitored the capacity for autophagosomes to fuse with lysosomes, using a mRFP-GFP-LC3 construct (31). This appears yellow in autophagosomes, but red upon fusion with the acidic compartment because of the accompanying reduction in pH, which quenches GFP fluorescence. We found that oleate pretreatment significantly increased the ratio of red:yellow puncta at both 8 and 48 h, suggestive of enhanced clearance (Fig. 3, A and B). The more modest effects of palmitate were not statistically significantly different from control. The fusion of membranes is influenced by their fluidity, which in turn depends upon the presence of unsaturated FA side chains in the membrane bilayer (32). We therefore used LC-MS to determine how FA pretreatment of β -cells alters the major lipid components of cellular membranes. Oleate reduced the saturation of both PC and PE by around 30% *versus* control, with significant but less profound decreases in PI and SM (Fig. 3C). In contrast, palmitate modestly enhanced the saturation of PE (20%), PC (15%), and SM (5%). Neither FA altered the saturation of PS (Fig. 3C), nor the overall mass of any of these lipids (not shown). Although these alterations represent total cellular membrane it is noteworthy that they are much more apparent in those lipids that are enriched in late endosomes/lysosomes (PC, PE, and SM) than in PI and PS, which are depleted in these compartments (32). Taken together these results suggest that oleate, in addition to stimulating upstream pathways, would also promote the clearance of LC3II via a combination of decreases in lysosomal pH and increases in membrane fluidity.

ER stress mediates autophagy in response to palmitate but not oleate

We next sought to determine the mechanisms contributing to the upstream pathway. We first addressed the relevance of ER stress, using the chemical chaperone PBA. As shown previously (33), this protected against induction of the markers CHOP, ATF4, and phospho-PERK in response to palmitate (Fig. S1, B–D), but more importantly it also inhibited LC3II accumulation (Fig. 4A). In keeping with our prior studies with this model (34), oleate did not stimulate ER stress (Fig. S1), nor did PBA block its capacity to augment LC3II (Fig. 4B). The results confirm previous findings that ER stress contributes to



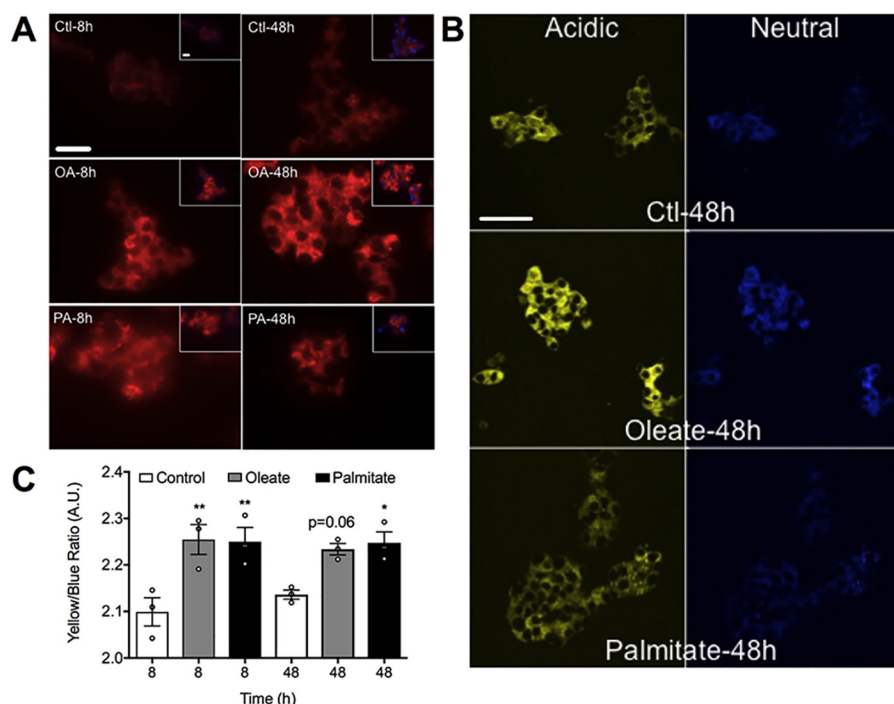


Figure 2. FAs enhance lysosomal amount and acidification. MIN6 cells were stained with (A) LysoTracker Red and co-stained with DAPI blue for nuclei ($n = 4$), or (B) LysoSensor yellow/blue following 8- or 48-h treatments with 0.4 mM oleate (OA) or 0.4 mM palmitate (PA) ($n = 4$), versus BSA-treated groups as the control (Ctl). Insets show merged images of LysoTracker and DAPI staining. Scale bar = 25 μ m. C, quantification of the yellow versus blue intensity ratio is shown as the mean \pm S.E. ($n = 4$). Statistical analyses were by one-way ANOVA with Sidak post hoc test. *, $p < 0.05$ and **, $p < 0.01$ versus the time-matched control.

the stimulation of autophagic flux due to palmitate (20, 25), but now show that this mechanism does not account for the response due to oleate.

