Insulin acutely decreases hepatic fatty acid synthase activity

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Summary

Insulin is viewed as a positive regulator of fatty acid synthesis by increasing fatty acid synthase (FAS) mRNA transcription. We uncover a new mechanism by which insulin acutely reduces hepatic FAS activity by inducing phosphorylation of the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) and its interaction with FAS. *Ceacam1* null mice (*Cc1*$^{-/-}$) show loss of insulin's ability to acutely decrease hepatic FAS activity. Moreover, adenoviral delivery of wild-type, but not the phosphorylation-defective *Ceacam1* mutant, restores the acute effect of insulin on FAS activity in *Cc1*$^{-/-}$ primary hepatocytes. Failure of insulin to acutely reduce hepatic FAS activity in hyperinsulinemic mice, including L-SACC1 transgenics with liver inactivation of CEACAM1, and *Ob*/*Ob* obese mice, suggests that the acute effect of insulin on FAS activity depends on the prior insulinemic state. We propose that this mechanism acts to reduce hepatic lipogenesis incurred by insulin pulses during refeeding.

Introduction

The carcinoembryonic-related cell adhesion molecule, CEACAM1, is a transmembrane glycoprotein expressed in liver but not in muscle and adipose tissue. It is phosphorylated by the insulin receptor (IR) kinase on Tyr488 in its cytoplasmic tail, and this phosphorylation requires an intact Ser503 residue (Najjar et al., 1995). When phosphorylated, CEACAM1 promotes insulin clearance by increasing receptor-mediated insulin uptake into endocytotic vesicles (Formisano et al., 1995; Li Calzi et al., 1997).

Biochemical evidence for a role of CEACAM1 in insulin clearance is buttressed by studies of L-SACC1 transgenic mice with inactivation of CEACAM1 in liver. These mice develop impaired insulin clearance, hyperinsulinemia, insulin resistance, visceral obesity with increased plasma free fatty acids (FFA), and dyslipidemia, including elevated plasma and hepatic triglyceride content (Poy et al., 2002b). Increased visceral obesity and chronically elevated FFA output contribute to altered insulin metabolism in L-SACC1 mice (Dai et al., 2004).

Chronically elevated levels of insulin increase the levels in liver of lipogenic enzymes, including fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) (Assimacopoulos-Jeannet et al., 1995; Bazin and Lavau, 1982; Shillabeer et al., 1992). This promotes hepatic lipogenesis and increases VLDL-triglyceride output and compensatory insulin secretion, which in turn leads to increased visceral adiposity. Thus, altered fat metabolism in liver could play a role in the pathogenesis of visceral obesity and insulin resistance in L-SACC1 mice. Growing evidence supports a central role for de novo synthesis of fatty acids (FA) and increased hepatic VLDL-triglyceride output in the pathogenesis of obesity and insulin resistance (McGarry, 2002; Zammit, 2002). In fact, mice overexpressing malonyl-CoA decarboxylase, the enzyme that degrades the malonyl-CoA pool that is routed to FA synthesis by FAS, exhibit increased insulin sensitivity and loss in body weight (An et al., 2004).

ACC converts acetyl-CoA to malonyl-CoA, which is in turn converted to saturated long-chain fatty acids by FAS (Wakil, 1989). Together with exogenous FA, these are converted to long-chain fatty acyl-CoA (LCFA-CoA) that may undergo β-oxidation or esterification to triglycerides or to phospholipids. In addition to its role as FAS substrate, malonyl-CoA inhibits carnitine palmitoyltransferase 1 (CPT1) to reduce mitochondrial fatty acid β-oxidation (Ruderman et al., 1999) and promote glycolysis (Randle, 1998). The sensitivity of CPT1 to malonyl-CoA is regulated by nutritional and hormonal factors (McGarry, 2002; Zammit, 2002). For instance, starvation increases fatty acid oxidation and gluconeogenesis in liver. Recovery of the sensitivity of CPT1 to malonyl-CoA progresses slowly upon refeeding despite rapid insulin surges to replete the glycogen stores (Shulman and Landau, 1992). In contrast, hepatic triglyceride output undergoes a rapid decrease by early insulin surges in refed normoinsulinemic (Malmstrom et al., 1998), but not hyperinsulinemic, humans (Lewis and Steiner, 1996). The mechanism of reduction in triglyceride secretion in response to acute insulin pulses has not been identified (Zammit, 2002).

Because FAS and ACC are key enzymes in the de novo syn-

thesis of FA, which essentially regulates the rate of hepatic triglyceride output, it is reasonable to hypothesize that insulin exerts its rapid effect on triglyceride secretion by acutely regulating FAS and ACC activity. Acutely, insulin activates ACC to promote malonyl-CoA synthesis via inhibiting AMP-activated protein kinase (Kim et al., 1989; Witters and Kemp, 1992), and the role of this regulation in energy balance and metabolism has been well documented (Kahn et al., 2005). In contrast to its positive long-term effect on FAS transcription (Fukuda et al., 1999; Paulauskis and Sul, 1989; Stapleton et al., 1990), acute regulation of FAS activity by insulin has not been described. Moreover, regulation of FAS by phosphorylation has not been well demonstrated. Direct phosphorylation of FAS by a serine/threonine kinase and indirectly through the activity of a phosphatase has been observed in mammary tumors (Hennigar et al., 1998).

In humans, FAS is expressed at high levels in liver and at lower levels in intra-abdominal adipose tissue, lungs, and mammary glands (Semenkovich, 1997). The superior hepatic FAS levels have been in part attributed to higher insulin levels in the portal circulation (Ward et al., 1990). Despite its high levels, hepatic FAS activity is not remarkable under physiologic conditions. In contrast, FAS activity is elevated in hyperinsulinemic, hypertriglyceridemic, and obese rodents (Assimacopoulos-Jeannet et al., 1995; Bazin and Lavau, 1982; Shillabeer et al., 1992). Conversely, treatment of obese mice with FAS inhibitors reduces body weight independently of leptin (Loftus et al., 2000). This suggests that downregulation of hepatic FAS activity plays an important role in regulating visceral adiposity and hepatic insulin action. We herein report that in normoinsulinemic states, acute rise in insulin levels reduces hepatic FAS activity by inducing CEACAM1 phosphorylation and binding to FAS.

Results

In vitro analysis of CEACAM1/FAS interaction

Using a phosphorylated CEACAM1 intracellular peptide as a probe to screen an expression library (Macgregor et al., 1990), we isolated three clones encoding the thioesterase domain and the acyl-carrier domains of FAS (nucleotides 6815–9136, accession number M76767) (Amy et al., 1989).

To investigate the CEACAM1/FAS interaction, we employed GST pull-down assays with peptides from the intracellular tail of wild-type (wt) and mutant CEACAM1 (Choice et al., 1998). In vitro phosphorylated fusion peptides were incubated with purified His-tagged recombinant FAS (Figure 1A) or with extracts from MDA-MB 468 breast cancer cells, which express FAS at high levels (Figure 1B). Recombinant insulin receptor kinase (IRK_β) increased phosphorylation of wt CEACAM1 (wt CC1) (Figure 1A, center, lane 4 versus lane 3) and elevated the level of its association with recombinant FAS, as demonstrated by immunoblotting (Ib) GST pellets with α-FAS antibody (Figure 1A, top, lane 4 versus lane 3). The absence of FAS in the GST control pellet (Figure 1A, top, lanes 1 and 2) indicates that the binding between FAS and the intracellular domain of CEACAM1 is specific. Similarly, IRK_β increased binding between the intracellular domain of wt CC1 and FAS derived from MDA-MB 468 lysates (Figure 1B, top, lane 4 versus lane 3). The increase in CEACAM1/FAS binding corresponded to increased CEACAM1 phosphorylation by IRK_β (Figure 1B, center, lane 4 versus lane 3). As previously shown (Choice et al., 1998), IRK_β phosphorylated the Y513F CEACAM1 peptide (Figure 1B, center, lane 10 versus lane 9). This, in turn, was correlated with an increased amount of FAS in the GST pellet (Figure 1B, top, lane 10 versus lane 9). The specificity of this binding is demonstrated by the failure of FAS to bind the GST moiety in control samples (Figure 1B, top, lanes 1 and 2). Consistent with our previous observations (Najjar et al., 1995), mutating Ser503 to Ala inhibited CEACAM1 phosphorylation by IRK_β (Figure 1B, center, lanes 8 versus lane 7 and 14 versus 13). Moreover, this mutation prevented the increase in CEACAM1 binding to FAS by IRK_β (Figure 1B, top, lane 8 versus 7 and 14 versus 13). In contrast, a Ser to Asp503 mutation preserved the ability of IRK_β to induce CEACAM1 phosphorylation (Figure 1B, center, lane 16 versus lane 15) and increase its binding to FAS (Figure 1B, top, lane 16 versus 15). Mutating Tyr488 to Phe, individually or in combination with Tyr513, prevented CEACAM1 phosphorylation (Figure 1B, center, lane 6 versus lane 5 and 12 versus 11) and the increase in its binding to FAS in the presence of IRK_β (Figure 1B, top, lanes 5 and 6 and 11 and 12). IRK_β did not phosphorylate His-tagged FAS even after 60 min (not shown), suggesting that FAS was not phosphorylated by IR. These data propose that CEACAM1 phosphorylation by IR is required for its binding to FAS. Although the identification of FAS in a screen with a CEACAM1 probe is consistent with direct binding of CEACAM1 to FAS, we cannot rule out an indirect association between these proteins.

