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Saturated Fatty Acids Induce c-Src Clustering within Membrane Subdomains, Leading to JNK Activation

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SUMMARY

Saturated fatty acids (FA) exert adverse health effects and are more likely to cause insulin resistance and type 2 diabetes than unsaturated FA, some of which exert protective and beneficial effects. Saturated FA, but not unsaturated FA, activate Jun N-terminal kinase (JNK), which has been linked to obesity and insulin resistance in mice and humans. However, it is unknown how saturated and unsaturated FA are discriminated. We now demonstrate that saturated FA activate JNK and inhibit insulin signaling through c-Src activation. FA alter the membrane distribution of c-Src, causing it to partition into intracellular membrane subdomains, where it likely becomes activated. Conversely, unsaturated FA with known beneficial effects on glucose metabolism prevent c-Src membrane partitioning and activation, which are dependent on its myristoylation, and block JNK activation. Consumption of a diabetogenic high-fat diet causes the partitioning and activation of c-Src within detergent insoluble membrane subdomains of murine adipocytes.

INTRODUCTION

Insulin resistance is a pathophysiologic condition caused by defective insulin signaling that can cause type 2 diabetes. Although insulin resistance has a strong genetic component (Kahn et al., 1996), it can be initiated and exacerbated by obesity (Ford et al., 1997). Obesity is also associated with low-grade chronic inflammation (Hotamisligil, 2010), whose hallmarks include enhanced production of inflammatory mediators, infiltration of activated macrophages into adipose tissue, and chronic JNK activation in liver, muscle, and fat tissue of obese individuals (Gregor et al., 2009) and experimental animals (Hirosumi et al., 2002; Solinas et al., 2006). Mouse studies identified adipocytes as an important cell type within which JNK activation causes cell-autonomous interference with insulin signaling (Sabio et al., 2008). Adipocytes store fat and exert both protective and adverse effects on glucose metabolism, depending on the quality and quantity of stored lipids (Virtue and Vidal-Puig, 2008). Not all lipids are equal in their metabolic and health effects; whereas saturated FA have a strong diabetogenic effect (Clandinin et al., 1991) and lead to JNK activation (Solinas et al., 2006), certain unsaturated FA and especially polyunsaturated FA (PUFA) are protective and can even reverse obesity-induced insulin resistance (Clandinin et al., 1991; Robinson et al., 2007; Storlien et al., 1987).

The JNKs belong to the mitogen-activated protein kinase (MAPK) group and are activated by physical stresses, such as UV light and heat shock, and receptor-mediated mechanisms, including TNF receptor 1 (TNFR1) and Toll-like receptors (TLR) 2 and 4 (Karin and Gallagher, 2005). Following activation, JNKs participate in many physiological and pathophysiological processes, including apoptosis, cell proliferation, cell migration, and cytokine production. Many of these effects depend on transcription factor activation, but JNKs also affect cell physiology through other substrates (Karin and Gallagher, 2005). For instance, JNKs phosphorylate insulin receptor substrates (IRS) 1 and 2 at serine (Ser) or threonine (Thr) residues and thereby attenuate their insulin-induced tyrosine (Tyr) phosphorylation, resulting in downmodulation of insulin action and diminished AKT activation (Aguirre et al., 2002; Solinas et al., 2006). JNK1-deficient mice are protected from obesity-induced insulin resistance (Hirosumi et al., 2002) due to loss of cell-autonomous IRS1/2 phosphorylation within adipocytes (Sabio et al., 2008). JNKs also contribute to insulin resistance by stimulating production of inflammatory mediators by myeloid cells (Solinas et al., 2007; Vallerie et al., 2008) and have neuronal effects that influence obesity and energy metabolism (Sabio et al., 2010). Several mechanisms were proposed to explain chronic JNK activation in obesity, including endoplasmic reticulum (ER) stress (Ozcan et al., 2004) and signaling through inflammation-associated receptors (Shi et al., 2006; Uysal et al., 1997). However, how obesity triggers ER stress remains to be determined and the mechanisms by which ER stress leads to JNK activation are not fully understood either, although they were proposed to depend on the RNA-dependent protein kinase PKR or TRAF2 (Hotamisligil, 2010). Other studies have

implicated the phosphoinositide 3-kinase (PI3K) p85α regulatory subunit (Taniguchi et al., 2007), the scaffolding protein JIP1 (Jaeschke et al., 2004), the lipid chaperone aP2 (Erbay et al., 2009), and the mixed lineage kinase MLK3 (Jaeschke and Davis, 2007). These studies, too, poorly explain JNK activation in fat depots during obesity.

In cultured cells, saturated FA such as palmitic acid (PA; C16:0) and stearic acid (SA; C18:0), which are elevated in plasma of obese individuals (Reaven et al., 1988), cause a spectrum of diabetes-related defects and activate JNK (Kharroubi et al., 2004; Solinas et al., 2006). Strong JNK activation is unique to long-chain saturated FA, whereas unsaturated FA are poor JNK activators and even inhibit JNK activation by saturated FA. These effects correlate with the pathophysiological effects of different FA types, suggesting that saturated FA may be physiologically relevant JNK activators. The exact mechanism through which saturated FA activate JNK in cells is unknown, although several studies suggest that FA may activate JNK via TLR2/4 (Shi et al., 2006; Tsukumo et al., 2007). Yet, JNK activation by PA does not require TAK1, a MAPK kinase kinase (MAP3K) that is essential for JNK activation by conventional TLR2/4 ligands (Jaeschke and Davis, 2007; Tseng et al., 2010). Moreover, saturated FA cause slow and sustained JNK activation, whereas JNK activation by TLR ligands is rapid and transient (Solinas et al., 2006; Tseng et al., 2010). The mechanism responsible for discrimination between saturated and unsaturated FA (some of which differ only by two hydrogen atoms) that accounts for their different effects on JNK activity and insulin signaling is even more mysterious.

Because FA incorporate into cellular membranes either in their original form or after conversion into other lipid species, they may exert receptor-independent effects on cell signaling and physiology. For instance, PA incorporation reduces membrane fluidity, whereas unsaturated FA and especially PUFA do not have such an effect (Clamp et al., 1997; Karnovsky et al., 1982; Luo et al., 1996; Rintoul et al., 1978; Stulnig et al., 1998, 2001; Webb et al., 2000). We therefore postulated that the membrane may play a key role in JNK regulation by FA and searched for membrane-associated protein kinases that are essential for JNK activation by saturated FA. We now show that a key mediator of JNK activation by saturated FA is c-Src, whose activation correlates with its FA-induced partitioning into intracellular membrane subdomains that can be isolated based on detergent insolubility (Karnovsky et al., 1982; Lingwood and Simons, 2007; Pike, 2004, 2009) or visualized by fluorescent microscopy. Our results provide a biochemical model that explains the differential effects of saturated and unsaturated FA on JNK activity and human health.