Oleate does not alter the AMPK or mTOR pathways

AMPK and mTOR are key responders to changes in nutritional status and play especially critical roles in multiple facets of β -cell biology (35–38). In particular they are established regulators of autophagy, and, indeed, these pathways have been previously implicated in the induction of autophagy due to oleate in other cell systems (28, 29). In our model, however, we observed no changes in the regulatory phosphorylation sites of AMPK due to FA pretreatment, nor in the phosphorylation status of its substrate, ACC (Fig. S3, A and B). AMPK plays an unusual role in β -cells where, in contrast to most other systems, its inhibition promotes autophagy (39). We were able to confirm this using the AMPK inhibitor, Compound C, which enhanced LC3II accumulation in a manner nonadditive to that of oleate (Fig. 5A). The latter response, however, was unaltered by either AICAR (Fig. 5B) or A769662 (Fig. 5C), respectively, even though these compounds stimulated AMPK as expected (Fig. S3C). This makes it unlikely that changes in AMPK mediate the observed effects of oleate on autophagy. Likewise, neither FA impacted on mTOR activation, as measured by the phosphorylation of either mTOR itself or its downstream sub-

strate, 4EBP (Fig. S4, A and B). We further tested the impact of the mTOR inhibitor, rapamycin, which was without effect in our model, either basally or in the presence of FAs (Fig. S4C). Its action was, however, confirmed directly as reduced phosphorylation of its downstream kinase p70S6K (Fig. S4D). Rapamycin is viewed as a canonical activator of autophagy, although its function in β -cells has almost always been assayed microscopically by visualization of autophagosomes (40, 41) rather than LC3II accumulation as we attempted. Regardless, the fact that we did detect effects of oleate, but not rapamycin using this assay, clearly argues against the involvement of mTOR inhibition in the mechanism of action of the FA.

Oleate-induced autophagy is independent of GPR40, oxidative stress, PKD, and PKC

We therefore looked to other potential mechanisms, including activation of the cell surface receptor, GPR40 (42, 43), which is abundantly expressed in β -cells and known to be ligated by unsaturated FAs (44). However, a GPR40 antagonist did not impact on oleate-stimulated autophagy (Fig. S5). Other possibilities include oxidative stress, and activation of various protein kinases, particularly PKD, the inhibition of which in β -cells has been previously linked to repression of autophagy (45). However, LC3II generation in response to oleate was unal-

Figure 1. Effect of unsaturated and saturated FAs on autophagic flux in MIN6 cells or mouse islets. A–E, levels of the autophagic marker LC3II in MIN6 cells after treatment with (A) oleate or palmitate, alone or combined, at the indicated concentrations for 48 h; or 0.4 mM oleate or 0.4 mM palmitate for (B) 8, (C) 24, or (D and E) 48 h ($n = 5–12$). F, levels of LC3II in isolated mouse islets with 48 h treatment of 0.4 mM oleate or 0.4 mM palmitate ($n = 4$). The BSA-treated group was used as the control. Where indicated MIN6 cells or islets were incubated with or without 50 μ M CQ for 2 h prior to cell lysis. Data shown are the mean \pm S.E. of the densitometric quantification. Statistical analyses were done with one-way ANOVA with Sidak post hoc test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$.

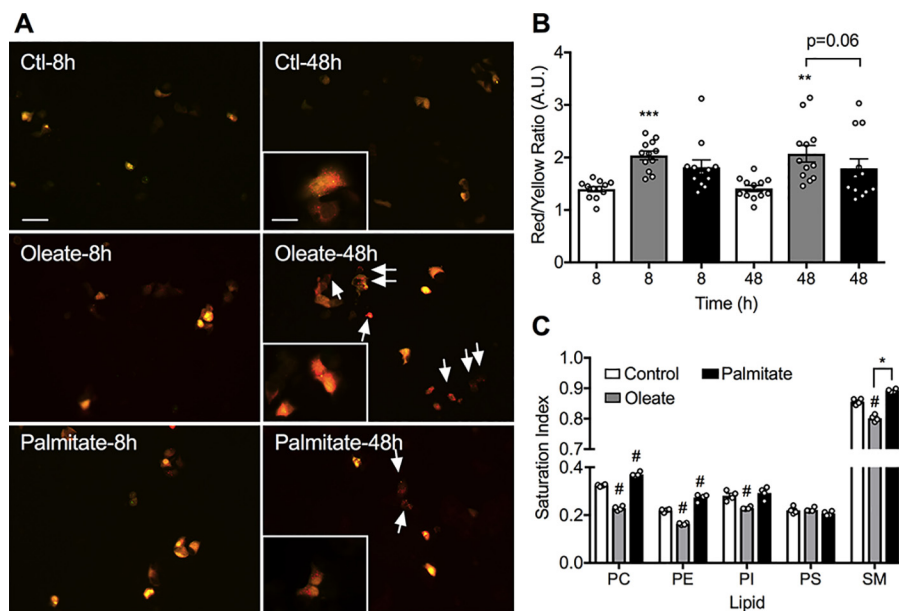


Figure 3. FAs increase autophagosome-lysosomal fusion. MIN6 cells were transfected with the mRFP-GFP-LC3 construct 24 h prior to treatment with 0.4 mM oleate (OA), 0.4 mM palmitate (PA), or BSA control (Ctl) for a further 48 h. **A**, representative fluorescence images illustrate the mRFP (red) and mRFP + GFP (yellow) signals; scale bar = 50 μ m. Insets are the magnified images, scale bar = 20 μ m. Arrows indicate the mRFP signal remaining after autophagosome-lysosomal fusion. **B**, quantification of red (fused) versus yellow (nonfused) and data shown are the mean \pm S.E. of the fluorescence intensity ($n = 4$). Statistical analyses were with one-way ANOVA with a Sidak post hoc test. **, $p < 0.01$, and ***, $p < 0.001$ versus the time-matched control. **C**, MIN6 cells were incubated with 0.4 mM oleate or 0.4 mM palmitate for 48 h before being subjected to MS analysis of the saturation of lipid species: PC, PE, PI, PS, and SM. Data shown are the mean \pm S.E. Statistical analyses were with two-way ANOVA with Tukey's post hoc test. #, $p < 0.0001$ versus control, *, $p < 0.001$.

