Adipose saturation reduces lipotoxic systemic inflammation and explains the obesity paradox

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Obesity sometimes seems protective in disease. This obesity paradox is predominantly described in reports from the Western Hemisphere during acute illnesses. Since adipose triglyceride composition corresponds to long-term dietary patterns, we performed a meta-analysis modeling the effect of obesity on severity of acute pancreatitis, in the context of dietary patterns of the countries from which the studies originated. Increased severity was noted in leaner populations with a higher proportion of unsaturated fat intake. In mice, greater hydrolysis of unsaturated visceral triglyceride caused worse organ failure during pancreatitis, even when the mice were leaner than those having saturated triglyceride. Saturation interfered with triglyceride’s interaction and lipolysis by pancreatic triglyceride lipase, which mediates organ failure. Unsaturation increased fatty acid monomers in vivo and aqueous media, resulting in greater lipotoxic cellular responses and organ failure. Therefore, visceral triglyceride saturation reduces the ensuing lipotoxicity despite higher adiposity, thus explaining the obesity paradox.

INTRODUCTION

While quantitative parameters such as body mass index (BMI) (1), waist circumference, and amount of visceral adipose (2, 3) are well-studied risk factors for acute disease severity, the impact of adipose composition on severity is unclear. Organ failure, the hallmark of acute pancreatitis (AP) severity, has been associated with long-chain non-esterified fatty acid (NEFA) lipotoxicity in humans (4–7). Clues to the role of adipose composition in severity come from studies from Western populations showing a BMI of >30 mostly, but not always (2, 3) associated with severity, while in Eastern populations, much lower BMIs, sometimes ≥23 (8, 9), are associated with severity. This “obesity paradox” (10) also occurs in other acute scenarios such as burns (11), acute heart failure (12), after trauma (13), cardiovascular surgery (14), and during critical illnesses (15) in which elevated pancreatic enzymes (16–18) and long-chain NEFA (19, 20) have been associated with severity. More recent studies associate such an enzyme leak (21) and dietary fat composition (22), along with elevated NEFA levels, to the severity (23) and mortality from coronavirus disease 2019 (COVID-19) (22, 24). However, the mechanisms determining NEFA generation and how NEFA mediate outcomes such as the cytokine storm (24) and organ failure (19, 20, 22) are unclear.

Most individual studies supporting the obesity paradox come from the Western Hemisphere (12–14), where dietary and adipose fat are more saturated (25, 26). We therefore aimed at understanding this problem in different populations, by modeling AP, wherein the pancreatic lipases leak into the surrounding fat (6, 7, 27) and hydrolyze the neutral lipids (7). Principal among these lipases is pancreatic triglyceride lipase (PNLIP), which enters visceral adipocytes by various mechanisms (7) and mediates lipotoxic systemic injury along with organ failure (7, 28) via the generated long-chain NEFA (7, 28) inhibiting mitochondrial complexes I and V (6). However, the impact of long-chain NEFA saturation on systemic inflammation is unknown.

RESULTS

Higher saturated fat intake is associated with SAP at a higher BMI

As seen in Fig. 1A and fig. S1, 20 reports from 11 countries used a BMI cutoff of ≥30 to stratify SAP. Six of these (blue countries and text in table) reported no increased risk (2, 3, 30–34), while the 14 (golden text and green countries) reported an increased risk of SAP at BMI of ≥30 (1, 6, 35–44). Six reports used a cut off BMI of 25 or
less (8, 9, 45–49). Adult AP typically occurs after the third decade (50, 51). Since the composition of visceral fat necrosed during AP may be influenced by the dietary fat composition over the preceding years (26), we analyzed dietary fat composition of different countries shown in Fig. 1A. The per capita fat consumption was calculated by averaging the yearly data (1970 to 2011) for each country. Countries with a BMI cutoff of ≥30 had higher per capita saturated fat consumption (Fig. 1B and fig. S1). While the amount of unsaturated fat consumption was the same (fig. S3A), unsaturated fat comprised a higher percentage of fat intake in the countries with reports having a cutoff BMI of ≤25 (pink countries; Fig. 1, A and C). Overall, there was a moderate correlation between the percentage of patients with SAP and the percentage of unsaturated fat intake (fig. S3B). On meta-analysis (Fig. 1D), a significantly increased risk of severity was noted for cutoff BMIs of ≤25 [pink shade, odds ratio (OR) 2.8, CI 1.3 to \( P = 0.008 \)] and also BMI of >30 (blue and greens, OR 2.7, CI 1.8 to 3.8, \( P < 0.001 \)). Publication bias was not detected on the basis of a Funnel plot (fig. S1C), an Egger’s regression, or a Begg and Mazumdar rank correlation. There were no differences in age, sex distribution, or etiology of AP between the two groups (fig. S1). While the effect of genes and comorbidities on %SAP cannot be commented on in these data, meta-regression showed that %unsaturated fatty acid (UFA) was able to explain 33% of the heterogeneity in the rate of SAP, and neither age, AP etiology, nor per capita gross domestic product (GDP) correlated with SAP. Furthermore, while per capita GDP of countries averaged for 5 years preceding publication was significantly lower in countries reporting SAP at BMIs of ≤25 ($15,768 ± 14,624 versus $32,272 ± 17,007, \( P = 0.018 \)) nor the proportion of patients with SAP (24 ± 12.5 versus 20.7 ± 8.4%, \( P = 0.892 \)) was different in the two BMI cutoff groups. Therefore, despite differences in income, the proportion of SAP and mortality were unchanged. This implied that quality of care was not different in the two groups of countries. Overall, these studies supported...
that severe pancreatitis occurred at a lower BMI in countries with a lower dietary saturated fatty acid (SFA) intake. Since we are not aware of the literature on how diet would affect the genetic background of a population over several generations to alter the severity of pancreatitis, we therefore went on to experimentally study whether and how altering visceral fat composition affected the development of SAP in the commonly used caerulein (CER) model of AP, which, in lean C57BL6 normal chow-fed mice, remains mild, self-limited, and without organ failure (27).

**Dietary unsaturated fat results in unsaturated visceral fat and worsens AP more than higher amounts of saturated visceral fat**

We first aimed at recapitulating the human studies showing that dietary fat composition can change adipose tissue composition in an animal model (26). Linoleic acid (LA; C18:2) is an unsaturated essential fatty acid (i.e., only available through diet) present at high concentrations in common FDA-recommended dietary fats (52) (e.g., cooking oils) and comprises a high proportion in pancreatic fat necrosis (FN) (5, 6). Being essential, LA’s concentrations in visceral fat parallel dietary intake (29). We thus formulated a diet enriched in LA (70% LA in a 45% fat diet) to represent high %UFA intake in dietary fat (red box in fig. S4A and Fig. 2A1, red columns) (26, 52). Similarly, a diet enriched in saturated fat was formulated, which had palmitic acid (PA; C16:0, PA 68% of a 47% fat diet; fig. S4B, green rectangle) replicating the 65 to 70% saturation of dairy fat. These diets spanned the range of %UFA intake in humans (16% in Australia and 79% in Japan), as shown in fig. S3A.

