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Chronic ethanol feeding impairs endothelin-1-stimulated glucose uptake via decreased $G\alpha_{11}$ expression in rat adipocytes

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Rachdaoui, Nadia, Becky M. Sebastian, and Laura E. Nagy. Chronic ethanol feeding impairs endothelin-1-stimulated glucose uptake via decreased $G\alpha_{11}$ expression in rat adipocytes. *Am J Physiol Endocrinol Metab* 285: E303–E310, 2003. First published April 8, 2003; 10.1152/ajpendo.00547.2002.—Chronic ethanol feeding decreases insulin-stimulated glucose uptake in rat adipocytes. Here, we show that chronic ethanol also decreases endothelin-stimulated glucose uptake. Endothelin-1 increased uptake of 2-deoxyglucose 2.4-fold in adipocytes isolated from pair-fed rats. However, in adipocytes isolated from rats that had consumed a diet containing 35% ethanol for 4 wk, endothelin-1 did not increase glucose uptake. Although endothelin-1 increased GLUT4 quantity at the plasma membrane in adipocytes from pair-fed rats, there was no increase in GLUT4 after chronic ethanol feeding. Loss of endothelin-1-stimulated glucose uptake after ethanol feeding was associated with a specific decrease in the quantity of $G\alpha_{11}$ in plasma membranes, with no change in $G\alpha_q$ quantity. Activation of proline-rich tyrosine kinase 2 (PYK2), a downstream target of $G\alpha_{q/11}$ that is required for endothelin-1-stimulated GLUT4 translocation in 3T3-L1 adipocytes, was also suppressed after chronic ethanol feeding. In contrast, activation of p38 MAPK by endothelin-1 was not affected by chronic ethanol exposure. These data demonstrate that chronic ethanol feeding suppresses endothelin-1-stimulated glucose uptake and suggest that decreased expression of $G\alpha_{11}$ coupled to impaired endothelin-1-dependent activation of PYK2 contributes to this response.

glucose transporter-4; insulin; G proteins; proline-rich tyrosine kinase 2; p38 mitogen-activated protein kinase

THE RATE-LIMITING STEP for glucose disposal in adipose and muscle is transport of glucose across the plasma membrane. Insulin-stimulated glucose transport in these tissues is mediated by the translocation of GLUT4, the insulin-recruitable isoform of the facilitative glucose transporter family, from an intracellular vesicular compartment to the plasma membrane (29). Insulin-stimulated GLUT4 translocation and glucose transport are dependent on the activation of phosphatidylinositol 3-kinase (PI 3-kinase) and phosphorylation of Akt (23). Although insulin is the predominant signal regulating glucose transport in adipocytes, several lines of evidence indicate that the PI 3-kinase pathway alone is not sufficient to stimulate glucose transport. For example, other agonists that activate PI 3-kinase in adipocytes, such as interleukin 4, do not stimulate glucose transport (14). Recent work has identified a second insulin-stimulated signaling pathway, involving activation of the small GTPase TC10 that, along with PI 3-kinase-dependent signaling, is required for GLUT4 translocation in adipocytes (3, 6). Activation of p38 mitogen-activated protein kinase (MAPK) also contributes to insulin-stimulated glucose transport (26, 27, 30). Studies utilizing pyridinyl imidazole inhibitors of p38 MAPK (e.g., SB-203580) indicate that p38 MAPK activation enhances insulin-stimulated transport activity of GLUT4 without affecting GLUT4 translocation (26, 30).

Recent evidence also implicates the action of $G\alpha_{q/11}$ in mediating both insulin (13, 15) and endothelin-1-stimulated (12, 21) glucose uptake in 3T3-L1 adipocytes. $G\alpha_{11}$, a member of the Gq family of heterotrimeric GTP-binding proteins, is highly expressed in adipocytes (5). Activation of endothelin A receptors by the vasoactive peptide endothelin-1 potently stimulates glucose transport in 3T3-L1 adipocytes via activation of $G\alpha_{q/11}$ (12, 36). Most studies find that endothelin-1-stimulated glucose uptake is independent of PI 3-kinase activity (5, 16, 36), although one report indicates that PI 3-kinase is involved (12). Instead, endothelin-1 increases glucose uptake via a mechanism that requires tyrosine phosphorylation of a number of effector proteins, including $G\alpha_{q/11}$ and the *src* family tyrosine kinase Yes, as well as the tyrosine kinase proline-rich tyrosine kinase 2 (PYK2) and the scaffolding protein paxillin (11, 21). The GTPase ADP ribosylation factor 6 and cortical actin polymerization also contribute to $G\alpha_{q/11}$-mediated GLUT4 translocation (5).