CEACAM1/FAS interaction in intact cells

To further investigate CEACAM1/FAS association, we carried out coimmunoprecipitation experiments in H35-BT cells, which express high levels of insulin receptors, FAS and CEACAM1 (Figure 2A). To this end, we immunoprecipitated (Ip) cell lysates with α-CC1 rat monoclonal antibody and analyzed immunoreactive proteins by immunoblotting with α-FAS (Figure 2A, top) or α-phosphotyrosine (α-pTyr) antibody (Figure 2A, bottom). To normalize for the amount of CEACAM1, membranes were reprobed with α-CC1 rat polyclonal antibody (Figure 2A, bottom). As previously reported (Najjar et al., 1995), CEACAM1 phosphorylation by the insulin receptor was observed 2 min after initiation of insulin treatment and progressively increased to reach a maximum after 5–10 min of treatment (Figure 2A, bottom). Coimmunoprecipitation analysis revealed that subsequent to initiation of its phosphorylation, CEACAM1 becomes associated with FAS, reaching a maximum at 7–10 min of stimulation with insulin and decreasing progressively during prolonged treatment for up to 90 min (Figure 2A, top). In contrast to FAS, CEACAM1 did not bind ACC in the absence or presence of insulin (not shown). These data indicate that in response to short-term treatment with insulin, CEACAM1 phosphorylation by the insulin receptor precedes its binding to FAS.

Internalization of CEACAM1 in response to insulin

To examine how the plasma-membrane protein CEACAM1 associates with the cytoplasmic protein FAS, we examined by confocal microscopy the internalization of CEACAM1 in rat hepatoma H4-II-E cells, derivatives of H35 cells, in the presence or absence of insulin. In the absence of insulin, CEACAM1 was distributed largely on the plasma membrane (Figure 2B, green stain—Insulin) as FAS was typically localized to the cytoplasmic region (Figure 2B, red dye). As evidenced by the

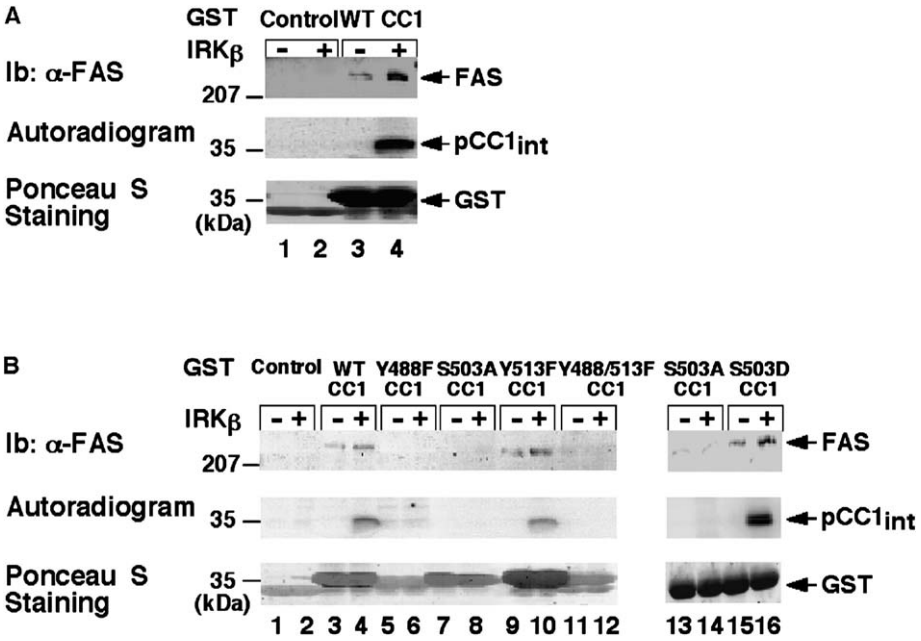


Figure 1. Insulin amplifies CEACAM1 binding to FAS in vitro

A and B) GST intracellular peptides of CEACAM1 ($CC1_{int}$) were incubated with (+) or without (–) IRK_β in the presence of [γ-^{32}P] ATP. Peptide-free GST was included as control to account for nonspecific association. Phosphorylated GST peptides were allowed to bind to purified His-FAS protein (**A**) or to cell lysates from MDA-MB 468 cells (**B**). The Sepharose pellets were analyzed by 6%–12% SDS-PAGE and immunoprobing with α-FAS antibody to examine FAS association with CEACAM1 (top). Phosphorylated CC1 (pCC1) was detected by autoradiography (center). The amount of GST peptides was measured by Ponceau S staining (bottom). Gels are representatives of at least three experiments.

minimal amount of overlap between the two fluorophores, CEACAM1/FAS colocalization was negligible in the absence of insulin (Figure 2B). Moreover, insulin treatment did not noticeably alter FAS distribution (Figure 2B). Instead, it stimulated the progressive cytoplasmic redistribution of CEACAM1 (Figure 2B), as evidenced by the higher than 90% localization of the green fluorophore in the cytoplasm and the merging of the fluorophores after 15–60 min of insulin treatment. Similar experiments using green FITC labeling revealed that, like FAS, the cytoplasmic distribution of insulin receptor substrate-2 (IRS-2) was comparable in insulin-treated and untreated cells (Figure 2B). This suggests that insulin treatment did not generally alter the cellular distribution of proteins under our experimental conditions and that its effect on CEACAM1 translocation into the cell is specific. The data propose that CEACAM1/FAS interaction requires CEACAM1 transport inside the cell, probably via clathrin-coated vesicles, as we have previously reported (Choice et al., 1998). The data are consistent with the notion that CEACAM1 phosphorylation precedes its cellular uptake and that this in turn promotes its binding to FAS.

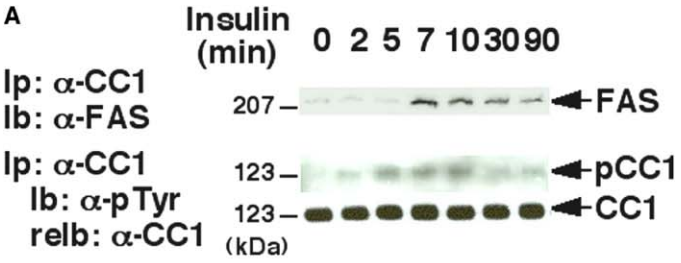
Effect of CEACAM1/FAS interaction on FAS activity

Next, we treated H35-BT cells with insulin for 0–90 min and investigated the effect of CEACAM1 association on FAS activity. As Figure 3A reveals, short-term insulin treatment decreased FAS activity, reaching a maximum effect by 15 min. Reversal of this effect occurred by 60 min, as the ability of insulin to induce CEACAM1 phosphorylation and its association with FAS was reduced (Figure 2A). This suggests that the effect of insulin is mediated by CEACAM1 phosphorylation. To investigate whether CEACAM1 phosphorylation is sufficient, we incubated His-tagged CEACAM1 purified from Sf9 insect cells (Phan et al., 2001) with IRKβ or buffer alone prior to the addition of purified His-FAS. FAS activity was assayed and normalized per activity in the absence of CEACAM1, and the remaining activity was plotted against CEACAM1 concentration (Figure 3B). As Figure 3B reveals, 250–500 pmol CEACAM1 reduced FAS activity by ~50% in the presence but not in the absence of IRKβ. This suggests that CEACAM1 phosphorylation is required to mediate the acute inhibition of FAS by insulin.

Acute effect of insulin on FAS activity in vivo

To investigate whether insulin downregulates FAS activity acutely in vivo, we fed overnight-fasted mice with glucose by gavage for 0–120 min (Figure 4A). Mirroring the biphasic increase in insulin secretion in response to glucose (Figure 4A, top graph), hepatic FAS activity underwent a biphasic decrease at 2 and 30–120 min post-glucose administration (Figure 4A, center graph). In contrast, this treatment had no effect on FAS activity in adipose tissue (Figure 4A, bottom graph). The ability of insulin to decrease hepatic FAS activity was paralleled by its ability to induce phosphorylation of the insulin receptor (pIR), as detected by immunoblotting the IR immunopellet (Ip) with α-pTyr antibody (Figure 4A, center gel set), and of CEACAM1 (pCC1) (Figure 4A, top gel set). As expected, CEACAM1 phosphorylation led to its increased interaction with FAS, as detected by coimmunoprecipitation (top gel set). The amount of FAS remained constant under these conditions (bottom gel set). This suggests that the acute effect of insulin on FAS activity depends on CEACAM1 phosphorylation rather than FAS content.