Mice fed these diets had a similar food intake (6.5 ± 1.4 g/day of UFA diet or 6.3 ± 0.5 g/day of SFA diet). This altered their visceral triglyceride composition, with LA and PA increasing to 40 to 45% (Fig. 2A1 and detailed in fig. S5) in the UFA- and SFA-fed groups, respectively. We previously showed that a normal chow diet with 5% fat diet (Purina 5053), which contains ≥70% UFA and <20% SFA

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**Fig. 2. Dietary and adipose fat composition and parameters of local, systemic severity of CER pancreatitis in UFA- and SFA-fed ob/ob mice.** (A1) Table comparing the fatty acid composition of fat pad triglycerides (TG) and diets of mice given the SFA- and UFA-enriched diets. Body weights (A2), body fat (A3), and body fat as a percentage of body weight (A4) in the SFA- and UFA-fed mice. Serum amylase (B1) and lipase (B2) in control (CON) mice and after 24 hours of AP (CER). Local pancreatic injury seen histologically in (C1) and quantified as acinar necrosis (C2) as a percentage of total parenchymal area and percentage of acinar necrosis adjacent to the FN (shown in yellow rectangles), termed as “% peri-fat acinar necrosis” (C3). Systemic injury measured as renal injury (TUNEL staining showing brown nuclei in (D1) and serum BUN in (D2), lung TUNEL positivity highlighted with arrows in (D3), and shown as number per high-power field (HPF) in (D4), along with shock (as measured by a drop in carotid pulse distention) in (E1), serum calcium (E2), and survival curve (E3) of mice with CER AP. NS, not significant.
consistent with FDA recommendations, results in fat pad triglyceride to be composed of 18 ± 6% PA and 31 ± 14% LA, with 35 ± 6% oleic acid (OA), i.e., C18:1 in ob/ob mice (7). On feeding the special diets, the LA concentrations in the fat pads of the UFA- and SFA-fed ob/ob mice correspondingly matched reports of those in human adipose tissue from Japan [≈40% (25)] and the United States [5% (26)]. By 8 to 14 weeks, the mice averaged 45.5 ± 0.5 g in both groups. CER AP reduced survival to 10% in the UFA group by day 3 versus 90% in the SFA group (P < 0.02; fig. S6). We then simulated the lower cutoff BMI (≤25) associated with SAP in countries with a higher %UFA intake. AP was initiated in UFA-fed mice weighing 20 to 30% less and having 35 to 40% less adipose tissue (Fig. 2, A2 to A4). AP increased serum amylase and lipase similarly in both groups (Fig. 2, B1 and B2) but was worse in the leaner UFA group. The SFA group has lesser pancreatic necrosis (5.8 ± 0.8 versus 16.8 ± 4.7%, P = 0.024; Fig. 2, C1 and C2) especially bordering FN, termed peri-fat acinar necrosis (2.7 ± 0.4 versus 6.9 ± 1.6%, P = 0.03; green outline, Fig. 2, C1 and C3). Consistent with SAP (53, 54), the UFA-fed mice had greater lung and renal tubular TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) positivity, higher serum blood urea nitrogen (BUN) levels (Fig. 2, D1 to D4), a greater decrease in carotid pulse distention (consistent with shock), and SAP-associated hypocalemia (55, 56) (Fig. 2, E1 and E2), resulting in 20% survival (P < 0.02 versus 90% in the SFA group; Fig. 2E3). The findings of SAP were replicated in UFA-fed C57BL6 mice with diet-induced obesity (DIO), but not the normal chow-fed mice (30.8 ± 0.7 g), which had mild pancreatitis (fig. S7, A to F) (27). However, we did not use DIO as the primary model since the time to weight gain (>5 months) was prolonged.

**Preferential lipolysis of unsaturated long-chain triglycerides worsens inflammation and systemic injury.** It has recently been shown that PNLIP mediates FN and severity of AP (7). To understand the worse outcomes in UFA-fed mice, we compared the necrosed gonadal fat pads in both groups (Fig. 3A). Grossly, the SFA group has reduced FN; however, the pancreatic amylase, lipase activity, and PNLIP protein (6- to 14-fold control) had a large increase in both groups (Fig. 3, B1 and B2, and fig. S8A).
The UFA mice had a larger cytokine mRNA increase in the fat pads during AP and interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor–α (TNF-α) proteins in the sera (Fig. 3, C1 to C4), which correlated with lower IkB-α (42 ± 15% of controls, P < 0.05) in the necrosed UFA fat pads (Fig. 3B2).

Since NEFA mediates cytokine increase (7), we measured serum NEFA to understand the underlying mechanism. The UFA group with AP had higher serum NEFA increase, especially UFAs, including C18:1 (OA) and C18:2 (LA) (Fig. 3, D1 to D4). This pattern has been noted during SAP-induced organ failure (7, 28, 53, 54) in both rodents (5, 6, 27) and humans (4). However, inexplicably, the increase in C18:1, C18:2, and C16:1 (Fig. 3, D1, D2, and D6) was muted in the SFA-fed mice, despite C18:1 being equal and C16:1 being higher in the fat pads of the SFA group (Fig. 2A1 and fig. S5). Similarly, C16:0 and SFA overall (Fig. 3, D5 and D7, and fig. S8B) increased more in the UFA group, despite the SFA group having more of these in the visceral triglyceride, serum at baseline. The proportion of SFA in general went down with AP (Fig. 3D8). This generalized reduction in SFA release suggested that SFAs in triglyceride are an unfavorable substrate for PNLIP.

We next directly investigated whether visceral triglyceride composition, irrespective of obesity or genetic background, affects its lipolysis and consequent severity during AP. For this experiment, the triglycerides of LA (C18:2), glyceryl trilinoleate (GTL), or PA (C16:0), i.e., glyceryl tripalmitate (GTP), were intraperitoneally given to lean mice with a genetically dissimilar background than the C57Bl6 mice, i.e., lean CD-1 mice. Two mechanistically distinct AP models—i.e., CER and IL12/18, both of which are mild in lean rodents (27, 57)—were then induced (Fig. 4 and fig. S9).

Mice given GTL or GTP alone exhibited normal behavior, with no change in physiologic or biochemical parameters. During AP, neither GTL nor GTP affected the initial serum amylase or lipase increase (Fig. 4, A1 and A2, and fig. S9, A1 and A2), which is similar
to ob/ob mice given UFA or SFA diets (Fig. 2, B1 and B2). The AP outcomes also paralleled the UFA and SFA groups (Figs. 2 and 3) with 0% survival in the GTL groups versus 80 to 90% in the GTP groups ($P < 0.01$; Fig. 4A3 and fig. S9A3). The GTL group had worse pancreatic necrosis (Fig. 4, B1 to B3, and fig. S9, B1 and B2), predominantly at the periphery of the lobules exposed to the lipolytically generated LA, resembling peri-fat acinar necrosis (Fig. 2, C1 to C3). The higher serum glycerol and corresponding NEFA in the GTL groups with AP (Fig. 4, C1 and C2, and fig. S9, C1 to C3) supported preferential unsaturated triglyceride lipolysis. Serum BUN elevations, renal tubular injury (Fig. 4, D1 to D4, and fig. S9, D1 and D2), lung injury (fig. S10, A and B), and profound hypotension noted as shock (fig. S10C) were only noted in the GTL groups with AP. Consistent with the small adipose tissue mass and visceral FN in lean mice (fig. S11, A1 and A2), there was no increase in serum resistin (Fig. 4E1 and fig. S9E1) and little or no increase in serum lactate dehydrogenase (LDH) (fig. S11, B and E). However, consistent with NEFA-driven inflammation (7), serum IL-6, TNF-α, and MCP-1 were higher in both AP models with GTL (Fig. 4, E2 to E4, and fig. S9, E2 to E4), perhaps due to the systemic lipotoxicity of LA noted as higher serum damage-associated molecular patterns (DAMPs), i.e., double-stranded DNA (dsDNA) and histone-complexed DNA fragments in the GTL groups with pancreatitis (fig. S11, C, D, F, and G). These findings suggested that unsaturated visceral triglyceride is hydrolyzed more than saturated triglyceride and worsens systemic inflammation and organ failure, consistent with what we note epidemiologically (Fig. 1). We thus went on to study the mechanistic basis of this in more detail.

**Saturation reduces long-chain triglyceride lipolysis by pancreatic lipases**

To understand how visceral triglyceride composition influences AP severity, we simulated the in vivo AP-associated lipase leak into fat using an in vitro system (Fig. 3, B1 and B2) (6, 7, 27). Acini release pancreatic enzymes into the surrounding medium under the basal state in vitro (58). Thus, we measured the hydrolysis of triglycerides added to the medium and biological responses of the NEFA generated. Addition of pure triglycerides GTP, glyceryl trioleate (GTO; the triglyceride of OA, C18:1), or GTO (300 μM each) to the stirred acinar medium in a quartz cuvette at 37°C resulted in 60 to 70% hydrolysis of GTO and GTO within 15 min but <10% of GTP (Fig. 5, A1 and A2). Lipolysis of GTL and GTO, unlike GTP or GTO (i.e., GTL + the lipase inhibitor orlistat at 50 μM) which has <15% lipolysis, also caused acinar mitochondrial depolarization ($\psi_m$) and cytosolic calcium (Cai) increase (Fig. 5, A3 and A4). Separately, on longer incubation (6 hours), 80 to 100% of GTL was hydrolyzed to glycerol (fig. S12A) versus 20 to 30% of GTP even at a 3x molar excess. GTL hydrolysis caused cytochrome c leakage, decreased ATP (adenosine 5’-triphosphate) levels, and increased LDH leakage and propidium iodide uptake (fig. S12, B to E). These studies showed that triglyceride saturation reduces its lipolysis and consequent biological effects.

