Decreased insulin-dependent glucose transport by chronic ethanol feeding is associated with dysregulation of the Cbl/TC10 pathway in rat adipocytes

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Sebastian, Becky M., and Laura E. Nagy. Decreased insulin-dependent glucose transport by chronic ethanol feeding is associated with dysregulation of the Cbl/TC10 pathway in rat adipocytes. Am J Physiol Endocrinol Metab 289: E1077–E1084, 2005.—First published August 16, 2005; doi:10.1152/ajpendo.00296.2005.—Heavy alcohol consumption is an independent risk factor for type 2 diabetes. Although the exact mechanism by which alcohol contributes to the increased risk is unknown, impaired glucose disposal is a likely target. Insulin-stimulated glucose disposal in adipocytes is regulated by two separate and independent pathways, the PI3K pathway and the Cbl/TC10 pathway. Previous studies suggest that chronic ethanol feeding impairs insulin-stimulated glucose transport in adipocytes in a PI3K-independent manner. In search of potential targets of ethanol that would affect insulin-stimulated glucose transport, we investigated the effects of 4 wk ethanol feeding to male Wistar rats on the Cbl/TC10 pathway in isolated adipocytes. Chronic ethanol feeding inhibited insulin-stimulated cCbl phosphorylation compared with pair feeding. Insulin receptor and Akt/PKB phosphorylation were not affected by ethanol feeding. Chronic ethanol exposure also impaired cCbl and TC10 recruitment to a lipid raft fraction isolated from adipocytes by detergent extraction. Furthermore, chronic ethanol feeding increased the amount of activated TC10 and filamentous actin in adipocytes at baseline and abrogated the ability of insulin to further activate TC10 or polymerize actin. These results demonstrate that the impairment in insulin-stimulated glucose transport observed in adipocytes after chronic ethanol feeding to rats is associated with a disruption of insulin-mediated Cbl/TC10 signaling and actin polymerization.

Muscle and adipose tissue are the major sites of glucose disposal in response to insulin. In these tissues, the phosphatidylinositol 3-kinase (PI3K) pathway regulates the translocation of the insulin-responsive glucose transporter GLUT4 from intracellular pools to the plasma membrane (PM). Insulin activates the tyrosine kinase activity of the insulin receptor, leading to autophosphorylation of the insulin receptor on several tyrosine residues and the phosphorylation of the insulin receptor substrate-1 (IRS-1) (27). The phosphorylated IRS-1 becomes a target for the regulatory subunit of PI3K, p85, activating the PI3K catalytic subunit p110 (35). The activation of PI3K leads to the downstream phosphorylation of Akt/PKB and atypical PKCζ, both shown to play a role in translocation of GLUT4 to the PM and glucose uptake (3, 4, 8, 25, 50, 53).

Although the PI3K pathway is necessary for insulin-stimulated glucose uptake, several lines of evidence suggest that this pathway alone is insufficient to mediate uptake (11, 14, 17, 26, 30, 37, 49, 57) and are reviewed in Ref. 45. A second insulin-signaling cascade implicated in glucose transport is the Cbl/TC10 pathway. In response to insulin, APS [adapter protein with pleckstrin homology (PH) and Src homology 2 (SH2) domains] is recruited to the autophosphorylated insulin receptor and binds via its SH2 domain (1, 28). APS is phosphorylated by the insulin receptor at Y618 (34), which becomes a docking site for the adapter protein cCbl. cCbl is found in the cytosol constitutively bound to the Cbl-associated protein (CAP) (28). Once associated with APS, the insulin receptor phosphorylates cCbl (28). This phosphorylation provides a binding site for the protein CrkII (44). CrkII is localized in the cytosol and is bound to C3G, a guanyl nucleotide exchange factor (GEF) (24). These proteins are recruited to lipid raft microdomains of the PM after insulin stimulation (5, 23). Also present in the lipid raft is the Rho family GTPase, TC10. C3G can function as a GEF for TC10, resulting in its activation. Studies utilizing dominant negative forms of CAP and TC10 implicate the Cbl/TC10 pathway in cortical actin polymerization/rearrangement, exocyst complex recruitment, and maximal glucose transport in response to insulin (6). A recent study argues against a role for Cbl/TC10 in insulin-stimulated glucose uptake in muscle cells (16). Furthermore, a recent study using short interfering (si)RNA strategies argues against TC10’s involvement in insulin-stimulated glucose uptake in 3T3-L1 adipocytes (33).