Short- and long-term ethanol exposure is associated with impaired glucose utilization. Ethanol consumption increases circulating glucose concentrations (8), glucose intolerance, and insulin resistance (25, 38), and chronic heavy alcohol consumption is an independent risk factor for the development of type 2 diabetes in some populations (9, 32, 34). However, the mechanisms for this disruption of glucose homeostasis by ethanol are not well understood. Adipocytes isolated from rats fed ethanol as part of the high-fat Lieber-DeCarli diet for 4 wk have impaired insulin-stimulated glucose uptake (22, 35). In many conditions, such as

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obesity, high-fat diets, or exposure to TNF-α, suppression of insulin-stimulated glucose transport is associated with impaired insulin receptor-dependent activation of PI 3-kinase (17, 24). However, chronic ethanol feeding was not associated with impaired insulin signaling to PI 3-kinase and Akt in isolated adipocytes (22). These data suggest that chronic ethanol targets alternative, PI 3-kinase-independent signaling pathways to suppress insulin-stimulated glucose transport. Because endothelin-1 stimulates glucose transport via mechanisms that are independent of PI 3-kinase (5, 16), we tested the hypothesis that chronic ethanol feeding impairs endothelin-1-stimulated glucose uptake and investigated the effects of chronic ethanol exposure on the signaling pathways contributing to endothelin-1-mediated glucose uptake in adipocytes.

MATERIALS AND METHODS

Materials

Male Wistar rats (150–160 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The Lieber-DeCarli ethanol diet was from Dyets (Bethlehem, PA). Antibodies were obtained from the following sources: rabbit polyclonal anti-GLUT4 (Biogenesis, Sandown, NH), anti-$G\alpha_s$ (Calbiochem, La Jolla, CA), anti-$G\alpha_{q/11}$ (Santa Cruz Technology, Santa Cruz, CA), anti-PYK2 (BD Transduction Labs, San Diego, CA), phosphospecific PYK2 antibodies (Biosource International, Camarillo, CA), phosphospecific Akt and Akt antibodies (Cell Signaling Technology, Beverly, MA), and monoclonal anti-phosphotyrosine (PY20; BD Transduction Labs). Goat anti-rabbit or anti-mouse IgG (Fab fragment) coupled to horseradish peroxidase and adenosine deaminase were from Roche (Indianapolis, IN). 2-Deoxy-[^{3}H]glucose was from Amersham (Arlington Heights, IL). Insulin and endothelin-1 were from Sigma (St. Louis, MO). All cell culture reagents were from GIBCO (Grand Island, NY).

Methods

Animal care and feeding. Rats were housed in individual cages in a temperature- and humidity-controlled room with a 12:12-h light-dark cycle. Animals were acclimatized for 3 days after arrival and provided with free access to Purina rat chow and water. Animals were then allowed free access to liquid diet (18) without ethanol for 2 days and then randomly assigned to the ethanol-fed or pair-fed groups. The ethanol-fed group was allowed free access to liquid diet with 17% of calories as ethanol for 2 days and then provided with diet containing 35% of calories from ethanol for 4 wk. Controls were pair fed a liquid diet that was identical to the ethanol diet except that maltose dextrins were isocalorically substituted for ethanol. Control diets had 18% of calories as protein, 35% as fat, and 47% as carbohydrates compared with the ethanol diets containing 18% protein, 35% fat, 12% carbohydrate, and 35% ethanol (18). Pair-fed rats were given the same amount of food that their ethanol-fed pair had consumed in the preceding 24 h. Procedures involving animals were approved by the Institutional Animal Care Board at Case Western Reserve University.

Uptake of 2-deoxy-[^{3}H]glucose. After the 4-wk feeding period, animals were anesthetized by intraperitoneal injection with pentothal sodium (0.2 ml/100 g), and epididymal fat pads were removed. Adipocytes were isolated by collagenase digestion as previously described (35), counted, and diluted to 5×10^5 cells/ml in phosphate-buffered saline with 1 mM $MgCl_2$, 0.68 mM $CaCl_2$, pH 7.4, 1 mg/ml BSA, 1 mM pyruvic acid, and 1 U/ml adenosine deaminase (incubation buffer). Adipocytes were stimulated with and without 10 nM endothelin-1 or 10 nM insulin for 30 min, and uptake of 2-deoxy-[1,2-^{3}H]glucose (final concentration 2.5 mM, 0.5 μCi/tube) was measured over 3 min for 2-deoxyglucose (35). In some experiments, adipocytes were pretreated or not with 5 μM SB-203580 for 15 min before being stimulated with endothelin-1. Nonspecific uptake was measured in the presence of 10 mM phloretin.