tered by the antioxidant NAC, nor by inhibitors of PKC (Go-6976) and PKD (CID-755673) (Fig. S6)

Reductions in cAMP contribute to oleate-stimulated autophagy, but via EPAC2 rather than PKA signaling

We investigated cAMP signaling more extensively because of the surprising initial finding that accumulation of this intracellular mediator was reduced by chronic exposure of MIN6 cells to oleate, but not palmitate (Fig. 6A). We therefore assessed the potential role of PKA, the cAMP-stimulated protein kinase. However, the effects of oleate on LC3II were not altered by, respectively, blocking or stimulating PKA, using either the inhibitor H-89 (Fig. 6B), or the long-acting cAMP analogue, 8-Br-cAMP (Fig. 6C). However, the latter is not selective for PKA, but can also activate EPAC, which serves as an exchange factor to stimulate small monomeric G-proteins. We therefore used, 6-Bnz-cAMP-AM, which shows selectivity for PKA activation (46) but this was without any effect on autophagic flux in mouse islets pretreated with oleate (Fig. 6D).

Focusing instead on the EPAC pathway, we found in preliminary studies that the general inhibitor (ESI-09), exhibited off-target effects resulting in disruption of ER stress signaling pathways (not shown). It has also been reported to cause protein denaturation (47). We therefore chose to knockdown *Epac2* (also known as *Rapgef4*), the most abundant and functionally relevant isoform found in β -cells (48). The response to oleate under these conditions was significantly further enhanced (Fig. 7A), although basal accumulation of LC3II was unaltered, possibly because the degree of knockdown was incomplete (Fig. 7B). To further assess this potential mechanism we made use of cell permeable cAMP analogue 8-pCPT-2-O-Me-cAMP-AM (8-CPT-AM), which unlike the 8-Br-cAMP employed

above, selectively activates EPAC and not PKA. Using mouse islets incubated in the presence of CQ for measurements of autophagic flux, we observed that activation of EPAC completely blocked the stimulatory effects of oleate pretreatment (Fig. 7C). These findings suggest that inhibition of EPAC2, secondary to reductions in its upstream regulator, cAMP, contributes to the mechanism whereby oleate stimulates autophagic flux in β -cells.

Discussion

It is now generally established that obesity and T2D are associated with enhanced accumulation of autophagic markers (7–9, 15). Because most analyses are undertaken under steady-state conditions, however, it has been difficult to establish whether these findings represent a stimulation of autophagy or an inhibition of autophagosomal clearance. More recent studies, incorporating CQ injection in mice, have strongly suggested that autophagic flux is indeed augmented by high-fat feeding (17, 18). Additional findings with knock-out mice would suggest that this most probably represents a beneficial adaptation to obesity, helping β -cells avoid ER stress and the cellular failure that occurs in the transition to overt T2D (10, 11). Therefore understanding the mechanisms whereby FAs stimulate autophagy in β -cells has become an important experimental goal. To date, the overwhelming majority of studies in this area have focused on palmitate, which is surprising from the viewpoint that autophagy is advantageous to β -cells, whereas palmitate is not. Hence we have focused on oleate. Our results show that oleate induced a delayed, proximal autophagic response (24 h), but promoted earlier increases in lysosomal pH and fusion with autophagosomes (8 h). This would constitute a favorable environment prior to signal initi-