Chronic ethanol feeding in rats decreases insulin-stimulated glucose transport and decreases GLUT4 surface accessibility in isolated adipocytes (41, 58) but does not impair the association of the p85 subunit of PI3K with tyrosine-phosphorylated pro-
teins or the serine phosphorylation of Akt/PKB (41). These data suggest that ethanol impairs insulin-stimulated glucose transport in adipocytes in a PI3K-independent manner. In search of potential targets of ethanol that could impair insulin-stimulated glucose transport, we focused on the PI3K-independent Cbl/TC10 pathway. Here, we show that chronic ethanol feeding decreased insulin-stimulated cellular (c)Cbl phosphorylation and its recruitment to the lipid raft. Furthermore, chronic ethanol feeding disrupted the critical cycle of GTP loading/GTP hydrolysis of TC10 by increasing activation of TC10 in the absence of insulin and preventing further activation in response to insulin. Chronic ethanol feeding also impaired insulin-stimulated actin polymerization, shown in other studies to be regulated by TC10 (18). These data demonstrate that impaired glucose disposal in adipocytes after chronic ethanol exposure is associated with a disruption in the Cbl/TC10 pathway at multiple sites.

**EXPERIMENTAL PROCEDURES**

**Materials.** Male Wistar rats weighing 170–180 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). Lieber DeCarli ethanol diet was purchased from Dyets (Bethlehem, PA). Antibodies were obtained from the following sources: anti-phospho-tyrosine (PY100), anti-phospho-Akt/PKB (Ser473), anti-Akt/PKB, and anti-actin from Cytoskeleton (Denver, CO); anti-caveolin-1 from BD Transduction Laboratories (San Jose, CA); anti-TC10 from Affinity BioReagents (Golden, CO); anti-transferrin receptor from Zymed (South San Francisco, CA); anti-cCbl and anti-insulin receptor β-subunit from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-tyrosine (4G10) from Upstate (Charlottesville, VA); anti-TC10 from Affinity BioReagents (Golden, CO); anti-transferrin receptor from Zymed (South San Francisco, CA); anti-caveolin-1 from BD Transduction Laboratories (San Jose, CA); and anti-actin from Cytoskeleton (Denver, CO). Maltose dextrin was purchased from BioServ (Frenchtown, NJ); Immunopure immobilized protein G-agarose beads from Pierce Biotechnology (Rockford, IL); Complete, EDTA-free protease inhibitor cocktail tablet, and enhanced chemiluminescence reagent from Roche (Indianapolis, IN); the cdc42 activation assay kit from Upstate; and all other reagents from Sigma (St. Louis, MO).

**Animal feeding and adipocyte isolation.** The chronic ethanol feeding model used in this study has been previously described (43). Briefly, upon arrival, rats were acclimated to their environment for 3 days and then introduced to a liquid diet for 2 days. Randomly selected rats assigned to the ethanol-fed group were provided an ad libitum liquid diet containing ethanol as 35% of total caloric value for 4 wk. Control rats were pair fed a liquid diet with maltose dextrin isocalorically substituted for ethanol. All animal procedures were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University. After the 4-wk ethanol feeding, rats were anesthetized by intraperitoneal injection with 0.075 (ethanol-fed rats) or 0.12 ml (pair-fed rats)/100 g body wt of a cocktail containing 10 mg/ml acepromazine, 100 mg/ml ketamine, and 20 mg/ml xylazine, and epididymal fat pads were removed. Adipocytes were isolated by collagenase digestion as previously described, except that bovine serum albumin (BSA) was excluded from the wash buffer (58). Isolated adipocytes were counted and adjusted to a cell density of 2 × 10^6 cells/ml, unless otherwise noted.