Insulin- and endothelin-1-stimulated PYK2 and p38 MAPK phosphorylation. Isolated adipocytes (~2×10^6 cells/ml) were treated with or without insulin or endothelin-1 for 2–10 min. For analysis of PYK2 tyrosine phosphorylation, cells were lysed for 15 min at 4°C in 10× lysis buffer [for a final concentration of 1% Nonidet P-40 and 1% Triton X-100 in 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, and protease inhibitors (Complete; Boehringer Mannheim) and phosphatase inhibitors (1 mM Na vanadate, 20 mM Na pyrophosphate, 100 mM NaF)]. Lysates were vortexed briefly and centrifuged for 2 min, and the infranatant below the fat cake was removed with a syringe. Samples were then normalized for protein content and separated by SDS-PAGE for Western blotting. For analysis of p38 MAPK phosphorylation, cells were lysed directly in Laemmli sample buffer, boiled for 5 min, and separated by SDS-PAGE for Western blot analysis.

Isolation of subcellular fractions. For the measurement of G protein quantity, isolated adipocytes were homogenized in 20 mM Tris, pH 7.4, 1 mM EDTA, and 255 mM sucrose with protease inhibitors (homogenizing buffer) by use of a Wheaton glass homogenizer with a tight-fitting pestle (clearance 0.05 μm). A plasma membrane-enriched fraction was prepared by centrifugation of homogenates at 16,000 *g* for 15 min. The protein content of the 16,000-*g* pellet was measured, and equal quantities were separated by either 10% polyacrylamide (for $G\alpha_s$) or 12.5% polyacrylamide ($G\alpha_{q/11}$) SDS-PAGE for Western blotting. Recovery of $G\alpha_q$ in the pellet compared with supernatant was followed to ensure complete recovery of plasma membranes; all immunoreactive $G\alpha_q$ was found in the 16,000-*g* pellet (data not shown).

To determine the subcellular localization of GLUT4, isolated adipocytes (1×10^6 cells) were incubated with or without 100 nM insulin or 10 nM endothelin-1 for 30 min at 37°C. Reactions were terminated by the addition of 2 mM KCN, and adipocytes were homogenized as described. Purified plasma membrane and low-density microsomes were isolated by differential centrifugation as previously described (22). In some experiments, plasma membrane-enriched fractions, isolated as described for measuring G protein quantity, were used to assess GLUT4 and GLUT1 translocation in response to endothelin-1 and insulin. Recovery of syntaxin 4, a plasma membrane protein, was followed to ensure complete recovery of plasma membranes (data not shown).

Western blotting. PVDF membranes were blocked with 5% nonfat dry milk or 3% BSA in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl) containing 0.1% Tween (TBS-T) for 2 h, washed twice with TBS-T, and then incubated with primary antibody overnight at 4°C. Membranes were washed again and probed with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse IgG Fab fragments for 1 h. Bound antibody was visualized using enhanced chemiluminescence reagent. Immunoreactive protein quantity was assessed by scanning densitometry; film exposure times were in the linear range of detectability. After probing for phosphorylated