We further tested the acute effect of insulin on hepatic FAS activity in vivo by feeding overnight-fasted mice regular chow for 1–10 hr. As Figure 4B reveals, insulin secretion was elevated at 1, 4, and 7 hr of refeeding, but more significantly at 4 and 7 hr (top graph), by comparison to fasted mice (F). FAS activity was reduced at 4 and 7 hr of refeeding in the liver (center graph) but not in the adipose tissue (bottom graph). Ad libitum randomly fed mice (Rand) exhibited no difference in hepatic



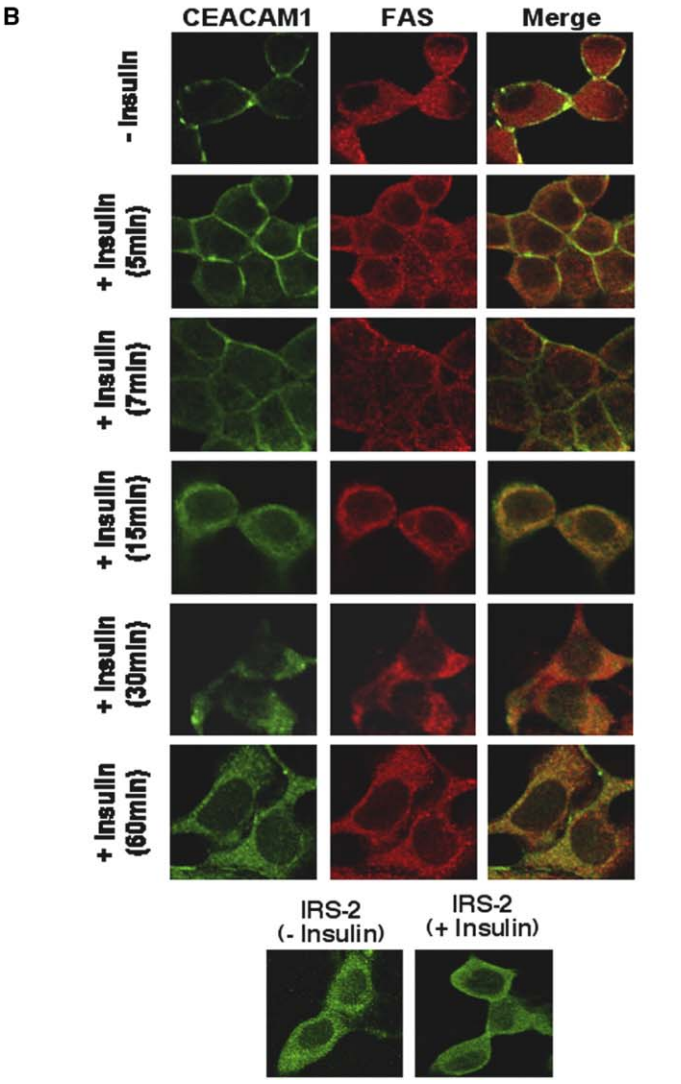
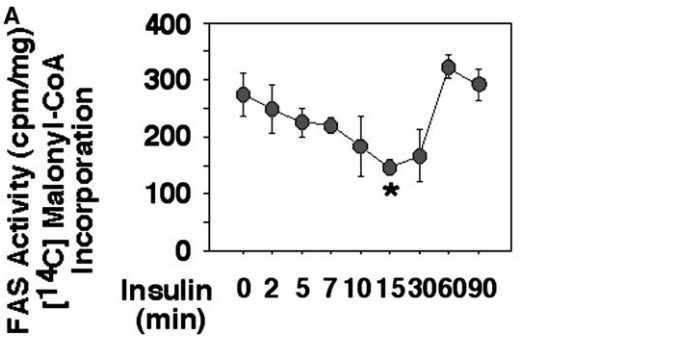


Figure 2. Insulin amplifies CEACAM1 binding to FAS in intact cells

A) H35-BT cells were serum-starved, treated without (0) or with insulin (100 nM) for 2–90 min, and lysed prior to immunoprecipitating (Ip) 100 μg of proteins with α-CC1 antibody for analysis by 6% SDS-PAGE and immunoblotting (Ib) with α-FAS antibody to detect coimmunoprecipitated FAS (top) and α-pTyr antibody to detect phosphorylated CEACAM1 (pCC1) (bottom). The latter was reprobed with α-CC1 antibody to account for the amount of CEACAM1 in the immunopellets (bottom). Gels are representatives of at least three experiments.

B) H4-II-E cells were serum-starved, incubated with α-CC1 monoclonal antibody, and fixed immediately (– insulin) or after incubation with insulin at 37°C from 5–60 min. Following incubation with polyclonal α-FAS antibody, cells were incubated with goat anti-mouse FITC and goat anti-rabbit Cy3 conjugated secondary antisera and analyzed by confocal microscopy. For IRS-2 detection, serum-starved cells were treated with (+) or without (–) insulin for 30 min, fixed, blocked, incubated with α-IRS-2 antibody, and labeled with green FITC.



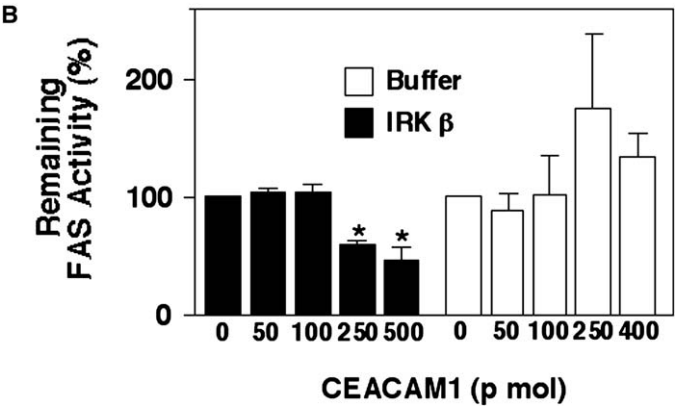


Figure 3. Short-term treatment with insulin reduces FAS activity

A) Serum-starved H35-BT hepatoma cells were treated with insulin for 0–90 min. FAS enzymatic activity was assayed by [^{14}C]-malonyl-CoA incorporation in triplicate as described in Experimental Procedures. Values are expressed as mean ± SEM. *$p < 0.05$ versus untreated. For simplicity, the x axis is not drawn to scale.

B) 0–500 pmol of purified His-CEACAM1 was phosphorylated by GST-IRK$_\beta$ (solid bars) or buffer (white bars). Thereafter, 1 pmol of His-FAS was added at 4°C for 60 min and FAS activity was assayed by [^{14}C]-malonyl-CoA incorporation in triplicate samples as described in Experimental Procedures and normalized per activity in the absence of CEACAM1. Values are expressed as mean ± SEM. *$p < 0.05$ versus 0–100 pmol CEACAM1.

FAS activity by comparison to fasting. The decrease in hepatic FAS activity occurred when CEACAM1 phosphorylation (pCC1) and association with FAS significantly increased in response to insulin (top gel set) and despite a modest increase in FAS protein content after 5 hr of refeeding (bottom gel set). Interestingly, the low level of insulin secreted at 1 hr of refeeding induced a modest receptor phosphorylation (pIR) (center gel set) but was not sufficient to trigger a significant increase in CEACAM1 phosphorylation and coimmunoprecipitation with FAS.

The acute effect of insulin on FAS activity depends on the insulinemic state

In order to examine the effect of the prior insulinemic state on the acute effect of insulin on FAS activity, we subjected the hyperinsulinemic insulin-resistant L-SACC1 transgenic mice to a fasting/refeeding paradigm similar to the one described above. Insulin secretion peaked at 2 hr and started to rise again at 5 hr of refeeding (Figure 5A, top graph). In contrast to normal