**Immunoprecipitation and Western blotting.** Four milliliters of isolated adipocytes were incubated with or without 10 nM insulin for 0–5 min at 37°C in a shaking water bath (100 rpm) and then immediately placed on ice and lysed by 3% Triton X-100 lysis buffer (50 mM Tris, 6.4 mM NaCl, 1 mM EDTA, 1 mM Na pyrophosphate, 1 mM activated Na vanadate, 10 mM NaF, and protease inhibitor cocktail at 1 Complete tablet/2.6 ml) for 30 min. Lysates were centrifuged in a microcentrifuge for 3 min at 4,000 × g, and the infranatant was removed and assayed for protein content. Three milligrams of protein from each sample were incubated with 6 μg of anti-cCbl antibody rotating overnight at 4°C and then incubated for 2.5–3 h rotating at 4°C with 50 μl of protein G-agarose beads. Immune complexes were pelleted in a microcentrifuge for 5 min at 500 g at 4°C, followed by two washes with ice-cold PBS (rotated at 4°C for 15 min for each wash). The final pellet was resuspended in 40 μl of Laemmli buffer (30 mM Tris, pH 6.8, 5% glycerol, 1% SDS, 2.5% β-mercaptoethanol, and 0.125 mg/ml bromophenol blue). All samples were boiled 5 min before being loaded onto a 6% SDS-polyacrylamide gel and transferred to PVDF membrane using a semidy transfer technique with continuous transfer buffer according to standard protocols. All blots were blocked with 3% BSA in Tris-buffered saline, pH 7.6, containing 0.1% Tween-20 before overnight incubation at 4°C with primary antibodies. After a 1-h incubation with secondary antibody and subsequent washes, bound antibodies were visualized using enhanced chemiluminescence reagent. Films were scanned, and the densities of immunoreactive proteins were analyzed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available at the Internet at http://rsb.info.nih.gov/nih-image/). Film exposure times were in the linear range of detectability.

**PM isolation.** Isolated adipocytes were counted and adjusted to a cell density of 1 × 10^6 cells/ml and were incubated with or without 10 nM insulin for 0–5 min at 37°C in a shaking water bath (100 rpm). Stimulation was terminated with 2 mM KCN, and cells were immediately placed on ice. PM fractions were obtained by differential centrifugation as previously described (41), with few exceptions. Briefly, cells were homogenized in 500 μl, 10 strokes in a glass-on-glass dounce with a loose-fitting pestle, and homogenates were centrifuged at 15,000 × g for 15 min at 4°C. Pellets were washed with 1 ml of homogenization buffer (20 mM Tris, pH 7.4, 1 mM EDTA, and 255 mM sucrose with added protease inhibitor cocktail at 1 Complete tablet/50 ml buffer) and resuspended in 500 μl of homogenization buffer and layered over a sucrose pad (20 mM Tris, pH 7.4, 1 mM EDTA, and 1.12 M sucrose) and centrifuged 100,000 × g for 70 min. The pellet was collected and centrifuged 16,000 × g, 15 min at 4°C. Pellets (isolated PM) were resuspended in 50 μl of homogenization buffer and assayed for protein content.