RESULTS

c-Src Is Required for JNK Activation by FA

Saturated FA that increase membrane order and melting temperature lead to JNK activation, whereas unsaturated FA capable of decreasing membrane order and melting temperature do not (Solinas et al., 2006). Optimal JNK activation by FA requires prolonged incubation periods and is much slower than receptor-mediated responses. We therefore hypothesized that the membrane is the most proximal sensor of FA and that membrane alterations may activate an associated protein kinase that triggers a signaling cascade leading to JNK. Although MLK3 was identified as the most upstream protein kinase that mediates JNK activation by saturated FA (Jaeschke and Davis, 2007), it is a MAP3K that is not membrane associated (Handley et al., 2007). Because many MAP3Ks act downstream to tyrosine kinases, we tested whether the Src group of membrane-anchored tyrosine kinases is involved in JNK activation by FA. Indeed, immortalized fibroblasts derived from mice that are deficient in three broadly expressed Src kinases—c-Src, Yes, and Fyn (or SYF) (Stein et al., 1994) —did not exhibit increased JNK1 activity upon incubation with either SA or PA (Figure 1A). A similar defect was exhibited by *c-Src*$^{-/-}$ fibroblasts, and reconstitution of *SYF*$^{-/-}$ cells with c-Src (SYF+Src) fully restored JNK1 activation. By contrast, reconstitution of *SYF*$^{-/-}$ cells with Yes or Fyn did not fully restore JNK activation by saturated FA (Figure 1B). c-Src was mainly required for JNK activation by saturated FA with acyl chains of at least 16 carbons, as lauric acid (LA; C12:0) and myristic acid (MA; C14:0) did not activate JNK in SYF+Src cells, and neither did the monounsaturated FA palmitoleic acid (POA; C16:1, n-7) or the PUFA eicosapentaenoic acid (EPA; C20:5, n-3) (Figure S1A available online). Knockdown of endogenous c-Src by shRNA in NIH 3T3 cells decreased JNK activation by PA (Figure 1C), and similar results were obtained in human HEK293T cells (Figure S1B). JNK activation by PA was restored by reconstitution of Src-deficient cells with wild-type (WT) Src, but not with an inactive (Y418F) mutant (Figure 1D). *SYF*$^{-/-}$ cells reconstituted with c-Src(Y527F), lacking the negative phosphorylation site recognized by Csk (Okada et al., 1991), displayed modestly enhanced JNK activation in response to PA, ruling out involvement of Csk-mediated c-Src phosphorylation in the response to saturated FA. By contrast, a compound c-Src(Y527F/G2A) mutant that also lacks the myristoylation sequence necessary for membrane targeting (Patwardhan and Resh, 2010) was highly compromised in JNK activation (Figures 1D and S1C). Pretreatment of SYF+Src cells with the Src family kinase inhibitor PP2 prevented JNK activation by PA, but the structurally similar control compound PP3 was ineffective (Figure S1D).

We also examined the role of c-Src in PA-induced MLK3 activation. NIH 3T3, SYF, and SYF+Src cells were treated with PA for up to 8 hr, and MLK3 activation was assessed by immunoblotting with an antibody that recognizes Thr277/Ser281 phosphorylation. Within 2 hr of PA addition, sustained MLK3 phosphorylation was detected in NIH 3T3 and SYF+Src cells, but not in *SYF*$^{-/-}$ cells (Figure 1E). JNK activation in the same cells was also monitored by immunoblotting and was found to parallel MLK3 activation. Curiously, *SYF*$^{-/-}$ cells contained lower amounts of MLK3 than 3T3 or SYF+Src cells, but the basis and significance of this finding are unclear.

We examined whether c-Src is needed for induction of ER stress, evaluated by induction of CHOP mRNA and IRE1α-catalyzed XBP1 mRNA splicing. Incubation of fibroblasts with either PA or SA induced CHOP mRNA and XBP1 mRNA splicing, but no role for Src kinases could be identified (Figure S1E). In accordance with these results, PP2 pretreatment of J774A.1 cells did not block PA-induced ER stress markers (Figure S1F). PP2,

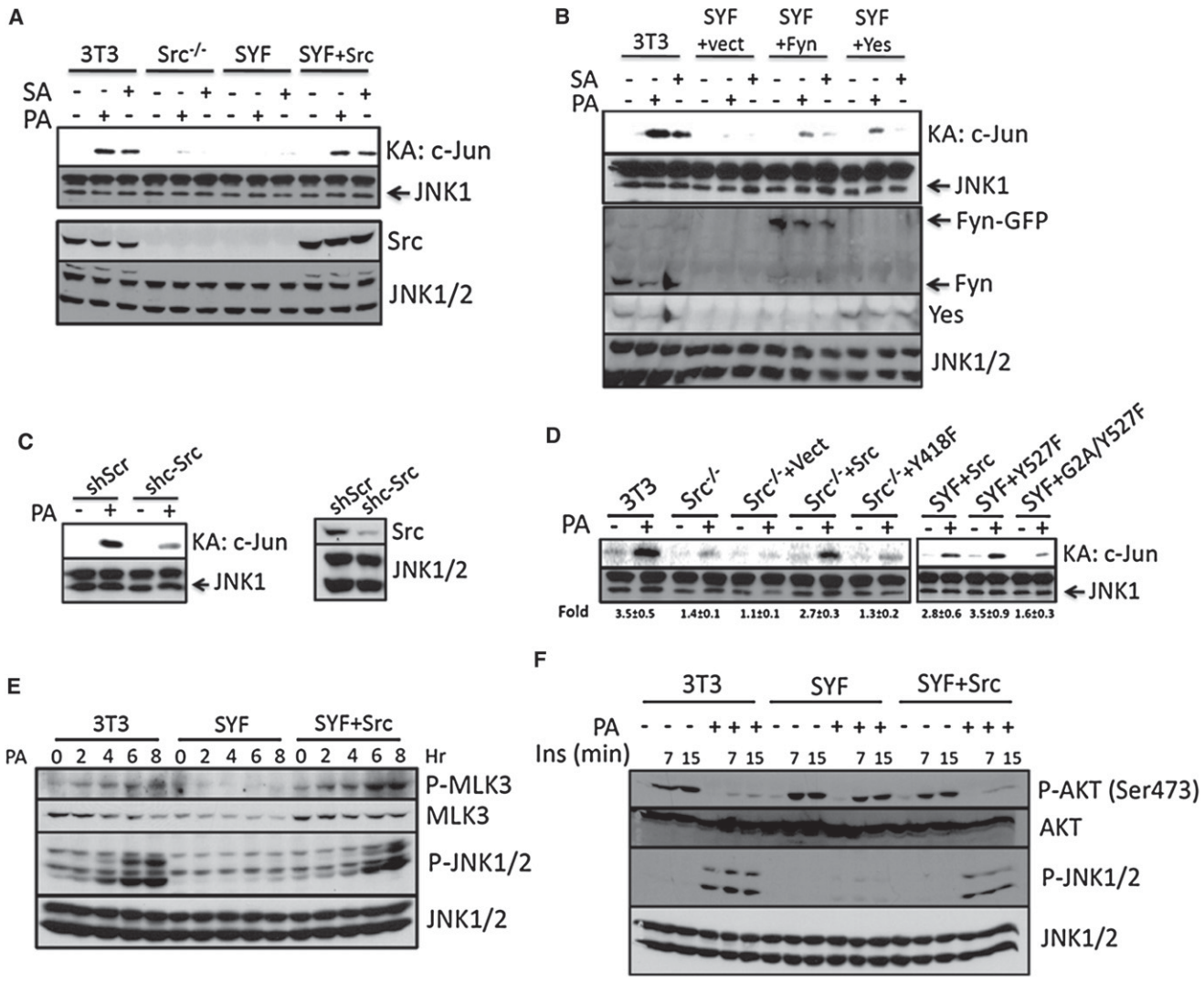

Figure 1. c-Src Is Required for JNK Activation and Insulin Resistance by Saturated FA

(A–D) JNK activation by FAs is c-Src dependent.

(A) NIH 3T3, *Src*$^{-/-}$, *SYF*$^{-/-}$, and SYF fibroblasts reconstituted with c-Src were treated for 6 hr with 500 μM palmitic (PA) or stearic (SA) acids loaded onto BSA. Whole-cell lysates were prepared and subjected to JNK1 immunecomplex kinase assay using GST-c-Jun (1–79) as a substrate and immunoblotting with indicated antibodies.