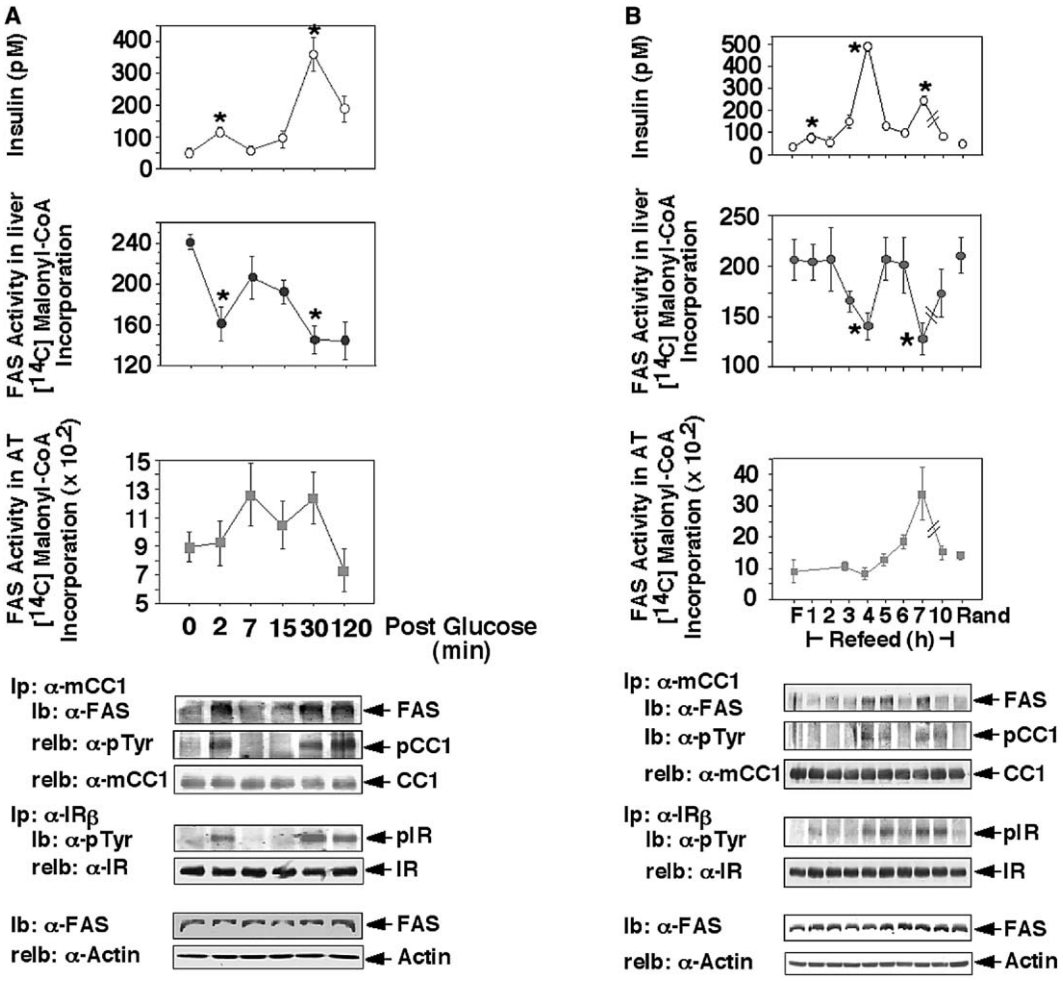


Figure 4. Insulin acutely decreases hepatic FAS activity in vivo

A) Two-month-old male mice were fasted overnight and given glucose by gavage for 0–120 min prior to drawing whole blood at indicated time points from retro-orbital sinuses to determine plasma insulin levels (top graph) and removal of liver and white adipose tissue (AT) (center and bottom graph, respectively) to assay FAS enzymatic activity by [^{14}C]-malonyl-CoA incorporation, as described in Experimental Procedures. Three to five mice were analyzed per time point and FAS assay was performed in quadruplet. Values are expressed as mean ± SEM. *$p < 0.05$ versus fasted (0). For simplicity, values on the x axis were not to scale. Liver lysates were immunoprecipitated (Ip) with α-mCC1 antibody, analyzed by 6% SDS PAGE, and immunoblotted (Ib) with α-FAS antibody to detect FAS binding to CEACAM1 and reimmunoprobed (relb) with α-mCC1 antibody to account for the amount of CEACAM1 in the immunopellets (top gel set). Lysates were also immunoprecipitated with α-IR$_\beta$ antibody prior to immunoblotting (Ib) with phosphotyrosine antibody (α-pTyr) and reprobing (relb) with α-IR antibody to account for the amount of IR in the immunopellets (center gel set). To measure total FAS content, liver lysates were analyzed by 6%–12% SDS-PAGE and immunoblotting with α-FAS antibody followed by reprobing with α-actin antibody (α-actin) (bottom gel set). These experiments were repeated on two sets of experiments using a different mouse from each refeeding time.

B) Male mice at 2 months of age were fed ad libitum (Rand) or fasted overnight (F) and refed for 1–10 hr prior to removal of blood to determine insulin levels (top graph) and liver and adipose tissue to assay FAS activity (center and bottom graphs, respectively). Insulin and FAS assays were performed on 3–5 mice in duplicate and quadruplet, respectively. Values are expressed as mean ± SEM. *$p < 0.05$ versus fasted (0). Hepatic FAS content (bottom gel set) and its interaction with CEACAM1 (top gel set) and insulin receptor phosphorylation (center gel set) were also analyzed by Western blotting, as in (**A**) above.

mice, insulin did not significantly decrease FAS activity in the liver of L-SACC1 mice (Figure 5A, bottom graph).

Because wild-type (wt) and L-SACC1 mouse lines commonly exhibited a substantial increase in insulin secretion at 7 hr of refeeding, we then compared FAS activity in these mice at this refeeding time. As expected, increases in insulin levels (by ~4-fold) caused an ~3-fold induction of hepatic FAS protein levels in refed (R7) wt by comparison to fasted (F) mice (Figure 5B, center gel set). Despite this increase, FAS enzymatic activity was reduced by ~50% compared to fasting (Figure 5B, graph, bar R7 versus F). This was associated with increased IR phosphorylation (pIR) and with an ~3-fold increase in the association of CEACAM1 with FAS, as demonstrated by the coimmunoprecipitation experiment (Figure 5B, top gel set, lane R7 versus F). In L-SACC1 mice, however, insulin release (by ~1.5-fold) failed to induce IR phosphorylation (Figure 5B, bottom gel set, lane R7 versus F) and FAS binding to CEACAM1 (Figure 5B, top gel set, lane R7 versus F), as well as to reduce FAS enzymatic activity (Figure 5B, graph, bar R7 versus F). As expected from hyperinsulinemia and obesity, steady-state fasting FAS levels (Figure 5B, center gel set) and activity (Figure 5B, graph) were higher in L-SACC1 by comparison to wt mice.

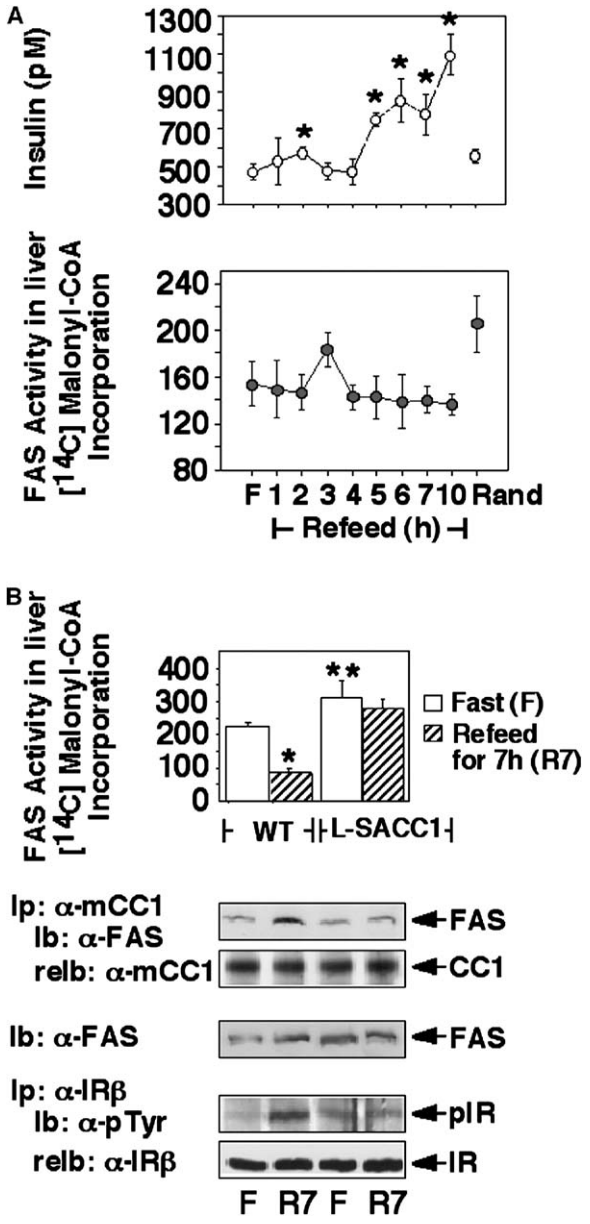


Figure 5. The acute effect of insulin on FAS activity is abolished in the hyperinsulinemic L-SACC1 mouse

A) Male L-SACC1 mice at 2 months of age were subjected to a fasting/refeeding paradigm similar to the one described in Figure 4B. At the end of fasting/refeeding, blood was drawn to measure insulin levels (top graph) and the liver removed to assay FAS activity (lower graph). Five to six mice per group were used for each time point, and the assays were performed in duplicate or quadruplet for insulin and FAS assays, respectively. Values are expressed as mean ± SEM. *$p < 0.05$ versus fasted.

B) Two-month-old wild-type (wt) and L-SACC1 female mice were fasted (F) and refed for 7 hr (R7), and the liver removed to determine FAS activity (graph), content (center gel set), and its interaction with CEACAM1 (top gel set), as above. Phosphorylation of the insulin receptor was also analyzed by immunoblotting with α-pTyr antibody as above (bottom gel set). FAS assay was performed in triplicate and insulin in duplicate. Values are expressed as mean ± SEM. *$p < 0.05$ versus fasted; **$p < 0.05$ versus wt fasted. SDS-PAGE analysis was repeated on two sets of experiments using a different mouse from each refeeding time.

Refeeding L-SACC1 mice did not augment FAS content above basal (Figure 5B, center gel, lane R7 versus F). Moreover, despite comparable levels of FAS in refed wt and L-SACC1 mice, FAS activity was lower in refed wt than L-SACC1 mice. Taken together, the data suggest that the acute effect of insulin on hepatic FAS activity is abolished in chronic hyperinsulinemia, probably due to reduced ability of insulin to activate the insulin receptor and induce CEACAM1 phosphorylation and interaction with FAS.

We then examined FAS activity in the hyperinsulinemic *Ob/Ob* obese mice to further investigate whether the negative acute effect of insulin depends on the insulinemic state. As expected from hyperinsulinemia (insulin levels ~9-fold higher than in lean mice), hepatic FAS steady-state levels (Figure 6D) and activity (Figures 6A and 6F lanes) are higher in *Ob/Ob* by comparison to their wt lean counterparts. Increases in insulin levels upon refeeding for 7 hr (by ~4-fold increase) did not significantly decrease FAS activity in *Ob/Ob* as it did to wt mice (Figure 6A, bars R7 versus F). The decrease in FAS activity in refed wt mice was associated with increased phosphorylation of the receptor (pIR) (Figure 6C, lane R7 versus F) and of CEACAM1 (pCC1) and elevated FAS/CEACAM1 coimmunoprecipitation (Figure 6B). In contrast, insulin did not increase receptor phosphorylation in refed *Ob/Ob* mice (Figure 6C, lane R7 versus F). Consequently, it failed to induce CEACAM1 phosphorylation and its association with FAS (Figure 6B, lane R7 versus F). Because hepatic CEACAM1 levels were unaltered in *Ob/Ob* mice (Figure 6E), the data suggest that the acute effect of insulin on FAS activity is abolished in hyperinsulinemia when insulin fails to activate its receptor and phosphorylate CEACAM1.