**Preparation of Triton-insoluble fraction.** Caveolin-enriched Triton-insoluble fractions were obtained using a technique adapted from Mastick and Saltiel (31). Isolated adipocytes (1–4 ml) were incubated with or without 10 nM insulin for 0–5 min at 37°C in a shaking water bath (100 rpm), immediately placed on ice, and lysed for 30 min with rocking at 4°C in 1% Triton X-100 lysis buffer (5 mM Tris pH 7.4, 15 mM NaCl, 1.6 mM EGTA, 2 mM Na pyrophosphate, 1 mM activated Na vanadate, 10 mM NaF, 1 mM benzamidine, 1 mM molybdenum, and protease inhibitor cocktail at 1 Complete tablet/500 ml buffer). Lysates were homogenized at 4°C in a glass homogenizer with a tight-fitting pestle for 10 strokes and then centrifuged in a microcentrifuge 2 min at 250 g to remove nuclei and float triglycerides. Infranatants were centrifuged for 20 min at 15,000 g to separate Triton-soluble material from Triton-insoluble pellets. Pellets were washed with 1% Triton X-100 lysis buffer, and the final pellet was resuspended by vortexing and triturating with 60 mM octyl β-D-thioglucopyranoside (OGT), dissolved in 1% Triton X-100 lysis buffer. All samples were normalized for protein before Western analysis. Triton-insoluble and soluble fractions of PM were prepared by incubating isolated PM in 1% Triton X-100 lysis buffer for 30 min rotating at 4°C and then centrifuged at 15,000 × g for 20 min to separate Triton-soluble material from Triton-insoluble pellets.

**TC10 activation assay.** One milliliter of isolated adipocytes at 1 × 10^6 cells/ml was incubated with or without 10 nM insulin for 0–5 min at 37°C in a shaking water bath (100 rpm). Active TC10 levels were measured using a GST pull-down assay kit, following the manufacturer’s instructions. Briefly, cells were lysed using a Mg^2+ lysis buffer (MLB, provided in kit), cellular debris was removed by centrifugation (5 min, 14,000 × g, 4°C), and lysates were incubated with PAK-1.
PBD-coupled agarose beads, which bind GTP-bound GTPases, for 45 min at 4°C with gentle agitation. Complexes were pelleted (10 s, 14,000 g, 4°C), washed three times with MLB, resuspended in 40 μl of 2× Laemmli buffer, and boiled for 5 min. Agarose beads were removed by centrifugation (10 s, 14,000 g), and samples were loaded onto 12% SDS-polyacrylamide gels for Western blot analysis. Quantity of TC10 pulled-down with the PAK-1 PBD-coupled agarose beads was assessed using a polyclonal antibody against TC10. Specific controls (GTP and GDP treated) were generated using lysates from unstimulated adipocytes from pair-fed rats according to manufacturer’s instructions (data not shown).

**Actin polymerization assay.** The quantity of Filamentous actin (F-actin) in cells was assessed using a G-actin/F-actin In Vivo Assay Kit (Catalog no. BK037; Cytoskeleton), following the manufacturer’s recommended protocol except for some adaptations required for working with adipocytes. Briefly, isolated adipocytes were counted, adjusted to a cell density of 1 × 10⁶ cells/ml, and treated with 10 nM insulin for 0–5 min in the presence of 1 μM phalloidin to stabilize existing and insulin-stimulated F-actin. Cells were lysed with the provided lysis buffer and homogenized with eight strokes using 21-gauge syringes at 37°C. The lysates were centrifuged 100,000 g for 60 min at 37°C. The supernatant (containing globular actin) was removed, and the pellets (containing F-actin) were resuspended in 500 μl of water with 2 μM cytochalasin D and incubated on ice for 60 min. To aid in resuspension, samples were sheared using 26 and 5/8th-gauge syringes. All samples were normalized for protein before Western analysis.

**Data analyses.** Because of limitations in the amount of tissue available from each animal, assays were conducted on adipocytes isolated from multiple feeding trials. Values reported are means ± SE. Statistical analyses were performed using either one-way ANOVA and differences between groups determined by the Tukey-Kramer multiple comparisons test (GraphPad InStat, San Diego, CA) or the general linear models program on the SAS statistical package for personal computers, and differences between groups were determined by least square means. Data were log transformed when necessary to produce a normal distribution.