(B) NIH 3T3 and *SYF*$^{-/-}$ fibroblasts transduced with empty vector, Fyn, or Yes expression vectors were treated with SA or PA and analyzed as above.

(C) NIH 3T3 cells were infected with lentiviral constructs carrying scrambled (Scr) or c-Src-specific shRNAs. After selection in puromycin-containing medium, cells were treated with PA, and JNK1 activity and c-Src expression were analyzed.

(D) NIH 3T3, *Src*$^{-/-}$ cells, and *Src*$^{-/-}$ cells transduced with empty vector or WT c-Src or c-Src(Y418F) expression vectors or *SYF*$^{-/-}$ cells reconstituted with WT, Y527F, or Y527F/G2A c-Src vectors were treated with PA-loaded BSA or BSA alone and analyzed as above. Fold increase in JNK1 activity is shown below and was determined by densitometric analysis of three similar but separate experiments.

(E) Src is required for MLK3 activation by PA. NIH 3T3, *SYF*$^{-/-}$, and SYF+Src cells were treated with PA-loaded BSA for the indicated time periods. Cell lysates were prepared, and phosphorylation of MLK3 and JNK1/2 was monitored by immunoblotting.

(F) PA-induced insulin resistance is c-Src dependent. NIH 3T3, *SYF*$^{-/-}$, and SYF+Src cells were pretreated with or without PA for 6 hr before treatment with 100 ng/ml insulin for 7.5 and 15 min. JNK activation and insulin-induced AKT phosphorylation were analyzed by immunoblotting.

See also Figure S1.

however, inhibited JNK activation and induction of TNF production in PA-incubated J774A.1 macrophages (Figure S1G and S1H). Importantly, in *SYF*$^{-/-}$ cells, PA treatment failed to inhibit insulin-induced AKT activation, but reconstitution with c-Src restored PA-induced inhibition of AKT Ser473 phosphorylation to the same magnitude seen in 3T3 fibroblasts (Figure 1F). c-Src expression also restored PA-induced JNK1/2 phosphorylation. Collectively, these results establish a critical role for c-Src in JNK activation by FA and inhibition of insulin-mediated AKT activation.

Saturated FA Induce Src Partitioning into Detergent-Insoluble Membrane Microdomains

Next, we examined whether c-Src activation is linked to changes in membrane structure. Membrane microdomains enriched in cholesterol, sphingolipids, and other lipids with saturated acyl chains, which presumably reduce membrane fluidity, can be isolated based on resistance to solubilization with cold nonionic detergents (Pike, 2009). Such membrane microdomains, sometimes referred to as lipid rafts, are postulated to be enriched in signaling proteins that can coalesce into larger assemblies (Janes et al., 1999; Pike, 2009). To examine whether saturated FA alter the membrane distribution of c-Src, SYF+Src cells were treated with PA, SA, or BSA alone for 4 hr before preparation of lysates that were solubilized with Triton X-100 at 4°C. Detergent-resistant membranes (DRMs) were isolated by equilibrium density gradient centrifugation (Lingwood and Simons, 2007). Using this technique, DRMs and their associated proteins float to the gradient's top (fraction 1), whereas solubilized membranes and cell debris remain in fraction 4 (Figure 2A). In BSA-treated cells, very little c-Src was present in the DRM fraction (fraction 1), and most of it was in fractions 3 and 4. However, upon incubation with PA- or SA-loaded BSA, the distribution of c-Src had significantly changed, and a substantial amount of the protein was in fraction 1 (Figures 2A and 2B). A similar enrichment within fraction 1 of cells treated with PA or SA was seen for c-Src phosphorylated at Tyr418, presumably an activated form of the kinase. Fraction 1 also contained flotillin-1 and flotillin-2, proteins that serve as DRM markers, but was devoid of calnexin, a membrane protein that is excluded from DRM (Lingwood and Simons, 2007). Unlike c-Src, the amounts of flotillin-1/2 within fraction 1 were not affected by incubation with either PA or SA (Figure 2B). Incubation with PA-loaded BSA also increased the amounts of JNK1/2, MLK3, and MKK4 within fraction 1 (Figures 2A and 2B). However, no significant effect on the distribution of p85 PI3K, a protein suggested to be involved in JNK activation and insulin resistance (Taniguchi et al., 2007), was seen (Figure 2B). Immunecomplex kinase assay with enolase as a substrate confirmed that incubation of cells with PA or SA increased the amount of active c-Src within fraction 1 (Figure 2C). PA treatment also enhanced the amount of Yes, but not Fyn, within fraction 1, but the effect on Yes was more subtle than the effect on c-Src (Figures S2A and S2B). The nonmyristoylated c-Src(G2A) mutant was mostly confined to the soluble fraction 4 and did not shift into the DRM fraction after PA treatment (Figures S2C). c-Src(G2A) also exhibited less Tyr418 phosphorylation before and after PA treatment. In contrast, a dually palmitoylated c-Src(S3C/S6C) mutant (Sandilands et al., 2007) was present within the DRM fraction and was Tyr418 phosphorylated even in BSA-treated cells, and PA treatment led to only a modest increase in its amount in fraction 1 relative to fraction 4 (Figures S2D). Consistent with their poor effect on JNK activity (Figure S1A), MA treatment led to a small increase in c-Src within the DRM fraction, and LA had no effect on the membrane distribution of c-Src (Figure 2D). Likewise, MA and LA were poorer inducers of CHOP mRNA in response to PA and did not stimulate XBP1 mRNA splicing (Figure 2E).

We also used a detergent-independent technique for isolation of putative lipid rafts that relies on physical disruption of cellular membranes in the presence of basic sodium carbonate buffer (Ostrom and Insel, 2006). This procedure yielded similar results to those described above, demonstrating PA-induced accumulation of c-Src and phospho-Tyr418 c-Src within the presumed lipid raft fraction, although enrichment of phospho-Tyr418 c-Src was more pronounced relative to the more modest enrichment of c-Src (Figures S2E and S2F). Compared to Triton X-100, a strong detergent that tends to solubilize all but the most ordered membrane subdomains, detergent-independent lipid raft isolation tends to yield larger membrane fragments that include highly ordered (and Triton X-100-insoluble) and less-ordered microdomains (Pike, 2004). Our results may therefore suggest that c-Src activation occurs within less-ordered microdomains, and once c-Src is activated, it is concentrated within a more ordered membrane compartment. An in vitro kinase assay using a peptide substrate demonstrated that the c-Src that segregated within the putative lipid raft fraction generated by the detergent-independent method was activated if isolated from PA-treated cells (Figures S2G). PA treatment led to no change in c-Src activity within raft-devoid fractions (Figures S2H).

Unsaturated FA Inhibit Src Redistribution and Activation

Mono- and polyunsaturated FA increase membrane fluidity (Clamp et al., 1997; Karnovsky et al., 1982; Luo et al., 1996; Stulnig et al., 1998, 2001; Webb et al., 2000) and antagonize cellular effects of saturated FA (Akazawa et al., 2010). We pretreated SYF+Src cells with 300 μM of the monounsaturated POA or the PUFA EPA for 15 min prior to treatment with PA and examined c-Src membrane distribution and activity, JNK activation, and ER stress markers. Pretreatment with either POA or EPA blocked PA-induced partitioning of total c-Src and phospho-Tyr418 c-Src into the DRM fraction while having little to no effect on flotillin-1/2 and calnexin distribution (Figures 3A and 3B). However, pretreatment with 300 μM SA did not prevent PA-induced c-Src redistribution, and as expected, even at this lower concentration, SA caused a small amount of c-Src redistribution on its own (Figure S3A). POA and EPA pretreatment also blocked PA-induced JNK activation (Figure 3C) as well as induction of ER stress markers by PA (Figure 3D). These results indicate that mono- and polyunsaturated FA block the effects of saturated FA on c-Src membrane distribution and activity, JNK activation, and ER stress. Given the opposing effects of unsaturated FA versus saturated FA on membrane fluidity, these findings support the hypothesis that altered membrane fluidity is key to the signaling effects of saturated FA.