A role for CEACAM1 in mediating the acute effect of insulin on FAS activity

Short-term insulin treatment significantly decreased FAS activity in primary hepatocytes from wt (439.4 ± 75.27 cpm/μg versus 674.2 ± 102.0; $p < 0.05$) but not L-SACC1 mice with liver overexpression of the phosphorylation-defective CEACAM1 mutant (767.1 ± 160.5 cpm/μg versus 678.7 ± 158.9). Because the short-term effect of insulin on FAS activity was retained in L-SACC1 hepatocytes even after they have been isolated from elevated levels of insulin, a functional CEACAM1 appears to be required for cells to respond intrinsically to acute changes of insulin levels.

To further test this hypothesis, we assayed FAS activity in *Cc1*$^{-/-}$ mice with null mutation of *Ceacam1*. As expected, CEACAM1 association with FAS was abolished in the *Cc1*$^{-/-}$ mice, as assessed by coimmunoprecipitation (Figure 7A). Increases in insulin secretion upon refeeding overnight-fasted mice for 7 hr (Figure 7B, top graph) did not reduce hepatic FAS activity in *Cc1*$^{-/-}$ as in wt mice (Figure 7B, bottom graph, bar R7 versus F). This occurred in parallel to the failure of insulin to induce insulin receptor phosphorylation (pIR) in *Cc1*$^{-/-}$, as opposed to wt mice (Figure 7B, top gel set, R7 versus F lanes).

However, because *Cc1*$^{-/-}$ mice are hyperinsulinemic (Figure 7B, top graph, plasma insulin levels of 538. 6 ± 49.4 in *Cc1*$^{-/-}$ versus 207.0 ± 20.6 in wt mice, $p < 0.05$), it is possible that the absence of the negative effect of insulin on FAS activity results from the chronically elevated insulin levels. Thus, we examined FAS activity in primary hepatocytes derived from these mice. Insulin treatment for 15 min significantly reduced FAS activity in wt but not in *Cc1*$^{-/-}$ hepatocytes (Figure 7C). Furthermore,

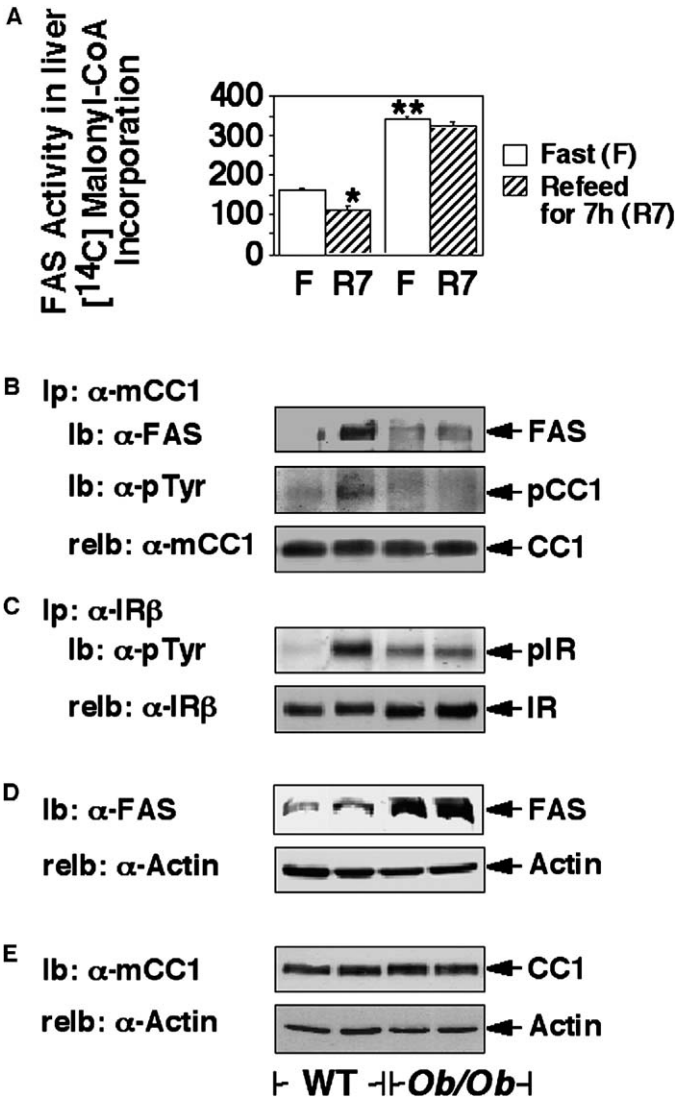


Figure 6. The acute effect of insulin on FAS activity is abolished in the hyperinsulinemic *Ob/Ob* obese mouse

A) Two-month-old lean (wt) and *Ob/Ob* obese male mice were fasted (F) and refed for 7 hr (R7), and the liver removed to determine FAS activity. The assay was performed in triplicate on 5–6 mice per group. Values are expressed as mean ± SEM. *$p < 0.05$ versus fasted; **$p < 0.05$ versus wt fasted.

B) Liver lysates were immunoprecipitated (Ip) with α-mCC1 and analyzed by immunoblotting (Ib) with α-pTyr antibody to determine phosphorylation of CEACAM1 (pCC1). CEACAM1 immunopellets were also immunoblotted with α-FAS to determine the interaction between these two proteins followed by reimmunoblotting (relb) with α-mCC1 to account for the amount of CC1 in the immunopellet.

C) Liver lysates were immunoprecipitated with α-IR_β antibody and analyzed for IR phosphorylation (pIR) as in (**B**) above.

D and E) To determine the content of FAS and CEACAM1, liver lysates were sequentially immunoblotted (Ib) with their respective antibodies and then with α-actin antibody to normalize for the amount of proteins on the gel. Gels are representatives of at least three experiments.

redelivering wt but not the S503A phosphorylation-defective *Cc1* mutant by adenovirus infection (Ad) restored the acute negative effect of insulin on FAS activity in $Cc1^{-/-}$ cells (Figure 7D, graph). Because infection with the adenovirus vector control (Ad *Luc*) did not yield a decrease in FAS activity in response to insulin, the negative effect of insulin on FAS activity in Ad-*wt Cc1* cells is specific to the wt isoform. Furthermore, insulin-stimulated receptor phosphorylation was intact in cells transduced with recombinant Ad-S503A CEACAM1 mutant (Figure 7D, bottom gel set). This emphasizes that acute regulation of FAS activity by insulin is a postreceptor event that is mediated by CEACAM1 phosphorylation.

Discussion

Altered fat metabolism plays an important role in the pathogenesis of obesity and type 2 diabetes. Since lipid metabolism and insulin clearance in liver modulate insulin action (Bergman, 2000; Massillon et al., 1997), it is important to understand whether they are regulated in a concerted manner. We have shown that CEACAM1 promotes insulin internalization (Choice et al., 1998; Formisano et al., 1995; Najjar et al., 1998) and that inactivation of CEACAM1 in liver impairs insulin clearance, alters fat metabolism, and leads to visceral obesity (Poy et al., 2002b). Thus, we investigated whether CEACAM1 directly regulates lipid metabolism. We report that CEACAM1 plays a significant role in insulin regulation of hepatic fatty acid synthesis.

In addition to its well-established chronic effect to increase FAS mRNA levels, we describe a novel acute lowering effect of insulin on hepatic FAS activity that is mediated by engaging CEACAM1-dependent insulin signaling pathways. Gavage feeding causes a biphasic decrease in FAS activity that mirrors insulin secretion in fasted normoinsulinemic mice. Similarly, refeeding mice with regular chow increases insulin surges after 1 hr, as has been shown (Zammit and Moir, 1993), but more significantly after 4 and 7 hr, and it causes a decrease in FAS activity. The decrease in FAS activity depends on the ability of insulin to induce CEACAM1 phosphorylation and binding to FAS, as highlighted by redelivering CEACAM1 to $Cc1^{-/-}$ primary hepatocytes. Moreover, it occurs despite a modest elevation in FAS protein content with prolonged refeeding, especially after 5 hr. Our data are in agreement with previous reports demonstrating that increases in FAS mRNA transcription begin only at about 4 hr but without elevation in FAS activity until about 8 hr of refeeding (Iritani, 1992).

In contrast to normoinsulinemic mice, insulin did not significantly reduce FAS activity in refed obese hyperinsulinemic mice (L-SACC1, $Cc1^{-/-}$, and *Ob/Ob*). This is consistent with blunted ability of insulin to activate insulin receptors in chronic hyperinsulinemia. Our data are in agreement with the acute downregulatory effect of hepatic triglyceride output by insulin and support the notion that hepatic fatty acid synthesis and triglyceride output are tightly regulated (Lewis and Steiner, 1996; Malmstrom et al., 1998).

The physiologic distribution of CEACAM1 to the liver, as opposed to the adipose tissue, and the dependence of the acute negative effect of insulin on CEACAM1 phosphorylation suggest that insulin acutely decreases FAS activity to dampen lipogenesis in liver during refeeding. With the liver being chronically exposed to high levels of insulin in the portal circulation (Ward et al., 1990) that could otherwise increase lipogenesis, acute downregulation of FAS activity could constitute a mechanism to maintain insulin sensitivity in liver.