**RESULTS**

Because chronic ethanol feeding impairs insulin-stimulated glucose uptake independently of the PI3K pathway (41), we examined the effects of chronic ethanol feeding on insulin regulation of the Cbl/TC10 pathway. Adipocytes isolated from ethanol- and pair-fed rats were treated with or without 10 nM insulin for 90 s. Cbl was immunoprecipitated from the lysates and Cbl tyrosine phosphorylation measured with an anti-phospho-tyrosine antibody. Insulin rapidly increased the phosphorylation of Cbl (Fig. 1A) in adipocytes isolated from pair-fed rats. Chronic ethanol feeding abrogated insulin-stimulated Cbl phosphorylation (Fig. 1A). Impaired insulin-stimulated Cbl tyrosine phosphorylation was not due to a reduction in Cbl expression, as there was no difference in total Cbl protein between adipocytes from pair- and ethanol-fed rats. The impaired insulin-stimulated tyrosine phosphorylation of Cbl was not due to a more general decrease in the ability of insulin to activate the insulin receptor tyrosine kinase domain, as insulin receptor autophosphorylation was maintained after chronic ethanol feeding (Fig. 1B). Consistent with a specific impairment in Cbl phosphorylation, the PI3K-dependent phosphorylation of Akt/PKB was unaffected by chronic ethanol feeding (Fig. 1B) (41).

Phosphorylated Cbl migrates to the lipid raft microdomains in the PM after insulin stimulation (5, 6, 23). If chronic ethanol decreases Cbl phosphorylation, we would expect to see reduced recruitment of Cbl to the lipid raft. To test this hypothesis, isolated adipocytes were treated with or without 10 nM insulin for 0–1.5 min and lysed. Lysates were subjected to immunoprecipitation (IP) with a polyclonal antibody directed against Cbl. Samples were probed with an anti-phospho-tyrosine (PY100) or anti-Cbl antibody. Representative blots are shown. Graph represents mean values ± SE; n = 4. Open bars, unstimulated; filled bars, insulin stimulated. Values with different letters are significantly different, P < 0.05. B: isolated rat adipocytes were stimulated with or without 10 nM insulin for 0–1.5 min and lysed. Lysates were separated by SDS-PAGE and analyzed with either anti-IRβ (insulin receptor β-subunit), anti-phospho-tyrosine antibody (4G10, for phospho-IRβ), anti-phosphospecific-Akt/PKB, or anti-Akt/PKB.

**Fig. 1.** Chronic ethanol exposure impairs insulin-stimulated Cbl tyrosine phosphorylation. A: isolated rat adipocytes were stimulated with or without 10 nM insulin for 0–1.5 min and lysed. Lysates were subjected to immunoprecipitation (IP) with a polyclonal antibody directed against Cbl. Samples were probed with an anti-phospho-tyrosine (PY100) or anti-Cbl antibody. Representative blots are shown. Graph represents mean values ± SE; n = 4. Open bars, unstimulated; filled bars, insulin stimulated. Values with different letters are significantly different, P < 0.05. B: isolated rat adipocytes were stimulated with or without 10 nM insulin for 0–1.5 min and lysed. Lysates were separated by SDS-PAGE and analyzed with either anti-IRβ (insulin receptor β-subunit), anti-phospho-tyrosine antibody (4G10, for phospho-IRβ), anti-phosphospecific-Akt/PKB, or anti-Akt/PKB.
Insulin-stimulated cCbl translocation to lipid raft is inhibited by chronic ethanol feeding. Isolated adipocytes from pair- and ethanol-fed rats were incubated with or without 10 nM insulin for 0–5 min and lysed. Lysates were subjected to detergent extraction (1% Triton X-100), and Triton-insoluble fractions were solubilized with 60 mM octyl-β-D-thioglucopyranoside (OTG), as described in EXPERIMENTAL PROCEDURES. Fractions were separated by SDS-PAGE and transferred to PVDF membrane for Western blot (IB) analysis using a polyclonal anti-cCbl antibody. Representative blots are shown. Graph represents mean values ± SE; n = 4. Open bars, unstimulated; checkered bars, 2 min of insulin; lined bars, 5 min of insulin. Values with different letters are significantly different, P < 0.01.