To determine whether saturated FA are preferentially incorporated into DRM, we treated cells for 2 hr with a mixture of ^{3}H-labeled and cold PA, isolated DRMs by the detergent-dependent method, and measured radioactivity in the different fractions. Most of the radioactivity was present in the DRM fraction (Figure 4A), especially when normalized to the protein content of each fraction (Figure 4B). To examine whether unsaturated FA incorporate preferentially into the detergent-soluble membrane fraction, we incubated cells with ^{3}H-labeled oleic acid (OA; C18:1). Most of the ^{3}H-OA-derived radioactivity was present in the soluble fraction 4 (Figures 4C and 4D). When cells were pretreated with POA and EPA prior to incubation with ^{3}H-PA, incorporation of ^{3}H-PA-derived radioactivity into

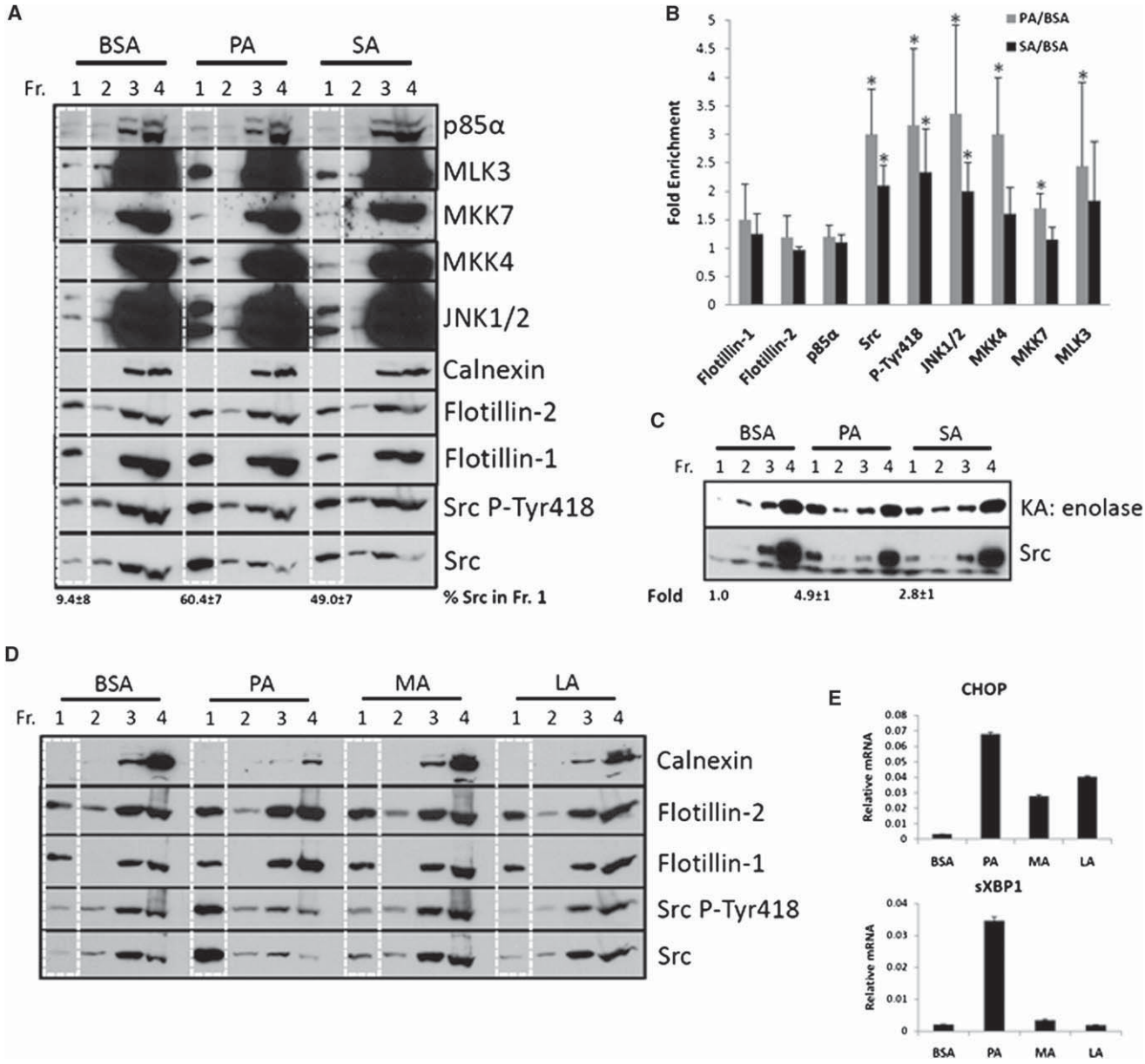

Figure 2. Saturated FA Induce c-Src Segregation into Detergent-Resistant Membranes

(A) SYF+Src cells were treated for 4 hr with BSA alone or PA- or SA-loaded BSA. Whole-cell lysates were solubilized with Triton X-100 at 4°C, and DRMs were isolated by equilibrium density gradient centrifugation. Fractions were collected from the gradient's top, such that fraction 1 represents the DRM fraction. Presence of the indicated proteins and phospho-Tyr418 c-Src in the different fractions was analyzed by immunoblotting. Flotillin-1 and flotillin-2 are lipid raft markers, whereas calnexin is a membrane protein that is excluded from lipid rafts/DRM. The percentage of c-Src in Fr. 1 is indicated underneath and was calculated by densitometric analysis of four separate experiments.

(B) FA-induced enrichment of different signaling proteins in the DRM fraction following PA or SA treatments. Results are averages ± SD of three to five experiments similar to the one in (A). *$p < 0.05$.

(C) c-Src kinase activity in different membrane fractions from cells treated with BSA or BSA plus saturated FA. Cells were treated and their membranes were fractionated as in (A). c-Src was immunoprecipitated from the different fractions and its activity measured using acid-activated enolase as a substrate. Average fold increase in c-Src activity (n = 4 for PA; n = 3 for SA) is indicated below.

(D) c-Src membrane redistribution is dependent on FA acyl chain length. SYF+Src cells were treated with 500 μM PA (C16), MA (C14), or LA (C12). The cells were fractionated as in (A), and the fractions were immunoblotted for the indicated proteins.

(E) SYF+Src cells were treated for 6 hr with either BSA alone or BSA loaded with PA, MA, or LA. Induction of CHOP mRNA or XBP1 mRNA splicing was analyzed by Q-RT-PCR. Results are averages ± SD. n = 3.

See also Figure S2.