We then studied the effect of saturation on the hydrolysis of mixed triglycerides in the acinar medium, since triglycerides in vivo are mostly saturated triglyceride and worsens systemic inflammation and organ failure, consistent with what we note epidemiologically (Fig. 1). We thus went on to study the mechanistic basis of this in more detail.

**Triglyceride saturation makes its lipolysis by PNLIP energetically and structurally unfavorable**

We then studied the interaction and hydrolysis of GTL, LLP, and LOP focusing solely on triglyceride hydrolysis by human PNLIP in phosphate-buffered saline (PBS; pH 7.4, 37°C, 150 mM Na). The hydrolysis, i.e., GTL > LLP > LOP, paralleled those in the acinar cell media (Fig. 5C1). We thus studied their interaction with PNLIP using isothermal titration calorimetry (ITC).

A single injection of human PNLIP (0.7 nmol/s) into a stable, sonicated, stirred, and 100 μM suspension of these triglycerides in PBS (at 300 s; fig. S13A) caused an endothermic interaction with a magnitude paralleling lipolysis, i.e., GTL > LLP > LOP, the enthalpy of which paralleled the raw heat data (Fig. 5, C2 and C3, and fig. S13B). The enzyme kinetics studied with multiple-injection experiments (fig. S13E) mirrored the above findings with the reaction rate ($V$; micromolars per second) of GTL > LLP > LOP (fig. S13, F and G). The calculated kinetic parameters (fig. S13G) for GTL hydrolysis are similar to those previously published (60) and are more favorable than for LLP or LOP. Addition of orlistat (50 μM) to PNLIP significantly reduced the $\Delta H$ and $V_{max}$ in both types of injections (fig. S13, C, D, H, and I), supporting the relevance of the parameters to lipolysis. The above experiments thus showed that increasing saturation makes the interaction of unsaturated triglycerides with PNLIP energetically unfavorable, resulting in reduced lipolysis.

To verify the experimental results in an independent unbiased manner, we undertook docking simulations using the open ligand conformation of the human PNLIP-procolipase complex (1LPA) (61) and studied access of the relevant triglycerides into the PNLIP ligand-binding domain (LBD) via the oxanion hole. Ser152, Asp175, and His263 compose the catalytic triad of human PNLIP and are critical in fatty acid liberation from triglycerides. The closed conformation (1LBP) (62) was not used since our goal was not to study molecular dynamics of pancreatic lipase activation by interfaces.

Previous in silico studies (63) found that orlistat, a potent lipase inhibitor, docks to pancreatic lipase with a GlideScore of $-6.90$ kcal/mol with root mean square deviation (RMSD) between 1.2 and 4.8 Å. This value served as a threshold to quantify strong binding. To verify this value, we docked orlistat to 1LPA with the induced fit protocol, and a similar GlideScore value was produced. Orlistat docked with a distance of 3.74 Å between the catalytic serine and the β-lactone ring that inhibits PNLIP hydrolysis. The induced fit protocol docked GTL into the 1LPA LBD with a GlideScore of $-7.16$ and with a distance of 4.04 Å between the hydroxyl group of Ser152 and the carbonyl C atom of the triglyceride’s glycerol backbone (Fig. 5D1). LLP docked with a GlideScore of $-7.42$ kcal/mol at a distance of 9.99 Å from Ser152, and LOP and GTP respectively produced GlideScores of $-1.33$ and $-1.58$ while being 12.42 and 11.98 Å from the catalytic serine (Fig. 5, D2 to D4). To verify the integrity of the docking simulation, the ligand present in the 1LPA crystal structure (61), dilauryl phosphatidyl choline (DLPC), was removed and then docked with the same induced fit docking protocol used for the three triglycerides. DLPC docked with a GlideScore of $-7.46$ (fig. S14A) and at a distance of 3.59 Å from the hydroxyl group of the catalytic serine,
which is 0.39 Å from its location in the crystal structure (3.20 Å). The resolution of 1LPA is 3.04 Å, so the variance seen is within the margin of error. DLPC inhibited the lipolysis of GTL added simultaneously in a dose-dependent manner (fig. S14B). This inhibition by DLPC, along with the redocking closely recapitulating the crystallographic findings, thus validates the docking simulation. Overall, these studies show that long-chain SFAs like palmitate make a triglyceride’s interaction with PNLIP structurally and energetically unfavorable, thus reducing its hydrolysis. We lastly went on to study how the fatty acids generated may result in cell injury and consequent organ failure.

Double bonds increase monomeric NEFA concentrations, signaling, and resulting organ failure

We first compared the ability of NEFA to directly induce inflammation and organ failure. LA [0.3% body weight (28, 53, 54)], OA (7), or PA (0.5%) body weight was instilled into the peritoneal cavity to simulate NEFA generated from visceral fat (2 to 10% body weight) lipolysis (64). Unlike LA and OA, there was no increase in circulating cytokines or organ failure (BUN increase) induced by PA (Fig. 6, A and B). The median survival for LA-treated mice was 18 hours and for OA was 64 hours, while the PA group had no adverse outcome over 3 days. Serum NEFA (which are predominantly albumin bound) and unbound NEFA (uNEFA; Fig. 6, C and D) increased significantly only in the LA and OA groups, along with levels of the DAMP and dsDNA (Fig. 6E) and a drop in serum albumin consistent with the hypoalbuminemia noted during experimental (fig. S15, A to C) and clinical SAP (55). This suggested that, unlike PA, both LA and OA with high uNEFA may directly injure cells, triggering DAMP release and downstream inflammation. This would also be consistent with the in vivo AP models (Figs. 3 and 4 and figs. S9 and S11, B to G) where higher NEFA were associated with worse outcomes.

We thus compared the ability of the most prevalent long-chain NEFA (C18:2, C18:1, and C16:0; Fig. 6, F1 to F3) to exist in a non-micellar monomeric form in aqueous media. On ITC at 37°C, we...
noted the critical micellar concentration (CMC) and therefore the aqueous monomeric NEFA concentrations to increase with the number of double bonds (Fig. 6, F1 to F3). The CMCs of PA (C16:0), OA (C18:1), and LA (C18:2) were respectively <8, ≈40, and ≈160 µM, which paralleled the uNEFA levels in vivo (Fig. 6D). The calorimetric results were then validated using ultracentrifugation. Both of these methods could be performed at room temperature (23°C; fig. S16) and showed that the nonmicellar NEFA concentrations in the infranatents of 500 µM NEFA in PBS (pH 7.4), spun at 105 g for 1 hour (fig. S16, A and B), were similar to the CMCs on calorimetry (fig. S16, C and D) and those shown previously using diphenyl hexatriene fluorescence spectroscopy (65). Thus, the CMC noted on calorimetrically at 37°C (Fig. 6, F1 to F3) are accurate.

The role of uNEFA in causing biological effects was then studied in cells. Addition of 100 µM LA (below CMC) caused a larger increase in Cai (Fig. 6G) and υm (Fig. 6H) than 100 µM OA (above CMC). PA caused no change in these. A total of 0.5% albumin (Alb) completely aborted the Cai increase by both LA and OA and prevented υm from progressing (Fig. 6, H and I). This effect of albumin proves that the Cai and υm increases were from the uNEFA. It also shows that Cai and υm changes result from distinct mechanisms of these uNEFA. The sustained υm elevation, without progression, despite albumin (Fig. 6, H and I) signifying irreversible uNEFA toxicity, is distinct from the complete reversal of Cai by albumin (Fig. 6G) and explains the cell death noted from LA or OA in previous studies (6). Therefore, the greater Cai and υm induced by LA than OA at 100 µM (Fig. 6, G and H) may be due to LA’s higher CMC (Fig. 6F3) and thus explains the higher uNEFA levels and shorter survival noted in LA-treated mice. We tested this by measuring the dose response of increase in υm over baseline...
(Δψm) 60 s after the addition of LA or OA to acini (Fig. 6J) or human embryonic kidney (HEK) 293 cells (Fig. 6K) to represent the pancreatic and kidney injury in vivo (Fig. 4, D1 to D4, and fig. S9, D1 and D2). Consistent with OA’s CMC (≈40 μM; Fig. 6F3), Δψm increased over baseline at 50 μM OA (*) and then plateaued (Fig. 6, J and K). Similarly, LA’s Δψm increased till 100 μM and ceased at 200 μM, consistent with its CMC of ≈160 μM. While LA’s Δψm equaled OA’s at 50 μM, it was more than OA’s (#) at concentrations of ≥100 μM. LA and OA (60 μM), unlike PA, also reduced endothelial barrier integrity (fig. S15, D and E), potentially explaining the hypotension (Fig. 2E1 and fig. S10C) hypothesized to be from vascular leak during SAP (66), along with explaining the hypoalbuminemia (fig. S15, A to C) (55) in severe AP, and the uNEFA increase noted (Fig. 6D).