The relevance of FAS activity to insulin action in metabolic control has been highlighted by the observation that treatment

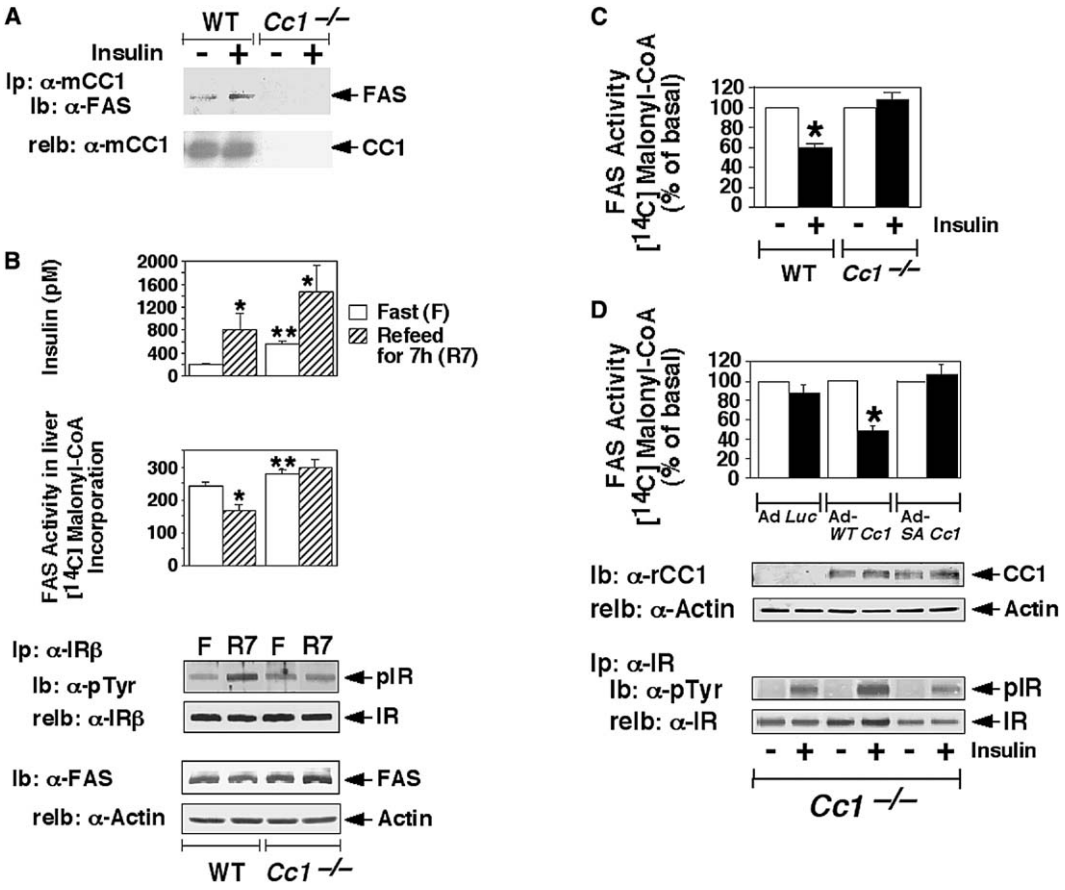


Figure 7. CEACAM1 phosphorylation mediates the acute negative effect of insulin on FAS activity

A) Primary hepatocytes from wt and *Cc1*$^{-/-}$ 3-month-old mice were treated with insulin (+) or with buffer (–) for 5 min. Cell lysates were immunoprecipitated (Ip) with α-mCC1 antibody and immunoblotted (Ib) with α-FAS antibody to detect FAS binding to CEACAM1 and reimmunoprobed with α-mCC1 antibody to account for the amount of CEACAM1 in the immunopellets.

B) wt and *Cc1*$^{-/-}$ female mice at 2 months of age were fasted overnight (F, white bars) and refed for 7 hr (R7, dashed bars). Mice were sacrificed and the blood removed to measure plasma insulin levels in duplicate (top graph), and the livers removed and homogenized to assay for FAS activity in triplicate (bottom graph). At least four mice were included in each group. Values are expressed as mean ± SEM. *$p < 0.05$ versus fasted; **$p < 0.05$ versus wt fasted. Phosphorylation of the insulin receptor was analyzed by immunoblotting α-IR$_\beta$ immunoprecipitates with α-pTyr antibody and normalizing with the amount of IR in the pellet, as assessed by reprobing (relb) with α-IR antibody (top gel set). FAS content was assessed by immunoblotting cell lysates with α-FAS followed by reprobing with α-actin antibody to normalize per the amount of proteins loaded (bottom gel set). These gels are representatives of three experiments.

C) Serum-starved primary hepatocytes from 3-month-old wt and *Cc1*$^{-/-}$ null mice were treated with insulin (solid bars) or buffer (white bars) for 15 min. FAS-specific activity was assayed in triplicate. Values are expressed as mean percentage of basal ± SEM. *$p < 0.05$ versus buffer.

D) Primary hepatocytes from wt and *Cc1*$^{-/-}$ null mice were infected with the adenovirus vector control (Ad *Luc*) or carrying rat *wt* and *S503A* phosphorylation-defective *Cc1* mutant (*SA Cc1*), treated with insulin (+) or buffer alone (–) for 15 min prior to analyzing lysates by immunoblotting (Ib) with α-rCC1 rat antibody and then with α-actin (top gel set). One hundred and fifty microgram cell lysates were immunoprecipitated (Ip) with α-IR antibody, analyzed on SDS-PAGE, and sequentially immunoblotted (Ib) with α-pTyr and α-IR antibody, with the latter being performed to normalize for the amount of IR (bottom gel set). FAS activity was also assayed in triplicate (graph). Values are expressed as mean percentage of basal ± SEM. *$p < 0.05$ versus buffer.

of obese mice with FAS inhibitors reduces body weight by regulating malonyl-CoA levels independently of leptin (Loftus et al., 2000). Inhibiting FAS in the arcuate nucleus also reduces food intake and promotes insulin sensitivity (Elmquist and Marcus, 2003; Gao and Lane, 2003; Mobbs and Makimura, 2002; Wortman et al., 2003), suggesting that FAS downregulation maintains insulin sensitivity. These observations are in agreement with the model proposed by our studies and emphasize the role of de novo synthesis of fatty acids in regulating insulin action in the brain-liver axis.

The current studies identify a new mechanism of the regulation of hepatic fat metabolism by insulin. The mechanism implicates CEACAM1 phosphorylation by the insulin receptor and its cellular uptake and binding to FAS. Increased redistribution of CEACAM1 in the cytoplasm of hepatoma cells in response to insulin is consistent with increased CEACAM1 endocytosis as part of the insulin endocytosis complex (Choice et al., 1998). This demonstrates the intertwined molecular mechanism of the effect of CEACAM1 on insulin and fat metabolism. Because FAS binding to CEACAM1 requires Tyr488 phosphorylation, as does the formation of CEACAM1/insulin receptor complex (Choice et al., 1998), it is possible that FAS binding to CEACAM1 increases simultaneously with the complex dissociation from the receptor. This suggests that CEACAM1 is not directly

involved in the postinternalization receptor trafficking events to a large extent, including receptor recycling, as we have previously reported (Li Calzi et al., 1997). Persistent CEACAM1 localization in the cytoplasmic compartment even after its phosphorylation and association with FAS were significantly reduced suggests that it may be involved with other cellular events. Whether CEACAM1 eventually recycles back to the plasma membrane is not known and analysis of its metabolism and function is beyond the scope of these current studies. Nonetheless, the data provide compelling evidence that CEACAM1 interacts with fatty acid synthase to tightly regulate fat metabolism and preserve insulin sensitivity in liver. In light of the dependence of CEACAM1/FAS interaction on insulin-induced CEACAM1 internalization, and the primary role of this mechanism in insulin clearance, the current data demonstrate that CEACAM1 is at the intersection of the pathways regulating insulin and fat metabolism in liver.

Experimental procedures

Generation of the *Ceacam1* null (*Cc1*$^{-/-}$) mouse

Exons 1 and 2 of *Ceacam1a* were replaced by a GK-*neo*^{r} cassette using homolgous recombination in ES cells to eliminate translation initiation (Nédellec et al., 1995). Generation of knockout mice was according to standard procedures (Hemmila et al., 2004). Animals were kept in a 12 hr dark-light cycle and fed standard chow ad libitum, unless otherwise indicated. Whole blood was drawn from anesthetized mice at indicated time points from retro-orbital sinuses to determine plasma insulin levels by radioimmunoassays (RIA) (Linco Research). All procedures are approved by the Institutional Animal Care and Utilization Committee.

Cell culture

MDA-MB 468 human breast cancer cells were maintained in Leibovitz's L-15 media (Invitrogen) at 37°C in a CO_2-free incubator, and H35-BT rat hepatoma cells (Taub et al., 1987) and their H4-II-E derivatives in Dulbecco-Modified Eagle's Medium (DMEM, Mediatech) and RPMI-1640 (Mediatech), respectively, at 37°C in a 5% CO_2 incubator. All media were supplemented with 10% fetal bovine serum (FBS, Mediatech), 100 U/ml penicillin, 10 μg/ml streptomycin, and 1% glutamine (Invitrogen), except for the medium of H35-BT, which was supplemented with 5% FBS and 5% new born calf serum.

Primary hepatocytes

As described in Poy et al. (2002b), male mice were anesthetized with sodium pentobarbital (30 μg/g body weight). Hepatocytes were isolated by a two-stage collagenase perfusion through the portal vein (Hanks/EGTA [0.5 mM] and 1 mg/ml collagenase D [Roche] in L-15 media supplemented with glucose [1 mg/ml]). The viability of hepatocytes was determined by Trypan Blue dye exclusion. Cells were plated at 4.5 × 10^{6} cells per 100 mm dish in 10 ml DMEM, supplemented with FBS (10% v/v) and penicillin/streptomycin (1% v/v) at 37°C. Cultures were refed with fresh DMEM medium after a 3 hr attachment period (Ruch and Klaunig, 1988).