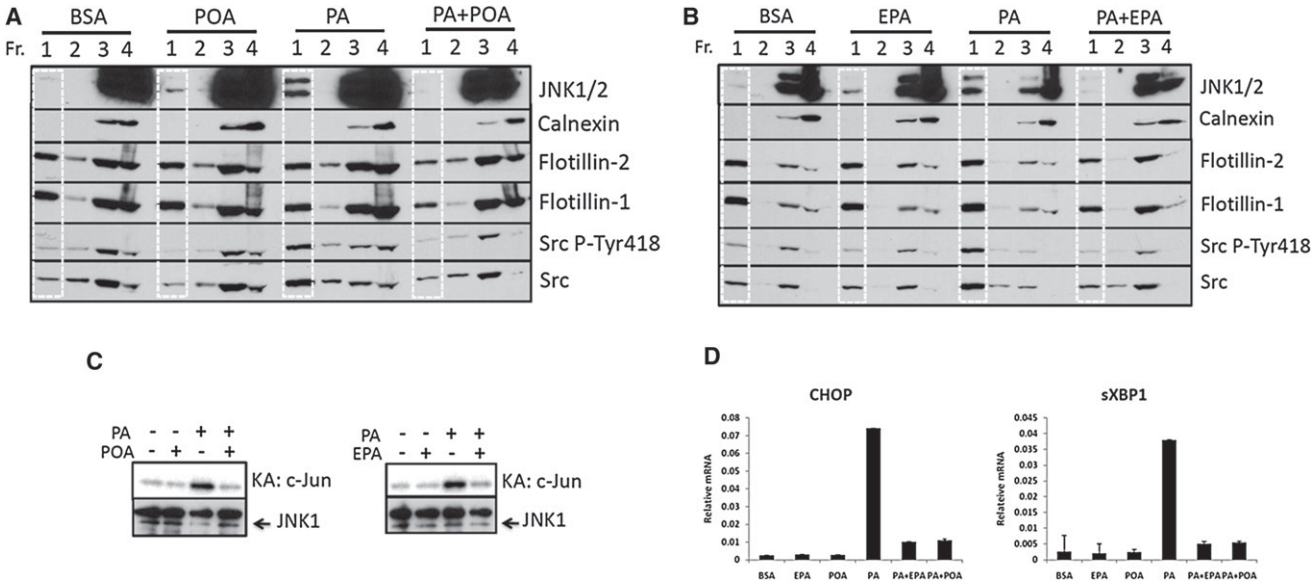

Figure 3. Mono- and Polyunsaturated FA Block c-Src Membrane Redistribution and Activation as well as JNK Activation and Induction of ER Stress Markers

(A–D) Unsaturated FA inhibit c-Src segregation and activation within DRM in response to PA. SYF+Src cells were pretreated with either monounsaturated palmitoleic acid (POA) (A) or polyunsaturated eicosapentaenoic acid (EPA) (B) for 15 min prior to addition of BSA or BSA loaded with PA. After 4 hr, membranes were solubilized and fractionated as in Figure 2, and distribution of the indicated proteins was determined by immunoblotting. c-Src and phospho-Tyr418 c-Src accumulated in the DRM fraction of PA-treated cells, and this was prevented by pretreatment with either POA (A) or EPA (B). (C) JNK activation and (D) induction of ER stress markers in the cells subjected to the above treatments were assessed as described in Figures 1 and 2. See also Figure S3.

the DRM fraction was significantly reduced (Figure 4E). In contrast, pretreatment with SA did not inhibit incorporation of ^{3}H-PA into the DRM fraction. OA behaved similarly to POA and EPA in that it blocked c-Src partitioning into the DRM fraction in response to PA (Figure S3B) and prevented PA-induced ER stress (Figure S3C) and JNK activation (Figure S3D). These results suggest that exposure of cells to PA results in direct effects on membrane structure, which eventually affect the distribution and activity of membrane-anchored c-Src.

DRM and Src Activation in Adipose Tissue of Obese Mice

Next, we checked whether consumption of high-fat diet (HFD) also results in c-Src partitioning and activation within DRM microdomains. C57BL/6 mice were placed on HFD for 16 weeks, and their brown adipose tissues (BAT) and white adipose tissues (WAT) were collected. The distribution of membrane proteins, including c-Src, within these tissues was analyzed by the Triton X-100 method described above. Consumption of HFD, but not normal chow (low-fat diet [LFD]), resulted in a dramatic increase of total c-Src and phospho-Tyr418 c-Src within fraction 1 of Triton X-100-solubilized BAT (Figure 5A). Consumption of HFD also increased the amounts of flotillin-1/2 within DRMs, suggesting a major expansion of such membrane microdomains in BAT, but as expected, calnexin remained excluded from the DRM fraction. Consumption of HFD also increased JNK1/2 and MLK3 within the DRM fraction but had little effect on the distribution of MKK4 and the p85α subunit of PI3K (Figure 5A). A Src kinase assay confirmed that HFD consumption resulted in c-Src activation and induced a large increase in the amount of activated Src within fraction 1 (Figure 5B).

Similar results were obtained by analysis of WAT. Although HFD consumption did not lead to an expansion of the DRM fraction, as indicated by the nearly identical distribution of flotillin-1/2 in membrane fractions of WAT from lean and obese mice, the amount of phospho-Tyr418 Src was higher in fraction 1 of WAT membranes isolated from obese mice (Figure 5C). Immunecomplex kinase assay using enolase as a substrate confirmed that there was a clear and significant increase in the amount of c-Src and c-Src catalytic activity in the DRM fraction of WAT from obese mice (Figure 5D). As expected, HFD consumption resulted in JNK1 activation in both BAT and WAT (Figures S4A and S4B). Increased JNK activation was accompanied by decreased AKT Ser 473 phosphorylation (Figures S4A and S4B).

Dasatinib (Sprycel) is a broad spectrum kinase inhibitor that potently inhibits c-Src (Agostino et al., 2010). Dasatinib is used for treatment of Imatinib-resistant chronic myelogenous leukemia (CML), but several case reports indicated that it had beneficial effects on blood glucose in diabetic patients with CML (Agostino et al., 2010; Breccia et al., 2008). We examined whether dasatinib improves glucose tolerance in mice rendered obese by feeding with a HFD for 16 weeks. Indeed, 3 hr of treatment with 30 mg/kg dasatinib resulted in improved glucose tolerance in obese mice (Figure S5). However, given the ability of dasatinib to inhibit several different tyrosine kinases, including the entire Src family, it is difficult to attribute this effect to c-Src itself.

Subcellular Distribution of c-Src after FA Treatment

Detergent-dependent and -independent methods of DRM or lipid raft isolation are informative but provide no direct evidence