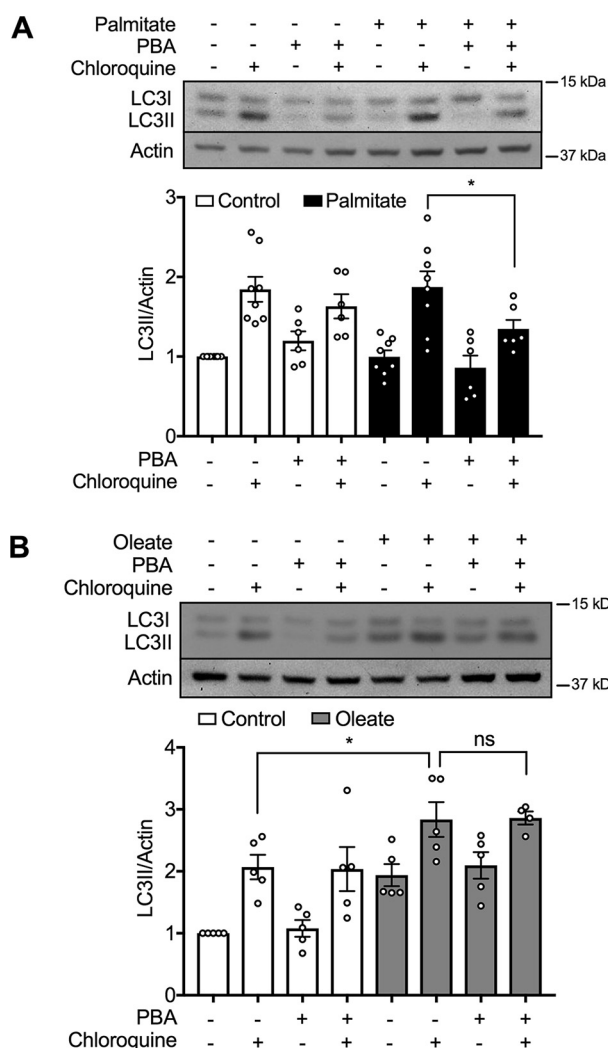


Figure 4. ER stress mediates induction of autophagy by palmitate but not by oleate. A and B, levels of LC3II in MIN6 cells after treatment with (A) 0.4 mM palmitate or (B) 0.4 mM oleate or BSA control, in the presence or absence of 2.5 mM PBA for 48 h, then incubated with or without 50 μ M CQ for 2 h prior to cell lysis ($n = 6$ and 5, respectively). Data shown are the mean \pm S.E. of the densitometric quantification. Statistical analyses were with one-way ANOVA with a Sidak post hoc test. *, $p < 0.05$.

ation, such that the resultant, mild increase in steady-state autophagy might contribute to the known protective effects of unsaturated FAs against cellular stress (49, 50). In contrast, canonical stimuli trigger a robust, fast and transient autophagic response, subjected to feedback (51), and often harmful if that feedback is overridden (52).

Although widely studied previously, the effects of palmitate on autophagy in β -cells are somewhat controversial. This is potentially explained by variations in experimental conditions, most notably the final concentration of free FAs, which depends critically on the molar ratio of exogenous FA to BSA, and how these are coupled (53). Our model involves pre-coupling at a ratio of around 3:1 (0.4 mM; 0.9% BSA), which equates to a free concentration in low micromolar, or moderately elevated physiological range (53). In our hands, this constitutes the minimal dose for 48-h treatments of palmitate and oleate to stimulate ER stress or autophagy, respectively (Fig. S1, Fig. 1A). Other variables across the different models include concentra-