GST pull-down assay

Unless indicated otherwise, GST-CEACAM1 fusion peptides were described previously (Najjar et al., 1995). The Ser503 to Asp mutation (S503D) was introduced by PCR. Primer sequences are available upon request. GST fusion peptides (10 μg) were phosphorylated by baculovirus-purified IRK$_{\beta}$ (Biomol, 1 U) or GST-tagged (Calbiochem, 1 U) in the presence of 70 μCi [γ-^{32}P] ATP, bound (60 min at 4°C) to equal amounts of MDA-MB 468 cell lysates, or 1 μg purified His-tagged FAS (Joshi and Smith, 1993; Joshi et al., 1997). Pellets were washed in HNTG buffer containing 0.5% Triton and analyzed by electrophoresis. Standard Western blotting techniques with anti-FAS antiserum were employed.

Western blotting

For coimmunoprecipitation, cells were treated with 100 nM insulin for 0–90 min unless otherwise indicated, prior to lysis and protein immunoprecipitation (Najjar et al., 1995). Mice were fasted and refed and the tissues removed and lysed. Following lysis, proteins (400 μg) were subjected to coimmunoprecipitation (Choice et al., 1998). In these experiments, a monoclonal HA4 c19 (Hybridoma Bank, Iowa) or an anti-mouse polyclonal antibody against BGP1 (α-mCC1; Ab-231) (Sadekova et al., 2000) was used to immunoprecipitate the rat and the mouse protein, respectively. Following immunoprecipitation, proteins were immunoblotted with α-FAS polyclonal antibody to detect the amount of FAS in the CC1 immunopellet, and with α-76 Ex/α-CC1 rat antibody (Poy et al., 2002a) or α-mCC1 to normalize for the amount of CEACAM1 in the immunopellet.

For phosphorylation, 1 μg of His-tagged FAS was phosphorylated by preactivated GST-IRK$_{\beta}$ (1 U) in the presence of [γ-^{32}P] ATP at RT for 10–60 min in phosphorylation buffer (above) prior to analysis by 6% SDS-PAGE and autoradiography. Insulin-treated cells or mouse livers were lysed, and 200 μg of lectin-purified proteins were immunoprecipitated with antibodies against CEACAM1 (α-CC1) or the β subunit of the insulin receptor (α-IR$_{\beta}$) (Santa Cruz) prior to analysis by SDS-PAGE and immunoblotting with α-phosphotyrosine antibody (α-pTyr) (Upstate Biotechnology Inc.) to examine the phosphorylation state. The amount of incorporated phosphotyrosine was normalized by the amount of proteins in the immunopellet, which was in turn determined by reprobing with α-CC1 or α-IR$_{\beta}$ antibody.

To determine the protein content, 75 μg lysates were analyzed directly by SDS-PAGE followed by sequential immunoblotting with their antibodies and then with α-actin (Sigma-Aldrich) to normalize for the amount of proteins loaded on the gel.

FAS enzymatic activity

Two-month-old male normal mice were fasted overnight (from 1700 to 0700 hr the next day) and refed regular chow for 1–10 hr or were given glucose (2 g/Kg body weight) by oral gavage for 0–120 min. Two-month-old male *Ob/Ob* mice (Jackson Laboratories) and L-SACC1 and *Cc1*$^{-/-}$ female mice were subjected to a similar fasting/refeeding paradigm. Following refeeding, mice were sacrificed, and their livers removed and homogenized in buffer containing 20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM DTT, and phosphatase and protease inhibitors (Najjar et al., 1995). Primary hepatocytes or H35-BT cells were grown in DMEM complete medium at a density of 4.5 × 10^{6} cells/10cc plate, serum-starved overnight, treated with 100 nM insulin for 0–90 min at 37°C, and lyzed, as above. The homogenate was centrifuged at 12,500 × g for 30 min at 4°C and FAS activity assayed in the postmitochondrial supernatant (Hennigar et al., 1998). Briefly, lysates were incubated at 37°C for 20 min with 166.6 μM acetyl-CoA, 100 mM potassium phosphate (pH 6.6), 0.1 μCi [^{14}C] malonyl-CoA, 25 nmol malonyl-CoA in the absence (negative controls) or presence of 500 μM NADPH. The reaction was stopped with 1 ml 1:1 chloroform:methanol solution and mixed at room temperature for 30 min. Following centrifugation at 12,500 × g for 20 min, the supernatant was vacuum-dried, and the pellet resuspended in 200 μl of water-saturated butanol. Two hundred microliters ddH_2O was added followed by vortexing and quick spinning for 1 min to remove the upper layer for re-extraction. Both butanol samples were pooled and washed with ddH_2O. The butanol layer was moved, dried, and counted for (Giaccari et al., 1998) incorporation. Protein concentrations of cell lysates were measured by the Bio-Rad protein assay. FAS activity was calculated as cpm of [^{14}C] incorporated/μg of cell lysates.

His-tagged baculovirus-purified CEACAM1 (Phan et al., 2001) was phosphorylated by buffer alone or GST-tagged IRK$_{\beta}$ at 25°C for 60 min in the presence of 70 μCi [γ-^{32}P] ATP. One picamole of His-tagged FAS (Joshi and Smith, 1993) was then added and the reaction mixed at 4°C for 60 min. FAS activity was assayed and normalized per activity in control samples without CEACAM1.

Confocal microscopy

H4-II-E rat hepatoma cells were serum-starved overnight and then incubated in DMEM medium (Invitrogen) containing 0.24 mg/ml of α-CC1/HA4 monoclonal antibody at RT for 2 hr (Lamaze et al., 2001; Oyarce et al., 2001). Cells were rinsed with PBS and fixed with cold methanol either immediately or following treatment with 100 nM of insulin at 37°C for different time periods. Cells were then blocked with 0.25% BSA and incubated at RT for 2 hr with polyclonal α-FAS antibody (2 μg/ml). Following incubation with the FAS antibody, cells were incubated with goat anti-mouse FITC and goat anti-rabbit Cy3 conjugated secondary antisera (Caltag Laboratories)

at RT for 1 hr and analyzed using an Olympus microscope coupled to a Bio-Rad Radiance 2100 confocal scanning laser. To label IRS-2, serum-starved cells were treated with insulin, fixed with cold methanol, and blocked in 25% BSA, as above. A rabbit polyclonal antibody against IRS-2 (Upstate Biotechnology) was added at 1/10–1/100 dilutions for an overnight incubation prior to green FITC labeling and confocal microscopy.

Quantitation of proteins and statistical analysis

Autoradiograms were scanned on an imaging densitometer (Bio-Rad), and the proteins were quantitated with SPECTRA MAX Plus (Molecular Devices) using the SoftMAX Pro Macintosh software program (Molecular Devices). Data were analyzed with Prism3 software (GraphPad Software, Inc.) using t test analysis. p values of less than 0.05 were considered statistically significant.

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References

Amy, C.M., Witkowski, A., Naggert, J., Williams, B., Randhawa, Z., and Smith, S. (1989). Molecular cloning and sequencing of cDNAs encoding the entire rat fatty acid synthase. Proc. Natl. Acad. Sci. USA *86*, 3114–3118.

An, J., Muoio, D.M., Shiota, M., Fujimoto, Y., Cline, G.W., Shulman, G.I., Koves, T.R., Stevens, R., Millington, D., and Newgard, C.B. (2004). Hepatic expression of malonyl-CoA decarboxylase reverses muscle, liver and whole-animal insulin resistance. Nat. Med. *10*, 268–274.

Assimacopoulos-Jeannet, F., Brichard, S., Rencurel, F., Cusin, I., and Jeanrenaud, B. (1995). In vivo effects of hyperinsulinemia on lipogenic enzymes and glucose transporter expression in rat liver and adipose tissues. Metabolism *44*, 228–233.

Bazin, R., and Lavau, M. (1982). Development of hepatic and adipose tissue lipogenic enzymes and insulinemia during suckling and weaning on to a high-fat diet in Zucker rats. J. Lipid Res. *23*, 839–849.

Bergman, R.N. (2000). Non-esterified fatty acids and the liver: why is insulin secreted into the portal vein? Diabetologia *43*, 946–952.

Choice, C.V., Howard, M.J., Poy, M.N., Hankin, M.H., and Najjar, S.M. (1998). Insulin stimulates pp120 endocytosis in cells co-expressing insulin receptors. J. Biol. Chem. *273*, 22194–22200.

Dai, T., Abou-Rjaily, G.A., Al-Share, Q.Y., Yang, Y., Fernström, M.A., DeAngelis, A.M., Lee, A.D., Sweetman, L., Amato, A., Pasquali, M., et al. (2004). Interaction between altered insulin and lipid metabolism in CEACAM1-inactive transgenic mice. J. Biol. Chem. *279*, 45155–45161.

Elmquist, J.K., and Marcus, J.N. (2003). Rethinking the central causes of diabetes. Nat. Med. *6*, 645–647.

Formisano, P., Najjar, S.M., Gross, C.N., Philippe, N., Oriente, F., Kern-Buell, C.L., Accili, D., and Gorden, P. (1995). Receptor-mediated internalization of insulin. Potential role of pp120/HA4, a substrate of the insulin receptor kinase. J. Biol. Chem. *270*, 24073–24077.