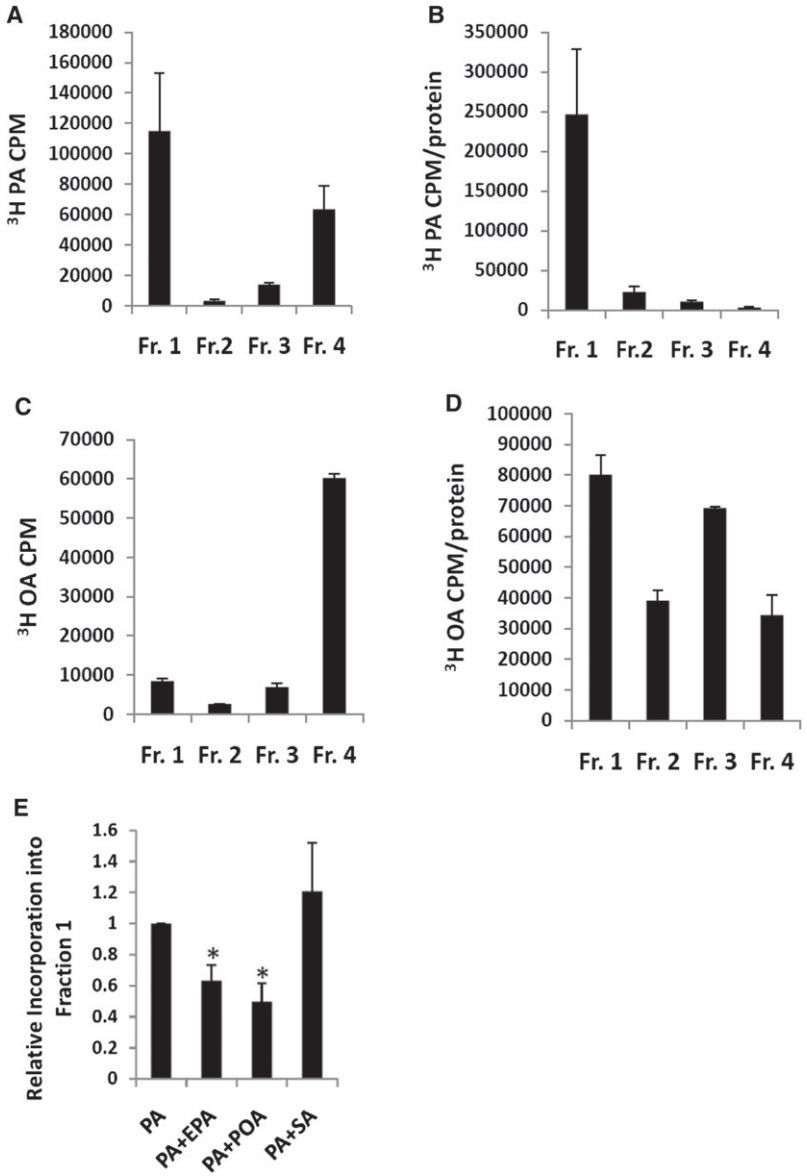

Figure 4. Saturated FA Are Preferentially Incorporated into DRM

(A and B) SYF+Src cells were treated for 2 hr with 200 μM ^{3}H-labeled PA and 300 μM cold PA loaded onto BSA. Cell lysates were prepared and membranes were solubilized and fractionated as in Figure 2. (A) The amount of ^{3}H in each fraction was determined by scintillation counting and (B) normalized to protein content. Results are averages ± SD. n = 3.

(C and D) SYF+Src cells were treated for 2 hr with 110 nM ^{3}H-labeled OA and 300 μM cold OA loaded onto BSA. Cells were fractionated as above, and (C) the amount of ^{3}H in each fraction was determined by scintillation counting and (D) normalized to protein content. Results are averages ± SD. n = 3.

(E) SYF+Src cells were pretreated for 15 min with 300 μM EPA, POA, or SA, followed by a 2 hr incubation with 200 μM ^{3}H-labeled PA and 300 μM cold PA loaded onto BSA. Cell lysates were solubilized and fractionated, and the relative amount of ^{3}H in fraction 1 was determined as above. Results are averages ± SD. n = 3; *p < 0.05.

See also Figure S3.

that the distribution of the protein in question within the membrane has changed prior to membrane solubilization and extraction. Furthermore, the membrane fractionation approach provides no information regarding the subcellular distribution of the protein in question. Previous studies of c-Src activation have demonstrated that c-Src is frequently targeted to ill-defined endosomal structures distinct from the plasma membrane (Sandilands et al., 2004; Seong et al., 2009; Wang et al., 2005). Furthermore, the lipid raft markers flotillin-1 and flotillin-2 are frequently found on organelles and endosomal membranes in addition to the plasma membrane (Browman et al., 2007; Rajendran et al., 2007). We used indirect three-color immunofluorescence and confocal microscopy to track the distribution of phospho-Tyr418 c-Src before and after treatment with PA or PA+POA relative to that of flotillin-1 and the lysosomal marker LAMP-1. The results indicated that, after 2.5 hr treatment with PA, Tyr418-phosphorylated c-Src was present in aggregates associated with perinuclear and vesicular membranes (Figure 6A). Phospho-Tyr418 c-Src aggregates frequently presented as distended rings, which were infrequent in BSA-treated control cells, or cells that were pretreated with POA prior to addition of PA. Flotillin-1 and LAMP-1 strongly colocalized both in control cells and in PA-treated cells. However, phospho-Tyr418 c-Src in control cells exhibited only weak colocalization with flotillin-1 or LAMP-1; but upon PA treatment, colocalization of phospho-Tyr418 c-Src with flotillin-1 and LAMP-1 was strongly enhanced (Figure 6A). POA pretreatment inhibited PA-induced colocalization of activated c-Src with flotillin-1 or LAMP-1 but had no effect on the relative distribution of the two markers.

To confirm these results, we used a detergent-independent postnuclear cell fractionation procedure and collected a fraction that was enriched for LAMP-1 (fraction 3) (Figure S6) and subjected it to lipid raft analysis using Triton X-100. This analysis indicated that the DRM fraction of the lysosomal membrane was strongly enriched for c-Src and phospho-Tyr418 c-Src after PA treatment (Figure 6B). However, only a small amount of c-Src and phospho-Tyr418 c-Src was present in fraction 1 of BSA-treated cells or cells preincubated with POA prior to PA addition. Consistent with the strong colocalization of LAMP-1 and flotillin-1 revealed by the immunofluorescence analysis, the DRM fraction of the lysosomal membrane contained both LAMP-1 and flotillin-1 (Figure 6B).

DISCUSSION

JNK activity is elevated in adipocytes of obese individuals (Gregor et al., 2009) and mice (Hirosumi et al., 2002; Solinas

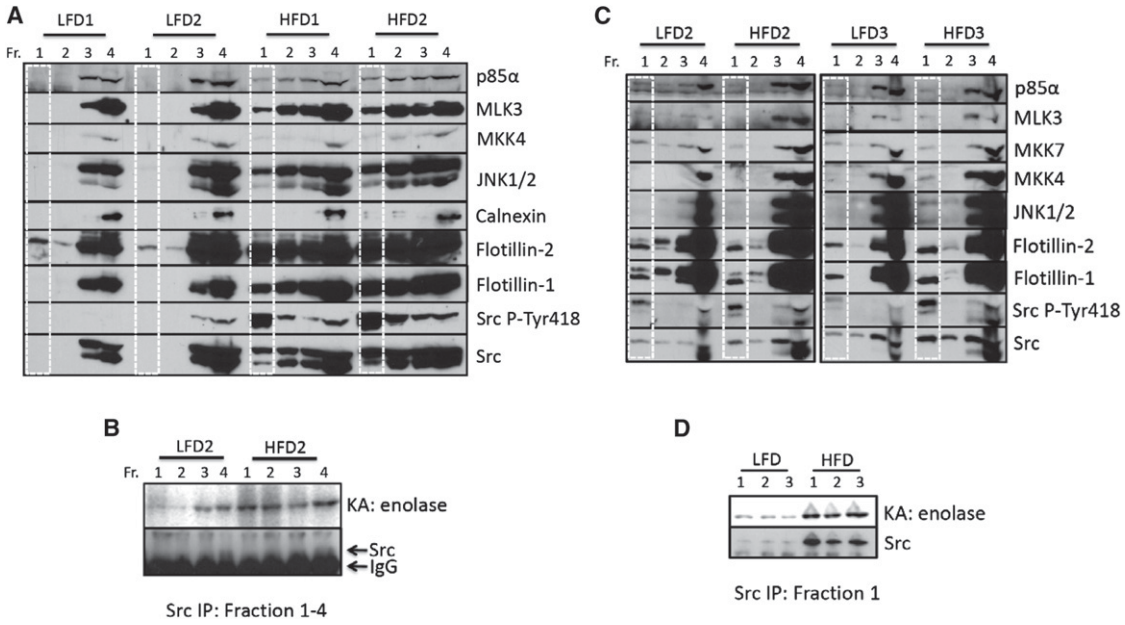

Figure 5. Consumption of High-Fat Diet Results in c-Src Segregation and Activation within DRM of Adipose Tissue

(A–D) Mice were kept on normal chow (LFD) or high-fat diet (HFD) for 16 weeks, after which brown (A and B, BAT) and white (C and D, WAT) adipose tissues were isolated. The tissues were homogenized, excess lipid was removed, and the lysates were adjusted to equal protein concentrations before cold Triton X-100 solubilization and fractionation as in Figure 2.