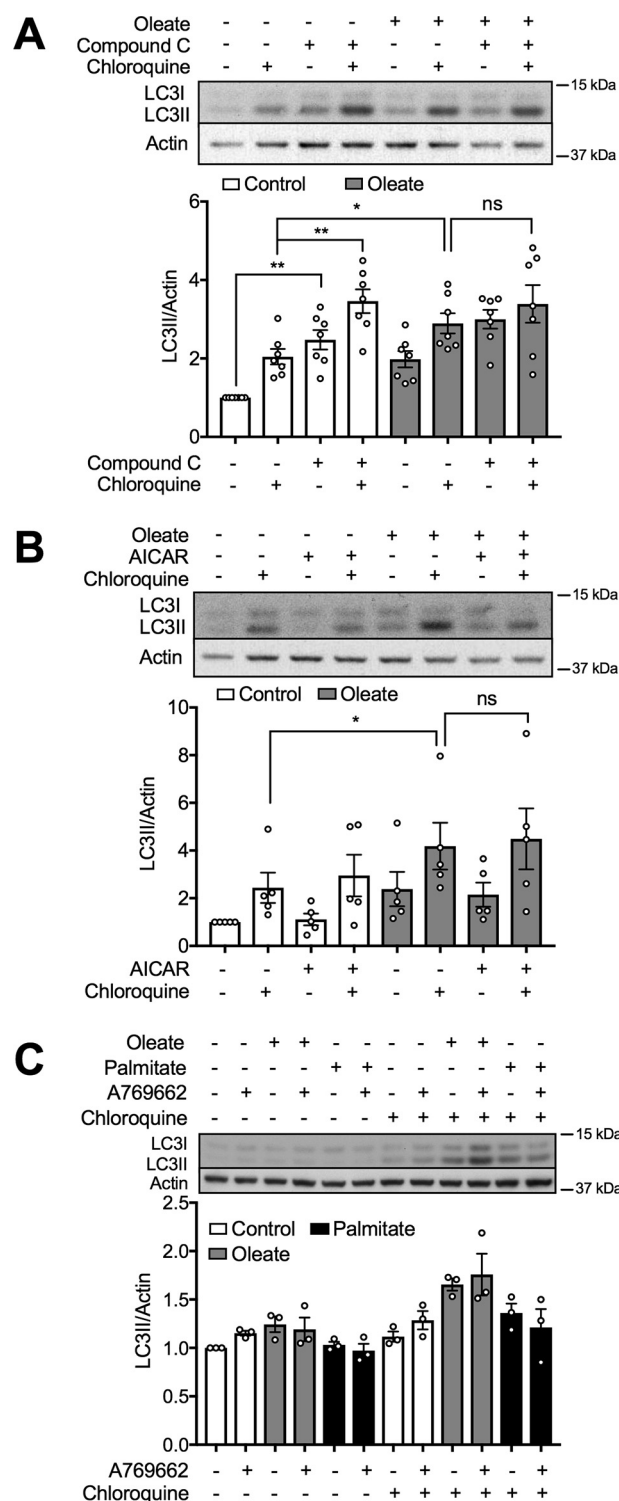


Figure 5. Inhibition of AMPK induces autophagy but oleate-activated autophagy is independent of AMPK. A and B, levels of LC3II in MIN6 cells after 48 h treatment with 0.4 mM oleate in the presence or absence of (A) 5 μ M Compound C, or (B) 500 μ M AICAR ($n = 7$ and 5, respectively). C, LC3II levels in MIN6 cells after a 48-h treatment with 10 μ M A769662 in the presence of 0.4 mM oleate or 0.4 mM palmitate. The cells were incubated with or without 50 μ M CQ for 2 h prior to cell lysis. Controls are (A and C) 0.1% DMSO in BSA or (B) BSA. Data shown are the mean \pm S.E. of the densitometric quantification. Statistical analyses were with one-way ANOVA with a Sidak post hoc test. *, $p < 0.05$ and **, $p < 0.01$.

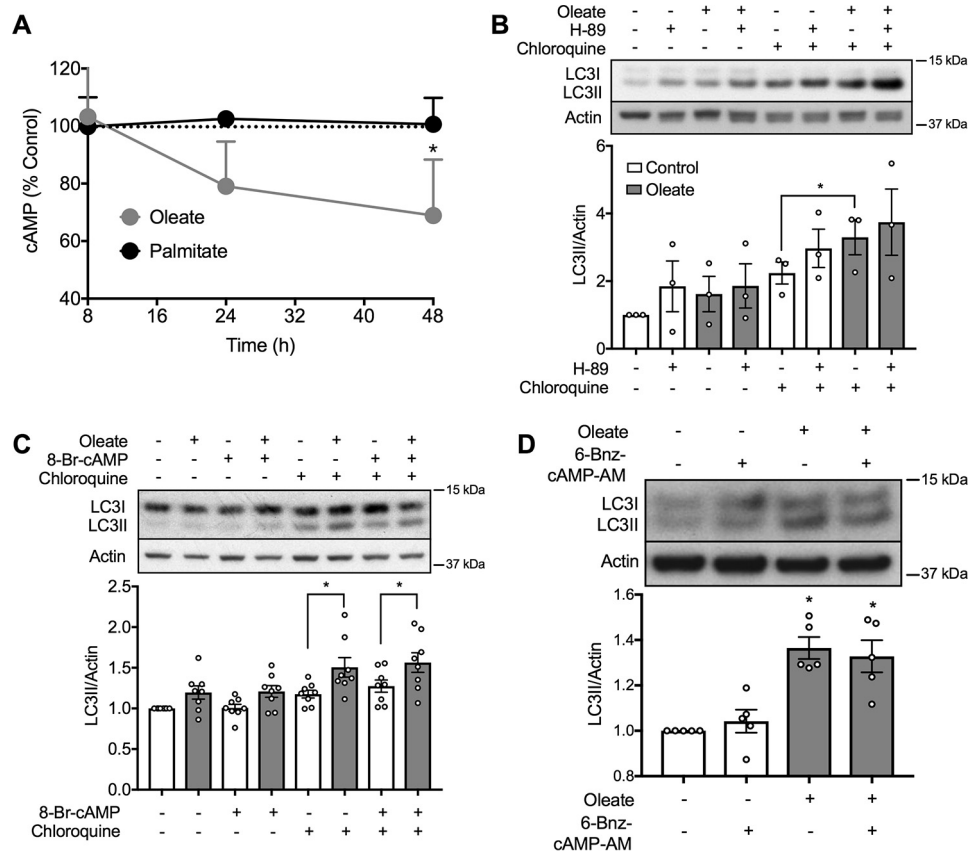


Figure 6. Oleate reduces cAMP levels and induces autophagy independent of PKA. A, MIN6 cells were treated with 0.4 mM oleate or 0.4 mM palmitate or BSA control, before lysis for assessment of cAMP levels ($n = 3$). Results are expressed as % of control (18.7 ± 0.3 fmol/mg of protein). B and C, levels of LC3II in MIN6 cells after 48 h treatment with 0.4 mM oleate or BSA control in the presence of (B) 50 μ M H-89 ($n = 4$), or (C) 100 nM 8-Br-cAMP-AM ($n = 9$). The cells were incubated with or without 50 μ M CQ for 2 h prior to cell lysis. D, levels of LC3II in isolated mouse islets with 48 h treatment of 10 μ M 6-Bnz-cAMP in the presence or absence of 0.4 mM oleate ($n = 4$). The islets were incubated with 50 μ M CQ for 2 h prior to cell lysis. The BSA-treated group was used as the control. Data shown are the mean \pm S.E. of the densitometric quantification. Statistical analyses were with one-way ANOVA with a Sidak post hoc test. *, $p < 0.05$ as indicated or versus control.