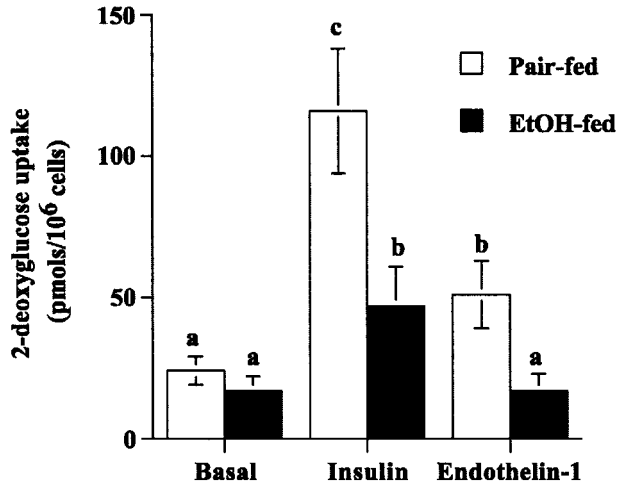

Fig. 1. Chronic ethanol (EtOH) feeding decreases insulin- and endothelin-1 (ET-1)-stimulated glucose transport in isolated adipocytes. Adipocytes were isolated from pair- and EtOH-fed rats. Adipocytes were stimulated or not with 10 nM insulin or 10 nM ET-1 for 30 min, and uptake of 2-deoxy-[^{3}H]glucose was measured over 3 min. Values represent means ± SE; n = 7–10. Values with different letters are significantly different ($P < 0.05$).

p38 or phosphospecific PYK2, membranes were stripped and reprobed with antibodies to total p38 or another phosphospecific PYK2 form. Total PYK2 was not well detected in stripped membranes and so was measured on separate membranes rather than on the same membranes as the phosphorylated forms of PYK2.

Statistical Analysis

Because of limitations in the amount of tissue available from each animal, assays were conducted on adipocytes isolated from multiple feeding trials. Each trial involved six rats per dietary treatment; adipocytes from two rats were pooled for isolation of purified plasma membrane and low-density microsomes. Values reported are means ± SE. Data were analyzed by Student's t-test or the general linear models program on the SAS statistical package for personal computer. Differences between groups were determined by least square means. Data were log transformed when necessary to produce a normal distribution.

RESULTS

We have previously reported that chronic ethanol feeding decreases insulin-stimulated glucose uptake in isolated adipocytes (22, 35). Interestingly, chronic ethanol-induced insulin resistance is not associated with impaired insulin-stimulated activation of PI 3-kinase or Akt (22). Because endothelin-1 stimulates glucose uptake in 3T3-L1 adipocytes in a PI 3-kinase-independent mechanism (36), here we have asked whether chronic ethanol feeding also impairs endothelin-1-stimulated glucose uptake. When adipocytes isolated from pair-fed control rats were stimulated with 10 nM endothelin-1 for 30 min, uptake of 2-deoxyglucose was increased 2.4-fold (Fig. 1). Ten nanomolar insulin increased 2-deoxyglucose uptake 6.2-fold over basal (Fig. 1). The stimulation of glucose uptake by endothelin-1 and by submaximal concentrations of insulin for 10 min was additive in adipocytes isolated from control rats. Uptake of 2-deoxyglucose in nonstimulated cells (n = 4–5) was 3.1 ± 1.0, 13.9 ± 3.7 after treatment with 10 nM endothelin-1, 11.6 ± 3.1 after treatment with 0.05 nM insulin, and 23.0 ± 7.4 after treatment with 10 mM endothelin-1 and 0.05 nM insulin together. All of these values differ significantly from each other ($P < 0.05$). In contrast, after chronic ethanol feeding, endothelin-1 did not stimulate glucose uptake, and insulin-stimulated glucose uptake was reduced by 60% compared with pair-fed rats (Fig. 1).

Both insulin and endothelin-1 increased GLUT4 protein at the plasma membrane in adipocytes isolated from pair-fed rats (Fig. 2). Insulin stimulation resulted in a more robust recruitment of GLUT4 to the plasma membrane compared with endothelin-1 (Fig. 2*A*), consistent with the greater increase in glucose uptake observed in response to insulin compared with endothelin-1 treatment. Stimulation with endothelin-1 decreased immunoreactive GLUT4 in low-density microsomes in pair-fed rats to 88 ± 8% of basal (Fig. 2*B*).