(A) Density gradient fractions of BAT membranes were analyzed for the indicated proteins by immunoblotting. Results show two different mice for each dietary condition and are representative of separate experiments in which three mice were analyzed.

(B) c-Src was immunoprecipitated from the gradient fractions of BAT isolated from mice LFD2 and HFD2, and its kinase activity was measured using enolase as a substrate.

(C) Density gradient fractions of WAT membranes from the indicated mice were analyzed for the indicated proteins by immunoblotting.

(D) c-Src was immunoprecipitated from fraction 1 of three different LFD- or HFD-fed mice, and its kinase activity was measured as above.

See also Figures S4 and S5.

et al., 2006). Chronic JNK activation is of physiological significance, as it contributes to insulin resistance, obesity, and production of inflammatory cytokines (Hirosumi et al., 2002; Sabio et al., 2010; Solinas et al., 2007). The exact mediators by which obesity activates JNK are unknown, but saturated FA such as PA and SA are potent JNK activators in cultured cells, whereas unsaturated FA have only a marginal effect (Solinas et al., 2006). In fact, some unsaturated FA, including POA, which differs from PA by only two hydrogens, and especially PUFA, inhibit JNK activation by saturated FA. Such observations invoke the existence of a sensor that discriminates between saturated and unsaturated FA, at times detecting the presence of a single double bond or the absence of two hydrogen atoms. Our results suggest that this sensor/receptor is none else but the membrane. Incorporation of saturated FA, whose acyl chains assume a rigid and straight conformation, into cellular membranes increases membrane order, resulting in reduced fluidity and higher melting temperature (Karnovsky et al., 1982). By contrast, the bent tails of unsaturated FA reduce membrane order and increase fluidity. We now show that saturated FA stimulate c-Src partitioning into membrane subdomains with increased rigidity that can be isolated based on resistance to detergent solubilization or increased density and that the c-Src that resides within such microdomains is more active than the general pool of c-Src. We also provide genetic evidence that c-Src is required for FA-induced JNK1 and MLK3 activation. Furthermore, mono- and polyunsaturated FA, POA, and EPA, respectively, do not alter the membrane distribution of c-Src on their own and block its partitioning and activation within DRMs in cells treated with PA. Correspondingly, POA and EPA prevent JNK activation by PA.

Although these results do not exclude the existence of a protein receptor that can discriminate between different FA forms, they favor a hypothetical model according to which FA discrimination is carried out by the membrane. In addition to decreasing membrane fluidity, saturated FA expand the lipid raft compartment, defined as a region of the membrane with decreased fluidity and resistance to nonionic detergents due to enrichment with cholesterol, sphingolipids, and saturated phospholipids (Pike, 2009). By contrast, membrane supplementation with unsaturated FA decreases lipid raft formation (Clamp et al., 1997; Karnovsky et al., 1982; Stulnig et al., 1998, 2001). Although the in vivo existence of lipid rafts remains controversial, such experiments suggest that saturated and unsaturated FA have opposing effects on membrane fluidity and/or structure that result in differential susceptibility to solubilization with nonionic detergents. It was also noted that cellular membranes from obese individuals have abnormal composition and decreased fluidity (Faloia et al., 1999; Watala and Winocour, 1992). Yet, the pathophysiological implications of these observations

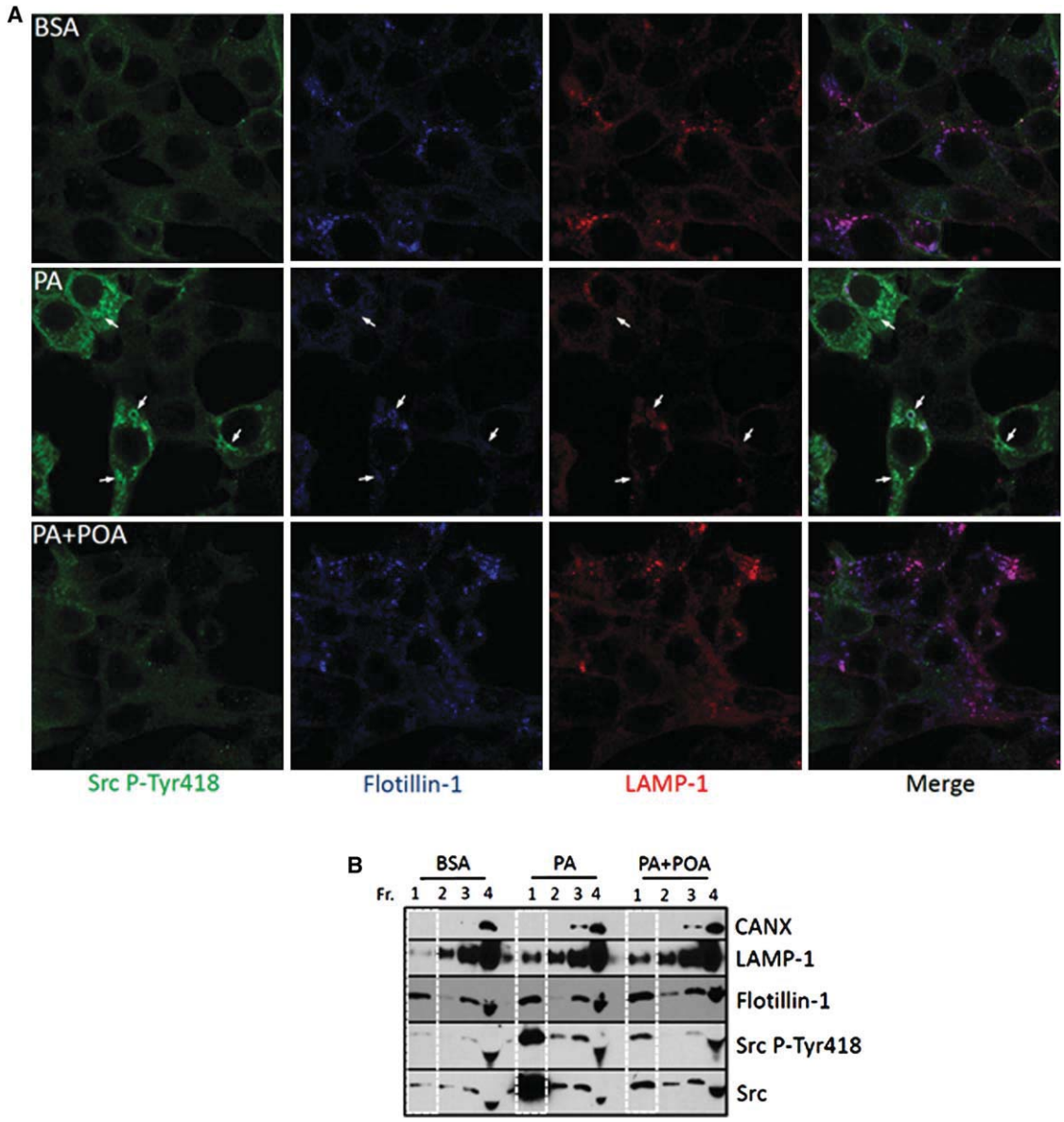

Figure 6. Subcellular Localization and Redistribution of Activated c-Src in FA-Treated Fibroblasts

(A) SYF+Src cells were treated for 2.5 hr with BSA, PA, or PA+POA before fixation and staining with antibodies to phospho-Tyr418 c-Src, LAMP-1, or flotillin-1. Three-color confocal images were acquired on a Leica SPE-2 confocal microscope. Magnification, 63×. Arrows indicate aggregates of phospho-Tyr418 c-Src that colocalize with flotillin-1 and LAMP-1.