Fukuda, H., Iritani, N., Sugimoto, T., and Ikeda, H. (1999). Transcriptional regulation of fatty acid synthase gene by insulin/glucose, polyunsaturated fatty acid and leptin in hepatocytes and adipocytes in normal and genetically obese rats. Eur. J. Biochem. *260*, 505–511.

Gao, S., and Lane, M.D. (2003). Effect of the anorectic fatty acid synthase inhibitor C75 on neuronal activity in the hypothalamus and brainstem. Proc. Natl. Acad. Sci. USA *100*, 5628–5633.

Giaccari, A., Morviducci, L., Pastore, L., Zorretta, D., Sbraccia, P., Maroccia, E., Buongiorno, A., and Tamburrano, G. (1998). Relative contribution of glycogenolysis and gluconeogenesis to hepatic glucose production in control and diabetic rats. A re-examination in the presence of euglycaemia. Diabetologia *41*, 307–314.

Hemmila, E., Turbide, C., Olson, M., Jothy, S., Holmes, K.V., and Beauchemin, N. (2004). Ceacam1a–/– mice are completely resistant to infection by murine coronavirus mouse hepatitis virus A59. J. Virol. *78*, 10156–10165.

Hennigar, R.A., Pochet, M., Hunt, D.A., Lukacher, A.E., Venema, V.J., Seal, E., and Marrero, M.B. (1998). Characterization of fatty acid synthase in cell lines derived from experimental mammary tumors. Biochim. Biophys. Acta *1392*, 85–100.

Iritani, N. (1992). Nutritional and hormonal regulation of lipogenic-enzyme gene expression in rat liver. Eur. J. Biochem. *205*, 433–442.

Joshi, A.K., and Smith, S. (1993). Construction of a cDNA encoding the multifunctional animal fatty acid sythase and expression in Spodoptera frugiperda cells using baculoviral vectors. Biochem. J. *296*, 143–149.

Joshi, A.K., Witkowski, A., and Smith, S. (1997). Mapping of functional interactions between domains of the animal fatty acid synthase by mutant complementation in vitro. Biochemistry *36*, 2316–2322.

Kahn, B.B., Alquier, T., Carling, D., and Hardie, D.G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab. *1*, 15–24.

Kim, K.H., Lopez-Casillas, F., Bai, D.H., Luo, X., and Pape, M.E. (1989). Role of reversible phosphorylation of acetyl-CoA carboxylase in long-chain fatty acid synthesis. FASEB J. *3*, 2250–2256.

Lamaze, C., Dujeancourt, A., Baba, T., Lo, C.G., Benmerah, A., and Dautry-Varsat, A. (2001). Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. Mol. Cell *7*, 661–671.

Lewis, G.F., and Steiner, G. (1996). Acute effects of insulin in the control of VLDL production in humans. Implications for the insulin-resistant state. Diabetes Care *19*, 390–393.

Li Calzi, S., Choice, C.V., and Najjar, S.M. (1997). Differential effect of pp120 on insulin endocytosis by two variant insulin receptor isoforms. Am. J. Physiol. *273*, E801–E808.

Loftus, T.M., Jaworsky, D.E., Frehywot, G.L., Townsend, C.A., Ronnett, G.V., Lane, M.D., and Kuhajda, F.P. (2000). Reduced food intake and body weight in mice treated with fatty acid synthase inhibitors. Science *288*, 2379–2381.

Macgregor, P.F., Abate, C., and Curran, T. (1990). Direct cloning of leucine zipper proteins: Jun binds cooperatively to the CRE with CRE-BP-1. Oncogene *5*, 451–458.

Malmstrom, R., Packard, C.J., Caslake, M., Bedford, D., Stewart, P., Yki-Jarvinen, H., Shepherd, J., and Taskinen, M.R. (1998). Effects of insulin and acipimox on VLDL1 and VLDL2 apolipoprotein B production in normal subjects. Diabetes *47*, 779–787.

Massillon, D., Barzilai, N., Hawkins, M., Prus-Wertheimer, D., and Rossetti, L. (1997). Induction of hepatic glucose-6-phosphatase gene expression by lipid infusion. Diabetes *46*, 153–157.

McGarry, J.D. (2002). Dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes *51*, 7–18.

Mobbs, C.V., and Makimura, H. (2002). Block the FAS, lose the fat. Nat. Med. *8*, 335–336.

Najjar, S.M., Philippe, N., Suzuki, Y., Ignacio, G.A., Formisano, P., Accili, D., and Taylor, S.I. (1995). Insulin-stimulated phosphorylation of recombinant pp120/HA4, an endogenous substrate of the insulin receptor tyrosine kinase. Biochemistry *34*, 9341–9349.

Najjar, S.M., Choice, C.V., Soni, P., Whitman, C.M., and Poy, M.N. (1998). Effect of pp120 on receptor-mediated insulin endocytosis is regulated by the juxtamembrane domain of the insulin receptor. J. Biol. Chem. *273*, 12923–12928.

Nédellec, P., Turbide, C., and Beauchemin, N. (1995). Characterization and transcriptional activity of the mouse biliary glycoprotein 1 gene, a carcinoembryonic antigen-related gene. Eur. J. Biochem. *231*, 104–114.

Oyarce, A.M., Steveson, T.C., Jin, L., and Eipper, B.A. (2001). Dopamine beta-monooxygenase signal/anchor sequence alters trafficking of peptidylglycine alpha-hydroxylating monooxygenase. J. Biol. Chem. *276*, 33265–33272.

Paulauskis, J.D., and Sul, H.S. (1989). Hormonal regulation of mouse fatty acid synthase gene transcription in liver. J. Biol. Chem. *264*, 574–577.

Phan, D., Han, E., Birrell, G., Bonnal, S., Duggan, L., Esumi, N., Gutstein, H., Li, R., Lopato, S., Manogaran, A., et al. (2001). Purification and characterization of human cell–cell adhesion molecule 1 (C-CAM1) expressed in insect cells. Protein Expr. Purif. *21*, 343–351.

Poy, M.N., Ruch, R.J., Fernström, M.A., Okabayashi, Y., and Najjar, S.M. (2002a). Shc and CEACAM1 interact to regulate the mitogenic action of insulin. J. Biol. Chem. *277*, 1076–1084.

Poy, M.N., Yang, Y., Rezaei, K., Fernström, M.A., Lee, A.D., Kido, Y., Erickson, S.K., and Najjar, S.M. (2002b). CEACAM1 regulates insulin clearance in liver. Nat. Genet. *30*, 270–276.

Randle, P.J. (1998). Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. Diabetes Metab. Rev. *14*, 263–283.

Ruch, R.J., and Klaunig, J.E. (1988). Kinetics of phenobarbital inhibition of intercellular communication in mouse hepatocytes. Cancer Res. *48*, 2519–2523.

Ruderman, N.B., Saha, A.K., Vavvas, D., and Witters, L.A. (1999). Malonyl-CoA, fuel sensing, and insulin resistance. Am. J. Physiol. *276*, E1–E18.

Sadekova, S., Lamarche-Vane, N., Li, X., and Beauchemin, N. (2000). The CEACAM1-L glycoprotein associates with the actin cytoskeleton and localizes to cell-cell contact through activation of Rho-like GTPases. Mol. Biol. Cell *11*, 65–77.

Semenkovich, C.F. (1997). Regulation of fatty acid synthase (FAS). Prog. Lipid Res. *36*, 43–53.

Shillabeer, G., Hornford, J., Forden, J.M., Wong, N.C., Russell, J.C., and Lau, D.C. (1992). Fatty acid synthase and adipsin mRNA levels in obese and lean JCR:LA-cp rats: effect of diet. J. Lipid Res. *33*, 31–39.

Shulman, G.I., and Landau, B.R. (1992). Pathways of glycogen repletion. Physiol. Rev. *72*, 1019–1035.

Stapleton, S.R., Mitchell, D.A., Salati, L.M., and Goodridge, A.G. (1990). Triiodothyronine stimulates transcription of the fatty acid synthase gene in chick embryo hepatocytes in culture. Insulin and insulin-like growth factor amplify that effect. J. Biol. Chem. *265*, 18442–18446.

Taub, R., Roy, A., Dieter, R., and Koontz, J. (1987). Insulin as a growth factor in rat hepatoma cells. Stimulation of proto-oncogene expression. J. Biol. Chem. *262*, 10893–10897.

Wakil, S.J. (1989). Fatty acid synthase, a proficient multifunctional enzyme. Biochemistry *28*, 4523–4530.

Ward, G.M., Walters, J.M., Aitken, P.M., Best, J.D., and Alford, F.P. (1990). Effects of prolonged pulsatile hyperinsulinemia in humans. Enhancement of insulin sensitivity. Diabetes *39*, 501–507.

Witters, L.A., and Kemp, B.E. (1992). Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5′-AMP-activated protein kinase. J. Biol. Chem. *267*, 2864–2867.

Wortman, M.D., Clegg, D.J., D'Alessio, D., Woods, S.C., and Seeley, R.J. (2003). C75 inhibits food intake by increasing CNS glucose metabolism. Nat. Med. *9*, 483–485.

Zammit, V.A. (2002). Insulin stimulation of triacylglycerol secretion in the insulin-replete state. Implications for the etiology of peripheral insulin resistance. Ann. N Y Acad. Sci. *967*, 52–65.

Zammit, V.A., and Moir, A.M. (1993). Monitoring the partitioning of hepatic fatty acids in vivo: keeping track of control. Biochem. J. *289*, 49–55.