Overall, these studies cumulatively validate that double bonds increase monomeric long-chain NEFA concentrations and signaling in an aqueous environment, thus enhancing their lipotoxicity.

**DISCUSSION**

Here, we find that a higher proportion of dietary unsaturated fat can worsen AP outcomes at a lower adiposity than seen in individuals with a higher proportion of saturated fats in their diet. We show that the higher likelihood of SAP associates with a higher unsaturated triglyceride content in visceral fat. This occurs because the presence of SFAs in triglycerides makes the interaction of the substrate with PNLIP structurally and energetically unfavorable. Moreover, the unsaturated NEFA generated by lipolysis can exist as monomers (65) in aqueous media, unlike saturated NEFA, resulting in injurious signaling, lipotoxic inflammation, and organ failure. This can potentially explain why higher dietary UFAs may result in worse AP in leaner animals and humans with lower BMIs compared to the more obese ones who consume a diet with higher proportions of saturated fat (Fig. 7), resulting in the obesity paradox.

We note that the obesity paradox in pancreatitis (10) holds true when the data are grouped and analyzed by BMIs using the World Health Organization (WHO) cutoffs (67) relevant to the countries from which the study originated (Fig. 1), including those that use BMI cutoffs of ≤25, which have a lower SFA and higher %UFA consumption in diet (Fig. 1, B and C). Socioeconomic and quality-of-care issues, age, sex, and etiology of AP are unlikely to have influenced our findings since the rate of SAP and mortality were the same in the >30 versus ≤25 BMI cutoff groups. We note that the SAP rates and %UFA intake have a moderate correlation (fig. S3B), and on meta-regression, %UFA intake explains 33% of the heterogeneity in SAP rates. Thus, other factors such as male sex, genetic background, and comorbidities that are not accounted for in the studies may contribute to the remaining heterogeneity in SAP rates.

The difference in BMI cutoffs set by the WHO is commonly attributed to a higher percentage fat per body mass in Asian populations compared to Caucasian populations (68). However, this is brought into question since visceral adiposity (3) that undergoes visceral FN during SAP (69) differs by ethnic groups (70) and in women versus men (71). We note that all visceral fat is not the same, since a higher proportion of diet-derived UFAs like LA make smaller

![Fig. 7. Schematic summarizing how dietary fat composition affects visceral fat necrosis and causes the obesity paradox.](http://advances.sciencemag.org/) The impact of consuming a Western diet enriched in saturated fat from dairy and red meat (left) or one enriched in unsaturated fat from vegetable oil and fish (right) are shown. In the event of AP, the relatively more saturated adipocyte triglyceride shown in blue ( left) or unsaturated triglyceride shown in red ( right) is exposed to pancreatic lipase, principal among which is PNLIP (zoomed circles). Despite being lesser in amount, the more unsaturated triglyceride is hydrolyzed more into the lipotoxic NEFA ( ), which are stable as monomers at higher concentrations than saturated NEFA, that form micelles ( ) at lower concentrations. This results in worse systemic inflammation, injury, and organ failure in those with a higher unsaturated fat consumption, despite having less adiposity. This pathophysiology can explain the obesity paradox.
amounts of visceral fat more prone to lipolysis (Figs. 2 and 3), generating higher concentrations of monomeric NEFA (Fig. 6), thus worsening outcomes in leaner populations (Fig. 1). The reverse holds true for SFAs, and this is relevant to studies from Western countries (72) that show AP severity to be independent of intra-abdominal fat amounts even when this fat is above the range in studies from Asian countries (71). This is exemplified by the six studies reporting a BMI of >30 to not be associated with severe AP—all of which came from countries with <40% UFA as dietary intake (Fig. 1C).

Our epidemiologic studies are limited by being retrospective, not involving dietary history, visceral fat sampling, or genetic studies, and by using nationwide dietary and economic data. We thus verified the conclusions in mice, cell, and pure enzyme models and physical chemistry. The use of two different strains of mice (i.e., C57bl/6 and CD-1) and two modes of increasing visceral triglyceride (i.e., obesity and intraperitoneal injection of triglyceride) experimentally support that acute triglyceride lipolysis is crucial in the pathogenesis of organ failure and weakens the argument in favor of the genetic or chronic effects of obesity such as insulin resistance and atherosclerosis in explaining these outcomes. The deleterious effects that we note with GTL are also seen with the triglyceride of OA but not with GTP during pancreatitis (5). These along with the deleterious effect of monomeric unsaturated NEFA (Fig. 6), lipase (16–18), and NEFA elevation (19, 20, 23), being associated with worse outcomes in other diseases states including COVID-19 (22, 24), perhaps support the general relevance of dietary and visceral fat unsaturation in causing the obesity paradox (11–15). A palmitate-enriched diet in ob/ob mice helped avoid the confounding effects of maligestion of a dietary saturated triglyceride by pancreatic lipases, which we note as reduced lipolysis of GTP, LLP, and PLP (Fig. 5, A2 and B1). We used PNLIP since recent studies show that adipocyte triglyceride lipase, PNLIPRP2, and carboxyl ester lipase are unlikely to mediate lipotoxic systemic inflammation (7, 53). Moreover, the similar lipolysis pattern in acinar media, which contains all three lipases (Fig. 5, A1 to A4 and B1 to B3) and PNLIP (Fig. 5, C1 to C3), lends credence of the concept to other lipases.

Stearoyl-CoA (coenzyme A) desaturase-1 (SCD-1) (73) is likely responsible for converting the dietary palmitate (C16:0) to palmitoleate (C16:1), which comprised 17% of the visceral triglyceride of SFA mice (Fig. 2A1 and fig. S5), despite palmitoleate being absent in the diet. SCD-1 is highly expressed in adipose tissue and can put a double bond in stearoyl-CoA or palmitoyl-CoA, thus forming oleate (C18:1) and palmitoleate from the palmitate in the diet. This may also explain the similar amounts of C18:1 noted in the visceral triglyceride of the UFA- and SFA-fed mice. Despite the potential influence of SCD-1 on our hypothesis, the similarity of dietary and visceral fat composition that we note in mice, the previous studies note in humans (26), along with the lack of known pathways to saturate C18:1, and our mechanistic data cumulatively support our conclusions.

An additional limitation may lie in our inability to resolve the lipolytic impact of stereochemical structure of the triglycerides (74), which we used in the racemic form rather than the enantiopure form. While this remains beyond our scope, the previous findings that pancreatic lipase hydrolyzes triglyceride without any stereoselectivity for the sn-1 or sn-3 position (59) suggest that the interference by palmitate is independent of these locations on the glycerol backbone. Last, the mechanisms underlying the exothermic changes (Fig. 5, C2 and C3) following the initial interaction of PNLIP and triglycerides remain unclear. The relative magnitude of these, i.e., GTL > LLP > LOP, mirror the initial interaction. Previous studies suggest that this may be due to binding of the generated LA to aromatic residues on PNLIP and further increasing its activity (75).

In summary, diet-induced visceral fat unsaturation increases lipolytic generation of unsaturated monomeric NEFA that cause cell injury, systemic inflammation, and organ failure. Long-chain SFAs like palmitate, however, interfere with this lipolysis, generating lower amounts of monomeric NEFA, making pancreatitis milder despite excess adiposity, thus explaining the obesity paradox (Fig. 7). The FDA guidelines to reduce saturated and increase unsaturated fat intake (https://www.fda.gov/files/food/published/Food-Labeling-Guide%-28PDF%29.pdf, appendix F) (26, 52) could therefore potentially contribute to worsening systemic inflammation and organ failure.