tions of glucose and serum, and duration of FA exposure. Species differences also play a role, with rat β -cells being more sensitive to palmitate exposure *versus* mouse or human β -cells because of their lower expression of desaturase enzymes (54). On the other hand, there is an overwhelming consensus that palmitate promotes β -cell apoptosis and ER stress, in all of these same highly variable models (26), suggesting that there is something inherently different about measuring autophagy as an end point, and unrelated to whether flux or steady-state levels are assayed. In our MIN6 model LC3II accumulation in response to palmitate varied over several years with differing passages and aliquots of cells. One interpretation of this variability is that palmitate exerts multiple and conflicting effects on autophagy, the balance of which determines the overall outcome. One positive input is almost certainly ER stress (20, 25). Thus we found that PBA inhibited autophagic flux in the presence of palmitate, reducing LC3II to levels below those of CQ alone (Fig. 4A). This implies that under conditions in which ER stress is counterbalanced, the residual effect of palmitate on autophagy is negative. Inhibitory inputs, potentially due to disruption of mitochondrial function (22, 24), might be especially prominent at higher molar ratios of palmitate:BSA and in rat β -cells, whereas in mouse cells and in milder models, activation predominates.

Our most important finding is that, despite its relatively neglected prior status, oleate is a more effective activator of autophagy in β -cells than palmitate *in vitro*, and thus potentially makes an important contribution in response to high-fat feeding *in vivo*. Oleate does not activate ER stress (26, 34), making this an unlikely mechanism for triggering autophagy, which we have now formally confirmed using the chemical chaperone PBA. We also ruled out a number of alternative mechanisms based on effects of FAs in other cell systems including PKC activation (27) and reactive oxygen species generation (28). Oleate has previously been shown to activate both GPR40 and PKD in β -cells (44), either of which routes might also stimulate autophagy (42, 43). However, inhibition of either of these pathways did not alter oleate-induced LC3II accumulation. Likewise we found no evidence for modulation of AMPK and mTOR, consistent with investigations of oleate-mediated autophagy on other cell types (28, 29). The fact that FAs did not activate AMPK in MIN6 cells is consistent with our earlier findings using mouse islets (55) but contrasts with other studies (56, 57), again probably explained by higher doses of FA and glucose than we employed. Palmitate treatment of β -cells has been previously reported to inhibit mTORC1 chronically (20), but stimulates in the short-term, in each case at concentrations higher

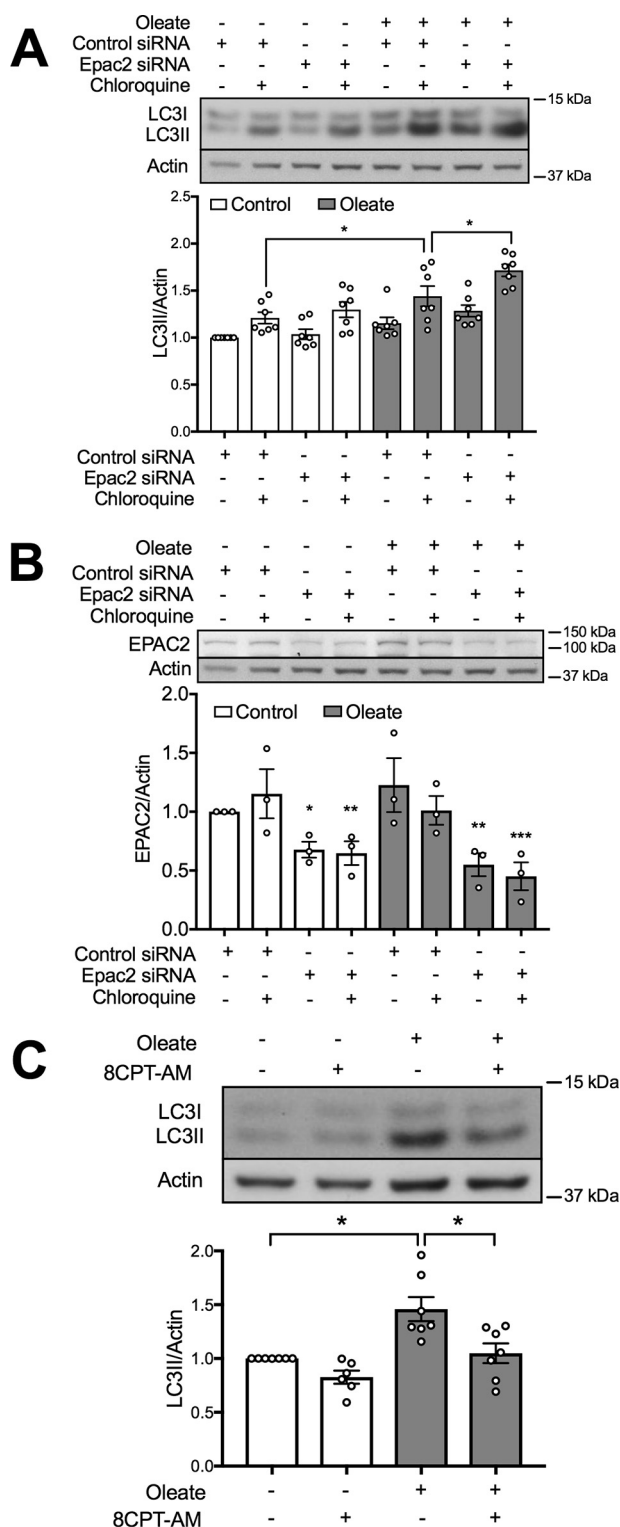


Figure 7. Oleate induces autophagy partially mediated by EPAC2. MIN6 cells were transfected with *Epac2* or the control siRNA, and then treated with 0.4 mM oleate. The cells were incubated with or without 50 μ M CQ for 2 h prior to cell lysis. Levels of (A) LC3II and (B) EPAC2 were examined in the same gel (same actin blot; $n = 7$). C, isolated mouse islets were treated with 0.4 mM oleate in the presence or absence of 10 μ M 8-CPT-AM or DMSO, then all were incubated with 50 μ M CQ for 2 h before cell lysis ($n = 4$). In all cases, BSA-treated groups served as the control. Data shown are the mean \pm S.E. Statistical analyses were with one-way ANOVA with a Sidak post hoc test. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ as indicated or versus control.

than those we have employed. Notably, oleate was ineffective (58).