To further characterize the effects of chronic ethanol on localization of TC10, PM fractions were prepared from isolated adipocytes and subjected to the Triton X-100 detergent extraction procedure to separate lipid raft and nonlipid raft domains specific to the PM. In the PM fractions, caveolin was enriched in the Triton-insoluble fraction, whereas the insulin receptor was detectable only in the Triton-soluble fraction (Fig. 4), similar to their distribution in the whole cell preparations (Fig. 3). In 3T3-L1 cells overexpressing human or murine TC10 isoforms, TC10 was constitutively resident in lipid rafts (7, 55). However, in primary rat adipocytes, only a relatively small quantity of TC10 was detected in the Triton-insoluble PM (lipid raft) fractions in untreated adipocytes (Fig. 4). There was no difference in baseline quantity or localization of TC10 between pair- and ethanol-fed rats (Fig. 4). Treatment with insulin resulted in a rapid increase in TC10 in the Triton-insoluble PM fraction in adipocytes from pair-fed rats (Fig. 4). In contrast, after chronic ethanol feeding, insulin treatment did not increase the amount of TC10 associated with lipid raft domains in the PM (Fig. 4).

Because insulin-stimulated cCbl and TC10 recruitment to the lipid raft is impaired after chronic ethanol feeding, we would expect to see a loss in insulin-stimulated TC10 activation in ethanol-fed rats. The relative amounts of active/GTP-bound TC10 in pair- and ethanol-fed rats were measured using a GST-PAK-1 PBD pull-down assay kit. Activation of TC10 was increased more than fourfold after 5 min of insulin stimulation in adipocytes from pair-fed rats compared with
unstimulated pair-fed adipocytes (Fig. 5). In contrast, after chronic ethanol feeding, the amount of activated TC10 present in adipocytes not treated with insulin was 13-fold higher than in adipocytes from pair-fed rats despite there being no change in total TC10 protein with chronic ethanol feeding (Fig. 5).

Stimulation with insulin failed to increase the amount of activated TC10 in ethanol-fed rats (Fig. 5). To ensure that a lack of insulin response in ethanol-fed adipocytes was not due to a saturation of the PAK-1 PBD-agarose beads at the high level of activation seen after ethanol feeding, we conducted control experiments using only 1/6th the amount of protein in the pull-down assay. Even with the lower input of protein into the assay, we were unable to detect an increase in GTP-bound TC10 after insulin-stimulation after chronic ethanol feeding (data not shown).

Insulin acts via TC10 to modulate actin polymerization, which is required for insulin-stimulated GLUT4 translocation and glucose uptake (18, 38, 52). Because chronic ethanol exposure affects TC10 recruitment to lipid rafts and dysregulates the activation/inactivation cycle of TC10, we asked whether chronic ethanol feeding also impaired insulin-stimulated actin polymerization. Adipocytes from pair- and ethanol-fed rats were isolated and treated with or without 10 nM insulin for 0–5 min. F-actin was separated from globular actin by differential centrifugation and the quantity of F-actin in each sample was determined by Western analysis. As expected, the quantity of F-actin was low in adipocytes from pair-fed rats, with a rapid increase in F-actin in response to insulin (Fig. 6). In contrast, after chronic ethanol feeding, F-actin quantity was already greater than in cells from pair-fed rats at baseline, and there was no further polymerization after insulin treatment. Increased F-actin after ethanol feeding was not due to increased expression of total actin; rather, chronic ethanol feeding shifted the proportion of F-actin to total actin within the adipocyte. The increase in F-actin at baseline after chronic ethanol feeding is consistent with an increase in active TC10 at baseline in these cells. Additionally, the failure of insulin to further activate TC10 after chronic ethanol feeding parallels the inability of insulin to further stimulate actin polymerization in adipocytes from ethanol-fed rats.