(B) SYF+Src cells were treated as above. Cells were lysed in detergent-free buffer, and postnuclear lysates were separated on a Percoll gradient under high-speed centrifugation. A band enriched for LAMP-1 was collected, immediately resuspended in buffer, and solubilized with Triton X-100 at 4°C and then fractionated on density gradients. Fractions were collected and examined for the indicated proteins by immunoblotting.

See also Figure S6.

remained unclear, and no direct effects on signaling proteins that lead to insulin resistance were found.

c-Src is a myristoylated protein that can be found both within and outside of lipid rafts, as well as within endosomes, before translocating to the plasma membrane (Arcaro et al., 2007; Mukherjee et al., 2003; Sandilands et al., 2004; Seong et al., 2009). c-Src can be activated within all of the aforementioned compartments, including rapid activation at nonraft regions of the plasma membrane and slower activation within putative lipid rafts (Seong et al., 2009). Activation can also occur within endosomes that transit from the perinuclear region to the plasma membrane (Sandilands et al., 2004). Obviously, c-Src distribution within cellular membranes is dynamic and is modulated by environmental conditions. Our results demonstrate that incubation of fibroblasts with PA increases the amount of c-Src that is present within the DRM fraction or membrane microdomains

of increased density that can be isolated by a detergent-free method. Imaging studies confirm that PA induces clustering of Tyr-418 c-Src within endosomal and lysosomal membranes. However, it is unclear whether c-Src translocates to specific membrane microdomains before or after its activation. Nonetheless, given the presumed higher protein density of the c-Src-containing microdomains, it is plausible that increased molecular crowding facilitates c-Src autophosphorylation and activation. Enrichment of c-Src within DRM was also seen in BAT and, to a lesser extent, in WAT of mice kept on HFD. It is plausible that BSA and other FA-binding proteins are taken up by the cell via a pinocytotic mechanism and thereby deliver saturated FA to the same intracellular endosomal and lysosomal compartments within which PA-induced c-Src activation has been observed.

Treatment of fibroblasts with PA also results in JNK1/2 and MLK3 recruitment to DRM. JNK1/2 were previously suggested to associate with lipid rafts in response to reactive nitrogen species (Wu et al., 2008) or following UV-C-induced ceramide accumulation (Charruyer et al., 2005), suggesting that such membrane microdomains may contain proteins that serve as platforms for formation of JNK-signaling modules. MLK3, the MAP3K responsible for JNK activation by FA (Jaeschke and Davis, 2007), is a soluble protein, and therefore its recruitment into the DRM fraction must be mediated through interactions with another protein, which could be either c-Src or a c-Src-binding protein. Importantly, c-Src activity is required for MLK3 and JNK1 activation and inhibition of insulin signaling in fibroblasts incubated with saturated FA. Moreover, incubation of fibroblasts with unsaturated FA prevents changes in c-Src distribution and inhibits both c-Src and JNK activation. These findings support the hypothesis that c-Src activation within membrane microdomains of reduced fluidity is a crucial event in the JNK-signaling cascade triggered by FA and are consistent with the model according to which the membrane is the primary sensor of FA structure.

Dietary n-3 PUFAs, such as EPA and docosahexaenoic acid (DHA; 22:6, n-3), are common in fish and marine mammals (Simopoulos, 2002). Epidemiological studies have shown that consumption of large amounts of foods rich in n-3 PUFAs reduces the incidence of type 2 diabetes and heart disease and improves glycemic control even in the face of high body mass index (Jørgensen et al., 2006; Kagawa et al., 1982; Kromann and Green, 1980; Thorsdottir et al., 2004). Other studies have implicated DHA, EPA, or fish oil in protection from insulin resistance and type 2 diabetes in rodents and humans (Browning et al., 2007; Luo et al., 1996; Neschen et al., 2007; Oh et al., 2010; Storlien et al., 1987). Monounsaturated FA also enhance insulin sensitivity, and POA was suggested to function as a protective lipokine (Cao et al., 2008; Ryan et al., 2000). Mono- and polyunsaturated FA are biologically active and have many pleiotropic effects that could account for their antidiabetic actions, including reduced adipose tissue inflammation (Oh et al., 2010; Todoric et al., 2006). EPA or DHA also inhibit JNK/AP-1 activation by various stimuli (Liu et al., 2001; Oh et al., 2010; Todoric et al., 2006). Several hypotheses were proposed to explain the antidiabetic effects of mono- and polyunsaturated FA, including altered eicosanoid production (Culp et al., 1980) and modulation of peroxisome proliferator-activated receptors (Neschen et al., 2007). In addition, unsaturated FA, but not saturated FA, activate anti-inflammatory G protein-coupled receptors such as GPR120 (Hirasawa et al., 2005; Oh et al., 2010). Activation of this receptor can decrease JNK activation in response to LPS in vitro, and GPR120-deficient mice are more insulin resistant on LFD and refractory to the insulin-sensitizing effects of PUFA. These data suggest that the anti-inflammatory activity of poly- and monounsaturated FAs may be independent of effects on membrane fluidity. However, involvement of GPR120 in the ability of unsaturated FA to block JNK activation by saturated FA has not been tested. Likewise, it is unknown whether saturated FA can competitively prevent GPR120 activation by unsaturated FA. Such a scenario would require GPR120 or similar receptors to bind many different FAs but be activated only by unsaturated FA. Until such a receptor is found, the opposing effects of saturated and unsaturated FA on c-Src, MLK3, and JNK1 activity are most parsimoniously explained by their differential effects on membrane fluidity and structure. Furthermore, it should be noted that the effects of saturated FA on JNK, MLK3, and Src distribution and activity are slow, requiring an hour or more to be detected, and therefore they seem inconsistent with standard receptor-mediated events that occur within much shorter time scales. The cellular uptake of saturated FA from FA-binding proteins and subsequent FA incorporation into biological membranes is also unlikely to be a rapid process.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are described in the Extended Experimental Procedures. In brief, most of the in vitro experiments were conducted using mouse fibroblasts that are wild-type, *c-Src*$^{-/-}$, *SYF*$^{-/-}$, or *SYF*$^{-/-}$ reconstituted with c-Src. Fibroblasts were incubated with low-endotoxin BSA that was delipidated and then loaded with different FA. JNK activation and phosphorylation were analyzed as described (Solinas et al., 2006). c-Src activation was analyzed either by immunecomplex kinase assays or by immunochemical detection of Tyr418 phosphorylation. Lipid rafts were isolated as previously described (Lingwood and Simons, 2007; Ostrom and Insel, 2006). c-Src activation and subcellular distribution were also analyzed by indirect immunofluorescence of formaldehyde fixed cells. Mice were kept on low-fat or high-fat diets as described (Solinas et al., 2007).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online

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