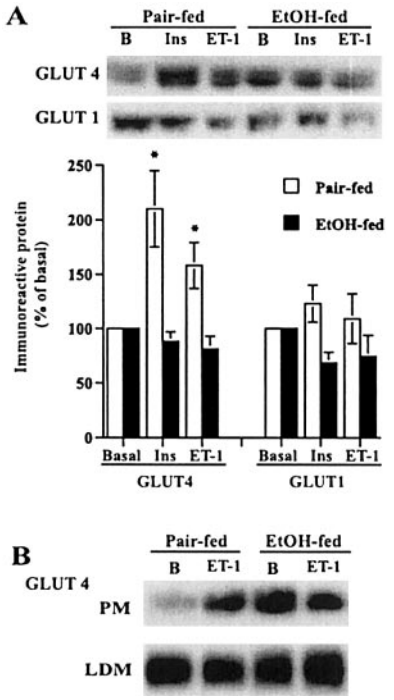

Fig. 2. Insulin- and ET-1-stimulated translocation of GLUT4 and GLUT1 to plasma membranes (PM) in adipocytes from pair- and EtOH-fed rats. Adipocytes were isolated from pair- and EtOH-fed rats and then stimulated or not with 100 nM insulin (Ins) or 10 nM ET-1 for 30 min. *A*: PM enriched fractions were isolated and used to quantify immunoreactive GLUT4 and GLUT1 by Western blotting. Immunoreactive GLUT4 at baseline (B) was 194 ± 36 (n = 9) in pair-fed and 271 ± 51 (n = 7) in EtOH-fed rats ($P < 0.05$). Immunoreactive GLUT1 at baseline was 90 ± 14 (n = 9) in pair-fed and 125 ± 23 (n = 5) in EtOH-fed rats. Values represent means ± SE. *$P < 0.05$ compared with basal. *B*: purified PM and low-density microsomes (LDM) were isolated as described (24), and GLUT4 quantity was measured by Western blot. Immunoreactive GLUT4 in PM was increased 1.55 ± 0.35-fold by ET-1 stimulation in pair-fed rats (n = 7, $P < 0.05$) but was not affected by ET-1 after EtOH feeding. Immunoreactive GLUT4 in LDM was decreased to 0.88 ± 0.08% of basal in pair-fed rats (n = 5) but did not change after EtOH feeding.

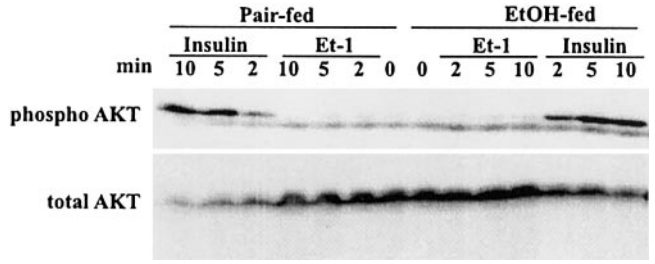

Fig. 3. Insulin, but not ET-1, increases phosphorylation of Akt (AKT). Adipocytes were isolated from pair- and EtOH-fed rats and then stimulated or not with 100 nM insulin or 10 nM ET-1 for 0–15 min. Cells were lysed as described in *Methods* and phosphorylated, and total Akt was visualized by Western blots. Figure is representative of experiments performed in 3 pairs of animals.

GLUT4 in high-density microsomes was not affected by endothelin-1 treatment (data not shown). We have previously reported (23) that chronic ethanol feeding decreases total GLUT4 expression in adipocytes by 30% compared with adipocytes from pair-fed rats. After chronic ethanol feeding, GLUT4 at the plasma membrane in nonstimulated cells (basal) was higher (271 ± 51 units of arbitrary density, $n = 7$) compared with adipocytes from pair-fed rats (194 ± 36, $n = 9$; Fig. 2*A*). Moreover, neither insulin nor endothelin-1 increased GLUT4 protein associated with the plasma membrane after ethanol feeding (Fig. 2, *A* and *B*). GLUT1 content in the plasma membrane did not change in response to treatment either with hormone or with chronic ethanol feeding (Fig. 2*A*).

Although the signal transduction cascade leading from activation of adipocytes with endothelin-1 to increased glucose transport is not completely understood, data from several groups indicate that endothelin-1-stimulated glucose transport in 3T3-L1 adipocytes is independent of PI 3-kinase (5, 16). Insulin rapidly increased the phosphorylation of Akt in adipocytes from pair-fed rats. However, endothelin-1 did not stimulate the phosphorylation of Akt (Fig. 3). After chronic ethanol feeding, insulin-stimulated Akt phosphorylation was normal (Fig. 3), consistent with our previous data showing that chronic ethanol feeding is not associated with impaired activation of PI 3-kinase or Akt by insulin (22). Taken together, these data suggest that endothelin-1-stimulated glucose uptake is independent of the PI 3-kinase/Akt-signaling pathway and, furthermore, that chronic ethanol feeding does not impair insulin-dependent activation of Akt.