Instead our findings suggested a role for a noncanonical activation pathway involving reductions in cAMP and inhibition of EPAC2. This is the predominant isoform expressed in β -cells where it acts in the amplification of insulin secretion (48). Perhaps under these conditions it would be advantageous to suppress autophagic clearance of insulin secretory granules and this has been previously shown to correlate inversely with secretory capacity in β -cells in other experimental settings (59). More broadly, EPAC isoforms have been implicated in both the stimulation (60, 61) and inhibition of autophagy (62), depending on context. Likewise cAMP, acting through PKA, can exert both positive and negative effects (63–65). This probably accounts for our observation that oleate-stimulated autophagic flux was suppressed by the EPAC selective activator, 8-CPT-AM, but by neither 8-Br-cAMP, which targets both EPAC and PKA pathways, nor the selective PKA agonist, 6-Bnz-cAMP-AM. Nevertheless, inhibition of EPAC probably does not account for the whole mechanism because knocking down *Epac2* was not sufficient in itself to stimulate autophagy, despite its stimulation overcoming the effects of oleate. Thus we conclude inhibition of EPAC2 is necessary but not sufficient for the activation of autophagy by oleate, suggesting that the FA must also exert additional effects that will require additional studies for elucidation. Likewise, the exact mechanism underlying EPAC2-suppressed autophagy is not clearly known, although EPAC and its downstream effector Rap2b, have been reported to be recruited to phagosomes, where they inhibited autophagy (62).

Our findings are not necessarily in dispute with the recent demonstration that GLP1 augments LC3II accumulation due to glucolipotoxicity (25), because the mechanism of action was not addressed in that study, and so might be independent of cAMP. Other data showing that GLP1 activates both mTORC1 (66) and AMPK (67) would also seem to exclude these protein kinases, suggesting that the mechanism linking GLP1 with autophagy in β -cells is likely to be unusual. It is probably also context dependent, because GLP1 inhibits LC3II accumulation due to tacrolimus dosing (68) in contrast to the situation with glucolipotoxicity (25). It is not surprising that β -cells would display a noncanonical mechanism for mediating the response to oleate, because they display multiple, unusual features in terms of autophagic regulation. Thus, in contrast to many tissues, inhibition of AMPK promotes LC3II accumulation (39), and starvation has even been linked to repression of autophagy (45). Moreover, high-fat feeding stimulates autophagic flux in pancreatic islets, but inhibits it in liver in the very same mice (17). It is also noteworthy that oleate and palmitate differentially regulate cAMP levels in β -cells, another novel finding of our study. The underlying mechanisms will also need to be investigated in future but could relate to the differences in transcriptional programs elicited by the two FAs (69).

In terms of downstream pathways, our results show that oleate and palmitate act very similarly on lysosomal compartment size and in lowering luminal pH. Palmitate has been previously shown to both enhance (22, 24, 25) and diminish (20) LysoTracker staining in β -cells, accompanied by indications of

impaired lysosomal function and fusion with autophagosomes (22–25). Proposed mechanisms included dysregulation of pH gradients (22) or membrane lipid composition (23). All of these studies, however, were conducted in rat β -cells, and employed higher concentrations of FAs and/or glucose than used here. Impaired mitochondrial function and depletion of ATP as observed in at least some of those reports (22, 24) would be expected to compromise lysosomal pH regulation. In contrast, effects of oleate on the size or function of the lysosomal compartment in β -cells have never been addressed, and reported impairments of fusion with autophagosomes were based on less direct protocols (22, 24) than the mRFP-GFP-LC3 reporter employed here. In our hands, oleate was better at promoting fusion of lysosomes and autophagosomes than palmitate. This is potentially explained by the observation that although both FAs lower lysosomal pH, which would be expected to be stimulatory, oleate additionally increased the phospholipid desaturation, a critical determinant of membrane fluidity and fusogenicity (32). In contrast, palmitate mildly increased saturation albeit to a lesser extent to that previously reported for rat β -cells, which thus displayed defective autophagic clearance (23).

In conclusion our major findings are that oleate is more effective at promoting autophagic flux than is palmitate; selectively impacts on autophagy via a noncanonical pathway involving the cAMP/EPAC2 pathway; and enhances fusion of lysosomes and autophagosomes consistent with decreases in both lysosomal pH and membrane phospholipid desaturation. Thus effects of unsaturated FAs, which are also present in high-fat diets, need to be taken into consideration when evaluating the autophagy in β -cells in models of obesity and diabetes.