**METHODS**

**Human data analysis**

**Literature search**

To explore the impact of BMI on AP severity, we conducted a literature review by searching the PubMed database from inception to January 2017. The terms “BMI” or “obese” and “acute pancreatitis” as well as “severe” or “severity” were used. A total of 118 studies were retrieved, as shown (fig. S2). Seventy-nine studies were excluded, as they were not related to the subject of this review, as were 12 studies that were reviews/meta-analyses/hypotheses. The remaining studies were extensively reviewed for eligibility criteria, which were as follows: (i) clear BMI cutoff to define obesity, (ii) clear report of the incidence of mild AP (MAP) and SAP in obese versus nonobese groups, and (iii) clear and satisfactory definitions of MAP and SAP. Twenty-seven studies met our eligibility criteria and were therefore included in this review (fig. S1). These are shown in different colors in Fig. 1A for descriptive purposes. The studies were then categorized into those using a BMI of 30 as a cutoff (n = 20, the one with a cutoff of >29 was included here) and those that used a cutoff BMI of ≤25 (n = 7; with text and country in pink) to define obesity’s association with SAP. The dietary fat consumption in the countries from which these papers originated was then extracted as described below and compared (fig. S1). The incidence of MAP and SAP in both obese and nonobese patients was extracted for the purpose of meta-analysis. Total number of patients was divided into MAP and SAP and then into those that were obese versus nonobese. In cases where this was not explicitly clear, the communicating author was contacted (30, 45), and the numbers so provided were included in the study. Papers that did not have adequate data or for reasons mentioned in the last column of fig. S1 and detailed in fig. S2 were excluded from the meta-analysis (n = 7). When more than one BMI cutoff was mentioned, the BMI used was the one at which the SAP risk increased.

**Statistical analysis**

The proportion of SAP in all pancreatitis was calculated and compared between obese and nonobese groups. Subgroup analysis was performed on the basis of the definition of obesity used in the different studies included. Random effects model was used to calculate pooled OR with 95% CIs. Heterogeneity was assessed using the I² measure and the Cochran Q statistic. Statistical analysis was performed using Comprehensive Meta-Analysis Software version 3.3.070 (Biostat, Englewood, NJ, USA). Publication bias was assessed for using Beggs and Mazumdar’s test as well as Egger’s regression intercept.
Dietary fat consumption of country from which paper originated

The per capita per year dietary fat consumption of each country was collected from the FAO website (http://www.fao.org/faostat/en/#data/FBSH), which is reported as kilograms per person per year. For purposes of simplicity, the chief sources of long-chain saturated fat (dairy, red meat, and palm oil) were analyzed separately from those of unsaturated fat (fish and vegetable oil). Fat from dietary sources were calculated by percentage weight (3.25% for whole milk, 20% from red meat, and 10% for fish) unless the food source was directly fat (e.g., cream, butter, ghee, cheese, vegetable oil, or palm oil). Data from the country from which each paper originated were included in the categories “BMI >30 not related to SAP,” “BMI >30 associated with SAP,” and cutoff “BMI ≤25” (shown as blue, green with golden text or symbols, and pink in Fig. 1, respectively). Each country was represented once in the category for a contributed paper (shown in Fig. 1, B and C). Comparisons were done by grouping the BMI of >30 not related to SAP and BMI of >30 associated with SAP into a grouped BMI of >30 category and by comparing this to the BMI of ≤25 category using a Mann-Whitney test. P < 0.05 was regarded as significant.

Reagents

Glyceroyl trilinolein (GTL) and other triglycerides including triolein (GTO), tripalmitin (GTP), and mixed triglycerides 1,2-dilinoleoyl-3-palmitoyl-rac-glycerol (LLP), 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol (LOP), 1,3-dipalmitoyl-2-linoleoylglycerol (PLP), 1,2-dioleoyl-3-palmitoyl-rac-glycerol (OOP), 1,3-dioleoyl-2-palmitoylglycerol (POO), 1,3-dipalmitoyl-2-oleoylglycerol (POP), linoleic acid (LA), palmitic acid (PA), oleic acid (OA), dimethyl sulfoxide (DMSO), and glycerol reagent were purchased from Sigma-Aldrich (St. Louis, MO). Palmitic acid (PA), oleic acid (OA), dimethyl sulfoxide (DMSO), and palm oil (POO) were purchased from Thermo Fisher Scientific, (Waltham, MA). Glycerol triilinolein (GTL) and other triglycerides including triolein (GTO), tripalmitin (GTP), and mixed triglycerides 1,2-dilinoleoyl-3-palmitoyl-rac-glycerol (LLP), 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol (LOP), 1,3-dipalmitoyl-2-linoleoylglycerol (PLP), 1,2-dioleoyl-3-palmitoyl-rac-glycerol (OOP), 1,3-dioleoyl-2-palmitoylglycerol (POO), 1,3-dipalmitoyl-2-oleoylglycerol (POP), linoleic acid (LA), palmitic acid (PA), oleic acid (OA), dimethyl sulfoxide (DMSO), and glycerol reagent were purchased from Sigma-Aldrich (St. Louis, MO). CET (Bachem AG, Bubendorf, Switzerland), IL12 (150 ng per dose per mouse; Bachem AG, Bubendorf, Switzerland) × 12 doses on two consecutive days as described before (27). IL12 (150 mg per dose per mouse; PeproTech) and IL18 (750 mg per dose per mouse; R&D Systems) pancreatitis was induced by giving IL12,18 as two doses 24 hours apart to lean ICR mice (10 to 14 weeks) as described previously (27).

Experimental diets

These were started immediately in ob/ob [B6.Cg-Leptob/]; Jax Stock #00632, the Jackson laboratory (Bar Harbor, ME) or C57bl/6 (to induce DIO) after weaning (age of 4 to 5 weeks), with an intent to change visceral fat composition. The diets are detailed in fig. S4 and were given ad libitum. Normal chow [Purina 5053 diet, LabDiet (www.lab supplierx.com/wp-content/uploads/2012/10/5053.pdf), which contains 5.0% of which ≤70% is UFA] and water were fed to control mice ad libitum. Mice were weighed periodically (1 to 4 weeks, depending on the rate of weight gain), and the body fat was measured using a Minispec Body Composition analyzer (Bruker Corporation, Billerica, MA) using nuclear magnetic resonance as previously described (27).

Experimental pancreatitis

The models used were C57BL/6 mice given the experimental diets or normal chow after 28 weeks. C57 pancreatitis was induced by hourly intraperitoneal injections of CER (50 μg/kg; Bachem AG, Bubendorf, Switzerland) × 12 doses on two consecutive days as described before (27). IL12 (150 ng per dose per mouse; PeproTech) and IL18 (750 mg per dose per mouse; R&D Systems) pancreatitis was induced by giving IL12,18 as two doses 24 hours apart to lean C57 mice (10 to 14 weeks) as described previously (27). Both agents were dissolved in PBS and given intraperitoneally. When studying the effect of intraperitoneal GTL (150 μl) or GTP (150 mg) in these models, either agent was given 4 hours after the first CER injection or second IL12,18 injections. NEFA were administered into the peritoneal cavity of mice as described previously (7, 22, 28).

Animal experiments

All animals were acclimatized for a minimum of 2 days before use. These were housed with a 12-hour light/dark cycle at room temperature, fed normal laboratory chow (ICR mice) or an experimental diet (see below), and were allowed to drink ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic (Scottsdale, AZ). Ex vivo studies were done on pancreatic acini harvested from ICR mice (Charles River Laboratories, Wilmington, MA). In vivo studies were done on genetically obese male ob/ob (B6.V-lepob/J; the Jackson laboratory, Bar Harbor, ME) mice given an experimental diet below or ICR mice. The mice were 7 to 14 weeks of age at the time of studies. There were six to eight mice in each group.