**DISCUSSION**

Chronic ethanol feeding in rats suppresses insulin-stimulated glucose transport in adipocytes (58). This insensitivity to insulin after chronic ethanol is independent of an effect of chronic ethanol on PI3K or Akt/PKB (41). In light of recent studies suggesting the involvement of a second signaling cascade, the Cbl/TC10 pathway, in insulin-stimulated glucose uptake in adipocytes, we hypothesized that this pathway might be a target for chronic ethanol. Here, we report that chronic ethanol feeding suppressed insulin-stimulated cCbl phosphorylation and caused a dysregulation of TC10 activation with an associated inhibition in actin polymerization. Impaired activation of the Cbl/TC10 pathway and actin polymerization in unstimulated pair-fed adipocytes (Fig. 5). In contrast, after chronic ethanol feeding, the amount of activated TC10 present in adipocytes not treated with insulin was 13-fold higher than in adipocytes from pair-fed rats despite there being no change in total TC10 protein with chronic ethanol feeding (Fig. 5).

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Chronic ethanol feeding impaired cCbl recruitment to the lipid raft in isolated adipocytes (Fig. 2), a step required for TC10 activation in response to insulin (6). In studying the lipid raft-associated proteins involved in the Cbl/TC10 pathway, we expected to see TC10 localized in the lipid raft fraction at baseline, as previous studies overexpressing human and murine isoforms of TC10 in 3T3-L1 cells found TC10 to be a resident protein of lipid rafts (55). These studies used confocal microscopy to visualize TC10 localization or extraction in Triton X-100, followed by sucrose density gradients, to demonstrate comigration of TC10 with caveolin-1 (7, 55). In our experiments in isolated adipocytes from pair-fed rats, using a similar detergent extraction method, we found the majority of TC10 to be associated with nonlipid raft fractions, with only a relatively small portion associated with lipid rafts (Figs. 3 and 4). In these primary adipocytes, insulin stimulation caused a rapid recruitment of TC10 into the lipid raft. These differences may be the result of overexpression of exogenous TC10 in the previous studies and/or differences in the localization of endogenous TC10 between 3T3-L1 adipocytes and primary adipocytes. Importantly, recruitment of TC10 to the lipid raft in response to insulin was completely prevented by chronic ethanol (Figs. 3 and 4).

One mechanism by which chronic ethanol could impair recruitment of cCbl and TC10 to the lipid raft would be via a disruption in the integrity of the lipid raft, either in the adipocyte itself and/or during the isolation procedure. However, this mechanism seems unlikely, because the distribution of caveolin (a marker for the lipid raft domain) and the transferrin receptor (a marker for nonlipid raft PM domains) between Triton X-100-soluble and insoluble fractions were not affected by chronic ethanol feeding (Fig. 3). Instead, it is more likely that chronic ethanol disrupts the molecular mechanisms mediating the regulated movement of proteins into and out of the lipid raft. Although it is clear from a number of studies in various cell types that ethanol can disrupt membrane protein trafficking (reviewed in Ref. 36), very little information is available about the potential specific effects of ethanol on recruitment of proteins to specific microdomains, such as lipid rafts, within the PM. Our data suggest that movement of proteins to lipid raft domains in response to insulin may be an important target of ethanol action in adipocytes.

Because chronic ethanol feeding impaired insulin-stimulated cCbl tyrosine phosphorylation as well as the recruitment of cCbl and TC10 to the lipid raft, we expected to see decreased activation of TC10. Surprisingly, we found a dramatic increase in the amount of active TC10 after chronic ethanol feeding in the absence of insulin (Fig. 5). The cycling between the GTP- and GDP-bound forms of a GTPase is critical to its function. A small portion associated with lipid rafts (Figs. 3 and 4). In these studies in other cell types, it is possible that ethanol disrupts the function of APS and/or another required adaptor protein, CAP, thus impairing the ability of the insulin receptor to interact with and phosphorylate cCbl.