Experimental procedures

Reagents and antibodies

Chemicals and reagents were from Sigma, unless otherwise indicated. Bafilomycin A1 was from Santa Cruz, mRFP-GFP-LC3 construct (31), NAC, PKC inhibitor (Go-6976), JNK Inhibitor II (for both JNK-1 and -2, Calbiochem), PKD inhibitor, Compound C, AICAR, EPAC inhibitor ESI 09, 8-Br-cAMP, and 8-pCPT-2-O-Me-cAMP-AM (Tocris). 6-Bnz-cAMP-AM, control, and Epac2 siRNA are from Dharmacon (Horizon Discovery Group Company, Lafayette, CO). Primary antibodies used in the study were: mouse monoclonal anti- β -actin and rabbit polyclonal anti-CHOP (Santa Cruz), anti-LC3, and anti-Lamp-1. Rabbit polyclonal anti-EPAC2, anti-ATF4, anti-phospho-AMPK, anti-AMPK, anti-phospho-ACC, anti-ACC, anti-phospho-4EBP, anti-4EBP, anti-phospho-mTOR (Ser-2448), anti-mTOR, anti-phospho-PERK, anti-phospho-p70S6K, and anti-p70S6K (Thr-389) were from Cell Signaling Technology (Danvers, MA).

Animals and islet isolation

All mouse studies were approved by the Garvan/St. Vincent's Hospital Animal Ethics Committee (AEC). C57Bl6 mouse islets were isolated by thermolysin and liberase digestion (Roche Diagnostics, IN) and gradient separation using Ficoll (GE Healthcare, Chalfont St. Giles, UK) as described previously (55).

Cell culture and treatment

The mouse MIN6 insulinoma cell line was routinely passaged and cultured as described previously (70), and was used at passages between 29 and 37 in this study. MIN6 cells were grown at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (25 mM glucose), supplemented with 10% fetal calf serum, 10 mM HEPES, 50 units/ml of penicillin, and 50 μ g/ml of streptomycin. Isolated islets were cultured in RPMI (11.1 mM glucose) at 37 °C and 5% CO₂, supplemented with 10% fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 50 units/ml of penicillin, and 50 μ g/ml of streptomycin, unless other treatments were used as indicated. MIN6 cells or isolated islets were exposed to palmitate or oleate (0.2 or 0.4 mM), which were precoupled to 0.92 g/100 ml of BSA or BSA-only controls in low (5.5 mM) glucose media (69). All treatments were performed with 5.5 mM glucose media. For plasmid transfection, cells were seeded in 12-well plates, and 100 nM mRFP-GFP-LC3 construct was present for 24 h. The media was changed to Dulbecco's modified Eagle's medium (25 mM glucose) and the cells incubated with the treatment indicated for 48 h, changing the media after 24 h. Knockdown experiments with control, or *Epac2* siRNA were performed as described previously (71), prior to oleate and CQ treatments.

Western blot analysis

MIN6 cells or islets were lysed and 10 μ g of protein was separated by a pre-cast 12% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary and secondary antibodies after blocking with 5% skimmed milk and developed with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific). Densitometric analysis was performed with the Adobe Photoshop CS4 software.

LysoTracker/LysoSensor staining

MIN6 cells were seeded onto FluoroDish™ (World precision instruments, Sarasota, FL) and lysosomes were visualized with LysoTracker Red DND-99 (ThermoFisher Scientific, L7528) and the lysosomal pH determined by LysoSensor Yellow/Blue DND-160 (ThermoFisher, L7545) as previously described (71). Images were taken by fluorescence microscopy.

Fluorescence microscopy

MIN6 cells were seeded onto the cover slide for treatments. Before subjecting to fluorescent imaging by LysoTracker/LysoSensor signaling, the live cells were washed with PBS three times and then mounted onto the slide (71). For the cells transfected with the mRFP-GFP-LC3 construct or for Lamp-1 staining, they were fixed in 4% paraformaldehyde. For the Lamp-1 staining, fixed cells were incubated with primary antibody followed by the Alexa 555-labeled secondary antibody (Molecular Probes) and then mounted with ProLong® Gold antifade reagent containing DAPI (Molecular Probes). The fluorescence signals were detected by fluorescence or confocal microscopy, respectively (models DM 5500 or DMI 6000 SP8; Leica Microsystems, Sydney, NSW, Australia).

Lipid profiling with MS

MIN6 cells were seeded at 1×10^6 cells/well in 6-well plates and then treated with FAs for 48 h. Lipids were extracted from whole cell homogenates and separated via LC before analysis by electrospray ionization-tandem MS as described previously (70, 72). The desaturation index was calculated from the ratio of the totals of saturated and unsaturated FA side chains in each phospholipid.

Intracellular cAMP measurement

MIN6 cells were seeded at 3×10^5 cells/well in 12-well plates and then treated with FAs for 48 h. The cells were washed with cold PBS three times and then lysed with 0.1 M HCl. The intracellular cAMP levels were assessed by the Direct cAMP enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY) following the manufacturer's instructions. The cAMP level was normalized to the protein concentration.

Statistical analysis

Data are expressed as mean \pm S.E. Multiple comparisons between groups were performed using ANOVA followed by Sidak or Tukey's post hoc test. Unpaired *t* tests were used when the differences between two groups were analyzed. A *p* value of less than 0.05 was considered statistically significant.

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