In vivo studies in mice

Experimental diets

These were started immediately in ob/ob [B6.Cg-Leptob/]; Jax Stock #00632, the Jackson laboratory (Bar Harbor, ME) or C57bl/6 (to induce DIO) after weaning (age of 4 to 5 weeks), with an intent to change visceral fat composition. The diets are detailed in fig. S4 and were given ad libitum. Normal chow [Purina 5053 diet, LabDiet (www.lab supplierx.com/wp-content/uploads/2012/10/5053.pdf), which contains 5.0% of which ≤70% is UFA] and water were fed to control mice ad libitum. Mice were weighed periodically (1 to 4 weeks, depending on the rate of weight gain), and the body fat was measured using a Minispec Body Composition analyzer (Bruker Corporation, Billerica, MA) using nuclear magnetic resonance as previously described (27).

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Histology and TUNEL staining

Pancreas, kidney, and lung tissue were fixed with 10% neutral buffered formalin and processed for embedding in paraffin. Sections (5 μm) were used for hematoxylin and eosin (H&E) staining and for TUNEL staining as described previously (5, 6, 27). Digital images of section were captured with a digital microscope Axiol Image M2 or Axi Observer Z1 (Zeiss, Oberkochen, Germany) with a 20× objective with 10 random images per section and quantified as number of TUNEL-positive nuclei/high field of lung tissue. Pancreatic necrosis was quantified on whole-pancreas H&E-stained sections after being examined by a trained morphologist blinded to the sample. Briefly,
all pancreatic parenchymal area was imaged using the PathScan Enabler IV slide scanner (Meyer Instruments, Houston, TX), and images were evaluated for total acinar area, total acinar necrosis, and acinar necrosis in direct proximity to the necrosed fat, i.e., peri-fat acinar necrosis as described previously (6, 27) in pixels for each pancreas. Percentage necrosis was reported as a percentage of the total acinar area for each pancreas.

**In vitro cell studies**

All data shown are from a three to five independent experiments.

**Use of lipids**

Triglycerides (e.g., GTL, LLP, LOP, and GTO) or NEFA were sonicated at 10× concentrations into the medium, and the only solvent used was 5% DMSO (as a 10× stock) for GTP and PLP or for palmitate, since these otherwise were insoluble and precipitated out in the medium. Solvents were otherwise avoided, since lipolysis in vivo takes place without solvents. These agents were added at a 1× concentration.

**Pancreatic acinar harvest and use**

Eight- to 12-week-old ICR mice (Charles River Laboratories, Wilmington, MA) were euthanized, and pancreatic acini were harvested as described (7, 28).

**Cell lines and use**

HEK293 cells were maintained in Eagle’s minimal essential media [American Type Culture Collection (ATCC), Manassas, VA] supplemented with 10% fetal bovine serum (ATCC, Manassas, VA) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA). All cell cultures were maintained in a humidified 5% CO₂ atmosphere in air at 37°C. Human endothelial cells (HUV-EC-C) were maintained in Kaighn’s modification of Ham’s F-12 medium (F-12K, ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (ATCC, Manassas, VA), 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA), heparin (0.1 mg/ml; Millipore Sigma, St. Louis, MO), and Corning endothelial cell growth supplement (50 µg/ml; Thermo Fisher Scientific).

**TEER and dextran permeability assay**

HUV-EC-C cells were grown as monolayers on a 0.4-µm polyester Transwell permeable membrane (Corning, NY) precoated with type IV collagen and then used for trans-endothelial electrical resistance (TEER) measurement and permeability tracer flux assay. Before studies, cells were suspended in Heps buffer (pH 7.4) (20 mM Heps, 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 10 mM sodium pyruvate, and 1 mM CaCl₂) and exposed to NEFA at the indicated concentration (60 µM). TEER measurements were made using EVOM2 Epithelial Voltomheter with an STX2 electrode (World Precision Instruments, Sarasota, FL). Results were presented as absolute values (Ω·cm²) after 2 hours of treatment. To evaluate the paracellular permeability of cultured HUV-EC-C cells, 10 kD of Dextran Alexa Fluor 647 (Thermo Fisher Scientific, Waltham, MA) was added to the upper chamber, and the increase in fluorescence was measured in the lower chamber 2 hours after stimulation with NEFA.

For in vitro calcium ion flux and mitochondrial depolarization studies, cells were loaded with fura-2 AM (5 µg/ml; Molecular Probes, Invitrogen) and 5,5′,6′,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 5 µg/ml; Enzo Life Sciences, Farmingdale, NY) at 37°C for 30 min in the media that they were cultured or harvested in. After this, these were stored on ice till just before use. Just before the experiment, the cells were washed and suspended in Heps buffer (pH 7.4) (20 mM Heps, 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, and 10 mM sodium pyruvate) as previously described (6, 7, 28). Triglycerides or NEFA were added after 2 min of stirring at a 1× concentration to the stirred cell suspensions in a quartz cuvette at 37°C, and changes in intracellular calcium concentrations were determined by alternate excitation at 340 and 380 nm, measuring emission at 510 nm. Mitochondrial inner membrane potential values (Ψm) were determined by excitation at 490 nm and alternate measuring emission at 530 and 590 nm using the F2100 Hitachi Fluorescence Spectrophotometer. The data were collected every 10 s. The first 2 min of data were averaged and subtracted, and the net increase was plotted as a function of time or NEFA concentrations. In some studies, excess (i.e., 0.5%) fatty acid–free bovine serum albumin was added to neutralize and thus determine the changes attributable to the uNEFA.

**Assays**

**Biochemical assays**

The assays (amylase, lipase, BUN, and calcium; Pointe Scientific, Canton, MI), DNA damage: Quant-iT PicoGreen dsDNA reagent (Life Technologies, Carlsbad, CA), LDH leakage using the Cytotoxicity Detection Kit (Roche, Mannheim, Germany), intracellular ATP measurement using ATP assay kit (CellTiter-Glo 2.0 Assay, Promega, Madison, USA), glycerol using free glycerol reagent, and standard (Sigma-Aldrich, St. Louis, MO) and colorimetric total free fatty acids using NEFA kit (FUJIFILM Wako Diagnostics, Mountain View, CA) were done as per the manufacturer’s protocol (5, 6, 27).

**uNEFA measurement by fluorescence**

uNEFAs were measured by the emission at 550/457 nm upon excitation at 375 nm and calculated as described in the ADIFAB2 kit (FKA Sciences LLC, CA, USA) protocol version 1.0 6-9-04. Standard concentration versus fluorescence ratio curves were made with known concentrations of either >99.5% purity LA, OA, or PA (1, 2, 5, 10, 20, 30, 50, and 100 µM) in DMSO to have these all in the pure monomeric form. Then, the unknown (e.g., mouse serum) was measured and reported as uNEFA (micromolar concentrations), based on the fatty acid standard curve.

**Cytokine/chemokine assays**

Serum cytokine/chemokine protein levels were assayed with a MILLIPLEX MAP Mouse Adipokine Magnetic Bead Panel (Millipore, Burlington, MA) according to the manufacturer’s recommendations on a Luminex 200 System (Life Technologies) and analyzed using the xPONENT software.

**Triglyceride lipid composition and NEFA analysis**

These were done at the Hormone Assay and Analytical Services Core (Vanderbilt University Medical Center). For tissue triglyceride composition, the Folch method (76) was used, and the lipids recovered in the chloroform phase were separated by thin-layer chromatography using Silica Gel 60A plates. The triglyceride fraction was methylated using the BF3/methanol method (77). These were analyzed using gas chromatography (Agilent 7890A) using a capillary column (SP2380, 0.25 mm by 30 m, 0.25-µm film; Supelco, Bellefonte, PA) and helium as a carrier gas. Methyl esters of the fatty acids were identified by comparing their retention times to those of known standard flame ionization detection. Internal lipid standards for quantification included dipentadecanoyl phosphatidylcholine (C15:0), diheptadecanoin (C17:0), trieicosenoin (C20:1), and cholesteryl eicosenoate (C20:1). The assays (amylase, lipase, BUN, and calcium; Pointe Scientific, Canton, MI), DNA damage: Quant-iT PicoGreen dsDNA reagent (Life Technologies, Carlsbad, CA), LDH leakage using the Cytotoxicity Detection Kit (Roche, Mannheim, Germany), intracellular ATP measurement using ATP assay kit (CellTiter-Glo 2.0 Assay, Promega, Madison, USA), glycerol using free glycerol reagent, and standard (Sigma-Aldrich, St. Louis, MO) and colorimetric total free fatty acids using NEFA kit (FUJIFILM Wako Diagnostics, Mountain View, CA) were done as per the manufacturer’s protocol (5, 6, 27).