Chronic ethanol feeding impaired cCbl tyrosine phosphorylation by the insulin receptor, rather than generally suppressing insulin receptor tyrosine kinase activity. The adaptor protein APS is required for cCbl tyrosine phosphorylation by the insulin receptor. Although chronic ethanol feeding did not change the level of APS expression in adipocytes (Fig. 3), it is possible that ethanol disrupts the function of APS and/or another required adaptor protein, CAP, thus impairing the ability of the insulin receptor to interact with and phosphorylate cCbl.

Fig. 6. Insulin-stimulated filamentous (F-actin) formation is disrupted in adipocytes from chronic ethanol-fed animals. Adipocytes from pair- and ethanol-fed rats were isolated and treated with or without 10 nM insulin for 0–5 min and lysed, and relative amounts of F-actin assessed using an antibody directed against actin. Whole cell lysates were used to determine total actin. Blots are representative of 6 repeats, and graph shown represents mean values ± SE; n = 5–6. Open bars, unstimulated; filled bars, insulin stimulated. Values with different letters are significantly different, P < 0.05.
Numerous studies have shown that the actin cytoskeleton is required for glucose uptake in adipocytes (12, 38, 40, 52). Because the Cbl/TC10 pathway has been implicated in the process of cortical actin reorganization at the PM (7), we hypothesized that actin polymerization might be a downstream event in this pathway targeted by chronic ethanol feeding. After chronic ethanol feeding, F-actin quantity was increased even in the absence of insulin, consistent with the greater quantity of TC10 found in the active/GTP-bound form (Figs. 5 and 6). Furthermore, insulin-stimulated actin polymerization was inhibited by chronic ethanol exposure (Fig. 6), again consistent with impaired activation of TC10 by insulin in adipocytes from ethanol-fed rats. These data are consistent with a recent study showing that constitutively active TC10 completely disrupts adipocyte cortical actin when expressed in 3T3-L1 cells (21).

A number of studies describe the development of insulin resistance during chronic ethanol exposure in humans and rodent models (9, 19, 39, 46, 51). Although chronic ethanol feeding suppresses insulin-stimulated glucose transport in isolated rat adipocytes (41, 58), it is not yet known whether impaired glucose transport in adipocytes contributes to insulin resistance in the intact organism. Recently, two groups have carried out hyperinsulinemic euglycemic clamp studies in rats chronically exposed to ethanol (39, 51). Both groups found that chronic ethanol exposure both increased hepatic insulin resistance, and decreased glucose utilization rates. However, neither study addressed the specific contributions of adipose tissue and/or skeletal muscle to impaired glucose disposal in vivo after chronic ethanol feeding. Despite the relatively minor contribution of adipose tissue relative to skeletal muscle in mediating whole body glucose disposal, studies in transgenic animals indicate that glucose transport capacity in adipose tissue can make significant contributions to whole body insulin sensitivity. For example, adipocyte-specific GLUT4−/− mice develop insulin resistance and glucose intolerance (32), whereas mice with adipose-specific overexpression of GLUT4 have enhanced insulin sensitivity (47). These studies suggest that impaired insulin-stimulated glucose uptake by adipose can contribute to a secondary insulin resistance in both skeletal muscle and liver (32). Studies are currently underway to determine the impact of impaired insulin-stimulated glucose uptake in adipocytes on in vivo insulin sensitivity in rats chronically fed ethanol.

In summary, we have identified the Cbl/TC10 pathway as a target for chronic ethanol action in rat adipocytes. Chronic ethanol feeding decreased insulin-stimulated tyrosine phosphorylation of c-Cbl, disrupted the recruitment of c-Cbl and TC10 to lipid raft microdomains, caused a marked increase in GTP-bound (active state) TC10, and inhibited insulin-stimulated actin polymerization. These data identify the Cbl/TC10 pathway as a specific target of ethanol action and suggest an association between disruption of the Cbl/TC10 pathway under pathophysiological situations, such as chronic alcohol consumption, and impaired insulin-stimulated glucose uptake in adipocytes.

**GRANTS**

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**REFERENCES**