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above. Dipentadecanoic acid (C15:0) was added as an internal standard. UFA amounts were calculated by adding individual C16:1, C18:1, C18:2, C18:3, C20:4, and C20:5 fatty acids. SFA amounts were calculated by adding individual C12:0, C14:0, C16:0, and C18:0 fatty acids.

Western blotting
Western blotting was performed as described previously (54). Briefly, cell lysate or fat pad lysate was prepared in radioimmunoprecipitation assay lysis buffer containing various protease inhibitors (complete and free of EDTA; Roche, Pleasanton, CA). Protein concentration (1 mg/ml) of the lysate was adjusted, boiled (10 min) in Laemmli buffer, and run on (10 mg per lane) 4 to 20% gradient polyacrylamide gels. Then, proteins were transferred from the gel to polyvinylidene difluoride membrane (Merck KGaA, Darmstadt, Germany) and blocked with 5% nonfat dry milk incubated with primary antibody of PNLIP (1:1000; Santa Cruz Biotechnology, Dallas, TX), 1xB-α (1:1000; Santa Cruz Biotechnology), α-tubulin (1:500; DSHB, University of Iowa, Iowa), cytochrome c (1:1000; Santa Cruz Biotechnology), and Cox-IV (1:1000; Thermo Fisher Scientific, Waltham, MA) separately. Then, blots were probed with horseradish peroxidase–labeled corresponding secondary antibodies (1:10,000; Millipore, Billerica, MA). Band intensity was visualized by chemiluminescence using the Pierce ECL 2 Western Blotting Substrate Kit (Thermo Fisher Scientific) and quantified by densitometry as described previously (79).

Isothermal titration calorimetry
The hydrolysis of triglycerides by hPTL was carried out on a Nano ITC (TA Instruments–Waters LLC, New Castle, DE) instrument and through Nano ITCRun Software v3.5.6. The experimental setup was equipped with a computer-controlled micro-syringe injection device, which allowed to inject small amounts of stock solution of even 0.1 μl to the sample chamber. Enzyme reaction was performed by two methods: either incremental injection of substrate to enzyme or continuous injection of enzyme to substrate.

Incremental injection
A total of 10 aliquots of freshly prepared, degassed GTL or LLP or LOP (0.4 mM, 5 μl per injection) were injected from a rotating syringe of speed 350 rpm into the ITC sample chamber containing hPNLIP (12 nM, ~500 U/liter activity) equilibrated at 37°C. The interval between two injections was 300 s. Similarly, individual triglyceride into PBS or PBS into hPNLIP was run as a control. To confirm the specificity, LLL was injected into hPNLIP having orlistat (50 μM orlistat; Cayman Chemical Company, Ann Arbor, MI) in a similar process.

Continuous injection
Degassed hPNLIP (45 μl, 120 nM) was injected during 900 s from a rotating syringe into the ITC sample chamber containing LLL or LLP or LOP (0.1 mM) equilibrated at 37°C. The control experiment was performed by injecting PBS into hPNLIP. hPNLIP-mediated hydrolysis of LLL was confirmed using hPNLIP with orlistat in the same experimental setup.

Calculation
The ITC raw data were analyzed using the NanoAnalyze v3.10.0 software. Maximum velocity at saturating substrate concentration ($V_{\text{max}}$) was determined. Michaelis constant ($K_m$), the measure of dissociation/affinity of enzyme-substrate complex, was calculated from the substrate concentration at $V_{\text{max}}/2$. Enzyme turnover rate ($K_{\text{cat}}$) was calculated by $V_{\text{max}}/\text{enzyme concentration}$. Substrate to product formation rate ($V$) was calculated by the equation $[K_{\text{cat}} \times \text{enzyme concentration} \times \text{substrate concentration}]/[K_m + \text{substrate concentration}]$. For each experiment, we calculated $V_{\text{max}}$, $K_m$, $K_{\text{cat}}$, and $V$ values and summarized as means ± SEM from three independent experiments.

From the raw data of continuous injection, enthalpy of the reaction was calculated from the delta heat change per mole. Interaction of triglyceride and hPNLIP followed by hydrolysis of triglyceride was graphically presented from three independent experiments.

ITC for CMC measurement experiments
We followed the method described previously (54). A total of 20 aliquots of degassed LA (0.7 mM, 2.5 μl per injection from PBS, pH 7.4), OA (0.34 mM, 2.0 μl per injection from PBS, pH 7.4), and PA (0.34 mM in 0.34% DMSO, 2.0 μl per injection from PBS, pH 7.4) were injected (10 μM per injection for LA and 4 μM per injection for both OA and PA) from a rotating syringe (350 rpm) into the ITC sample chamber containing PBS equilibrated at 37°C. Double-distilled water was used in a reference electrode chamber. The interval between two injections was kept at 200 s. The control experiment was performed by injecting PBS into PBS and DMSO (0.34%) into PBS.

Ultracentrifugation studies
A total of 500 μM C18:1, C18:2, and C18:3 were probe-sonicated into PBS (pH 7.4) at room temperature. For C16:0 and C18:0, 150 mM stocks were prepared in pure DMSO, and these were then serially sonicated and diluted down to 500 μM (0.34% DMSO) in PBS (pH 7.4) at room temperature. A total of 4.8 ml of sample was loaded in OptiSeal 4.9-ml tubes (Beckman Instruments Inc., Palo Alto, CA) in a NVT90 fixed-angle rotor (Beckman Instruments Inc., Palo Alto, CA) and spun at 105g for 1 hour at room temperature in a Beckman Coulter Optima L-100 XP Ultracentrifuge (Beckman Coulter Inc., IN, USA). The bottom of the tubes was then pierced with a 25-gauge needle, and 1.0 ml of the liquid was removed. NEFA concentrations in these were measured using the colorimetric NEFA kit (FUJIFILM Wako Diagnostics, Mountain View, CA).

Molecular docking
Protein Data Bank structure 1LPA (interfacial activation of the lipase-procolipase complex by mixed micelles revealed by x-ray crystallography) was downloaded from the RCSB protein data bank (80) (rcsb.org) and then imported to the Schrödinger Maestro (Schrödinger Release 2019-4; Maestro, Schrödinger LLC, New York, NY, 2019). Using the protein preparation wizard (Schrödinger Release 2019-4: Protein Preparation Wizard; Epik, Schrödinger LLC, New York, NY, 2016; Impact, Schrödinger LLC, New York, NY, 2016; Prime, Schrödinger LLC, New York, NY, 2019), 1LPA was pre-processed at pH 7.4, and a restrained minimization was completed by converging when the RMSD between iterations was less than 0.30 Å. The processed 1LPA structure was rendered in an aqueous environment at pH 7.4 in the presence of 150 mM sodium ions and 150 mM chloride ions by using the Desmond System Builder (Schrödinger Release 2019-4: Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, 2019; Maestro-Desmond Interoperability Tools, Schrödinger, New York, NY, 2019) within Schrödinger Maestro to mimic the physiological environment that the enzyme functions in.

All structures to be docked (GTL, GTP, LLP, LOP, and DLPC) were downloaded as SDF files from PubChem (81) and imported to Schrödinger Maestro. The LigPrep (Schrödinger Release 2019-4: LigPrep, Schrödinger LLC, New York, NY, 2019) feature was used to obtain the ionization states of the ligands at pH 7.4 and to generate tautomers of the ligands. An induced fit docking (Schrödinger Release 2019-4: Induced Fit Docking protocol; Glide, Schrödinger LLC, New York, NY, 2016; Prime, Schrödinger LLC, New York, NY, 2019) was used to find the best docked conformations based on a scoring function. The 5 highest scoring docked conformations were selected for each ligand and subjected to NMR analysis.
NY, 2019) was performed by selecting the four triglycerides as the ligands to be docked and designating the 1,2-didodecanoyl-sn-glycero-3-phosphocholine ligand from the 1LPA crystal structure as the centroid of the workspace ligand around which the receptor grid was generated. The ligand diameter midpoint box was constructed with dimensions of X, 10 Å; Y, 10 Å; and Z, 10 Å; while the receptor grid was generated with dimensions of X, 30 Å; Y, 30 Å; and Z, 30 Å. No constraints were imposed to ensure that the docking simulation was unbiased. The induced fit docking used the OPLS3e force field (X, 10 Å; and Y, 10 Å; and Z, 10 Å; was generated. The ligand diameter midpoint box was constructed.

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**REFERENCES AND NOTES**

Independent variables for in vivo studies are shown in box plots in which the mean (dotted line), median (solid line), and in vitro data are shown as bar graphs reported as means ± SEM. Line graphs were used for continuous variables. All values are reported as means ± SD. Significance levels were evaluated at P < 0.05. Data for multiple groups were compared by one-way analysis of variance (ANOVA) versus controls, and values significantly different from controls were shown as (asterisks) or with the P value mentioned above the corresponding conditions. When comparing two groups, a t test or Mann-Whitney test was used, depending on the normality of distribution and shown as (asterisks) when significantly different; alternately, P values are shown in the graph. Graphing was done using SigmaPlot 12.5 (Systat Software Inc., San Jose, CA).

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**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/5/eabd6449/DC1

View/request a protocol for this paper from Bio-protocol.

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J. Martinez, J. Sánchez-Payá, J. M. Palazón, J. R. Aparicio, A. Picó, M. Pérez-Mateo, Obesity: A prognostic factor of severity in acute pancreatitis. Pancreas 19, 15–20 (1999).

30. C. D. Johnson, S. K. C. Toh, M. J. Campbell, Combination of APACHE-II score and an obesity score (APACHE-O) for the prediction of severe acute pancreatitis. Pancreatology 4, 1–6 (2004).

31. G. I. Papachristou, D. J. Papachristou, H. Avula, A. Silvia, D. C. Whitcomb. Obesity increases the severity of acute pancreatitis: Performance of APACHE-O score and correlation with the inflammatory response. Pancreas 6, 279–285 (2006).

32. B. de Waee, B. Vannierlo, Y. Van Nieuenhove, G. Delvaux, Impact of body overweight and class I, II and III obesity on the outcome of acute biliary pancreatitis. Pancreas 32, 343–345 (2006).

33. L. Sempere, J. Martínez, E. de Madaria, B. Lozano, J. Sánchez-Paya, R. Jover, M. Pérez-Mateo, Obesity and fat distribution implicate a greater systemic inflammatory response and a worse prognosis in acute pancreatitis. Pancreatology 8, 257–264 (2008).

34. R. Hegazi, A. Raina, T. Graham, S. Rohlif, P. Canta, H. Kandil, S. J. O’Keefe, Early jejunal feeding initiation and clinical outcomes in patients with severe acute pancreatitis. JPEN J. Parenter. Enteral Nutr. 35, 91–96 (2011).

35. J. P. B. Davis, K. M. Eltawil, B. Abu-Wasel, M. J. Walsh, T. Topp, M. Molinari, Effect of obesity and decompensative laparotomy on mortality in acute pancreatitis requiring intensive care unit admission. World J. Surg. 37, 318–332 (2013).

36. J. Katouchova, J. Bober, P. Harbulak, A. Hudak, T. Gajdzik, R. Kalanin, J. Radonak, Obesity as a risk factor for severe acute pancreatitis patients. Wien. Klin. Wochenschr. 126, 223–227 (2014).

37. E. G. Górriz, R. Calderon, P. Montes Teves, E. Monge Salgado, Bisap-O: Obesity included in score BisAP to improve prediction of severity of acute pancreatitis. Rev. Gastroenterol Perú 32, 251–256 (2012).

38. J. Suazo-Barahona, R. Carmona-Sánchez, G. Robles-Díaz, P. Mikele-García, V. Gargas-Vorácková, L. Uscanga-Domínguez, M. Peláez-Luna, Obesity: A risk factor for severe acute biliary and alcoholic pancreatitis. Am. J. Gastroenterol. 93, 1324–1328 (1998).

39. Y. P. Yeung, B. Y. Lam, A. W. Yip, APACHE system is better than Ranson system in the prediction of severity of acute pancreatitis. Hepatobiliary Pancreat. Dis. Int. 5, 294–299 (2006).

40. Y. Yashima, H. Isayama, T. Tsujino, R. Nagano, K. Yamamoto, S. Mizuno, H. Yagioka, K. Kawakubo, T. Sasaki, H. Kogure, Y. Nakai, K. Hirano, N. Sashihara, M. Tada, T. Kawanbe, K. Koike, M. Omata, A large volume of visceral adipose tissue leads to severe acute pancreatitis. J. Gastroenterol. 46, 1213–1218 (2011).

41. F. Yang, H. Wu, Y. Li, Z. Li, C. Wang, J. Yang, B. Hu, Z. Huang, R. Ji, X. Zhan, H. Xie, L. Wang, M. Zhang, C. Tang, Prevention of severe acute pancreatitis with octreotide in obese patients: A prospective multi-center randomized controlled trial. Pancreas 41, 1206–1212 (2012).

42. S. R. Thomson, W. S. Hendry, G. A. McFarlane, A. J. Davidson, Epidemiology and outcome of acute pancreatitis. Br. J. Surg. 74, 398–401 (1987).

43. D. Yadav, A. B. Lowenfels, The epidemiology of pancreatitis and pancreatic cancer. Gastroenterology 144, 1252–1261 (2013).

44. T. L. Blasbalg, J. R. Hibel, C. E. Ramsden, S. F. Majchrzak, R. R. Rawlings, Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. Am. J. Clin. Nutr. 93, 950–962 (2011).

45. B. Khatau, R. N. Trivedi, P. Noel, K. Patel, R. Singh, C. de Oliveira, S. Trivedi, V. Mishra, M. Lowe, V. P. Singh, Carboxyl-ester lipase may not mediate lipolytic injury during severe acute pancreatitis. Am. J. Pathol. 189, 1226–1240 (2019).

46. C. de Oliveira, B. Khatau, A. Bag, B. El-Kurdi, K. Patel, V. Mishra, S. Navina, V. P. Singh, Multimodal transgastric local pancreatic hyperstimulation reduces severity of acute pancreatitis in rats and increases survival. Gastroenterology 156, 735–747.e10 (2019).

47. S. L. Blamey, C. W. Imrie, J. O’Neill, W. H. Gilmore, D. C. Carter, Prognostic factors in acute pancreatitis. Gut 25, 1340–1346 (1984).

48. C. W. Imrie, I. S. Benjamin, J. C. Ferguson, A. J. McKay, I. Mackenzie, J. O’Neill, L. H. Blumgart, A single-centre double-blind trial of trasylol therapy in primary acute pancreatitis. Br. J. Surg. 65, 337–341 (1978).

49. M. Pini, J. A. Sennello, R. J. Cabay, G. Fantuzzi, Effect of diet-induced obesity on acute pancreatitis induced by administration of interleukin-12 plus interleukin-18 in mice. Obesity (Silver Spring) 18, 476–481 (2010).

50. J. A. Williams, M. Korec, R. L. Dormer, Action of secretagogues on a new preparation of functionally intact, isolated pancreatic acini. Am. J. Physiol. 235, 517–524 (1978).
Analysis of data were facilitated and carried out by B.K., B.E.-K., K.P., C.R., P.N., M.C., J.R.Y., S.K., A.G., M.L., and V.P.S.; while B.K., D.O.F., M.L., and V.P.S. critically evaluated the manuscript. Interpretation of data was done by B.K., B.E.-K., K.P., C.R., M.L., and V.P.S. Manuscript was drafted by B.K., B.E.-K., K.P., C.R., A.G., and V.P.S. Statistical analysis was done by B.K., B.E.-K., K.P., M.C., and V.P.S. Funding was obtained by V.P.S. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 2 July 2020
Accepted 11 December 2020
Published 29 January 2021
10.1126/sciadv.abd6449

Citation: B. Khatua, B. El-Kurdi, K. Patel, C. Rood, P. Noel, M. Crowell, J. R. Yaron, S. Kostenko, A. Guerra, D. O. Faigel, M. Lowe, V. P. Singh, Adipose saturation reduces lipotoxic systemic inflammation and explains the obesity paradox. Sci. Adv. 7, eabd6449 (2021).
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Sci Adv 7 (5), eabd6449.
DOI: 10.1126/sciadv.abd6449