The heterotrimeric G protein $G\alpha_{11}$ is a critical mediator of endothelin-1-stimulated glucose uptake (12, 21). $G\alpha_{11}$ and $G\alpha_q$ have also been implicated as modulators of insulin-stimulated glucose uptake (13, 15). Therefore, we investigated the effect of chronic ethanol feeding on expression of $G\alpha_{q/11}$ proteins in rat adipocytes. Immunoreactive quantities of $G\alpha_s$, $G\alpha_{11}$, and $G\alpha_q$ were measured by Western blot in plasma membrane enriched fractions prepared from adipocytes isolated from pair- and ethanol-fed rats. Although the quantity of $G\alpha_s$ increased twofold after chronic ethanol feeding (Fig. 4) (22, 35), the quantity of $G\alpha_{11}$ was decreased to 30% after chronic ethanol feeding compared with pair-fed animals (Fig. 4). There was no effect of chronic ethanol feeding on $G\alpha_q$ expression (Fig. 4).

The downstream elements in the endothelin-1/$G\alpha_{q/11}$ pathway leading to glucose uptake in adipocytes are not completely understood. Tyrosine phosphorylation of $G\alpha_{q/11}$ (11) as well as that of tyrosine kinase PYK2 and the scaffold protein paxillin (21) is increased in response to endothelin-1 stimulation in 3T3-L1 adipocytes. A dominant negative construct of PYK2 [calcium-dependent protein kinase-related nonkinase (CRNK)] inhibits endothelin-1-stimulated GLUT4 translocation in 3T3-L1 adipocytes, demonstrating a functional role for this kinase in endothelin-1-stimulated glucose uptake (21). We hypothesized that, if the chronic ethanol-induced decrease in $G\alpha_{11}$ contributes to impaired endothelin-1-stimulated glucose uptake after chronic ethanol, then endothelin-1-stimulated tyrosine phosphorylation of PYK2 should also be decreased after chronic ethanol feeding. We first characterized the effects of insulin and endothelin-1 on PYK2 tyrosine phosphorylation in adipocytes isolated from control rats. Insulin rapidly increased the tyrosine phosphorylation of proteins migrating at the apparent molecular weights of insulin receptor substrate-1 and the insulin receptor β-subunit (Fig. 5). Endothelin-1 did not stimulate tyrosine phosphorylation of these peptides (Fig. 5). Using antibodies specific for Tyr^{402} and Tyr^{881} of PYK2, we show that endothelin-1, but not insulin, increased tyrosine phosphorylation of PYK2 (Fig. 5). Both insulin and endothelin-1 stimulated p38 MAPK phosphorylation, but the response to endothelin-1 was more robust than the response to insulin (Fig. 5).

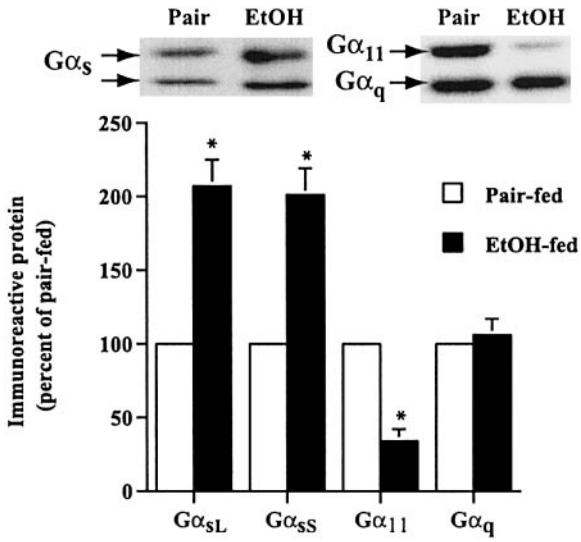

Fig. 4. Chronic EtOH feeding decreases expression of $G\alpha_{11}$ in adipocyte PM. Adipocytes were isolated from pair- and EtOH-fed rats. PM enriched fractions were then prepared as described in *Methods*, and expression of $G\alpha_s$ and $G\alpha_{q/11}$ was measured by Western blot. Antibody to $G\alpha_s$ recognized both the short ($G\alpha_{sS}$) and the long form ($G\alpha_{sL}$) of $G\alpha_s$. Antibody to $G\alpha_{q/11}$ recognized both $G\alpha_{11}$ (apparent molecular mass 43 kDa) and $G\alpha_q$ (apparent molecular mass 42 kDa). Figure shows a representative Western blot ($n = 7$, $^* P < 0.05$ compared with pair fed).

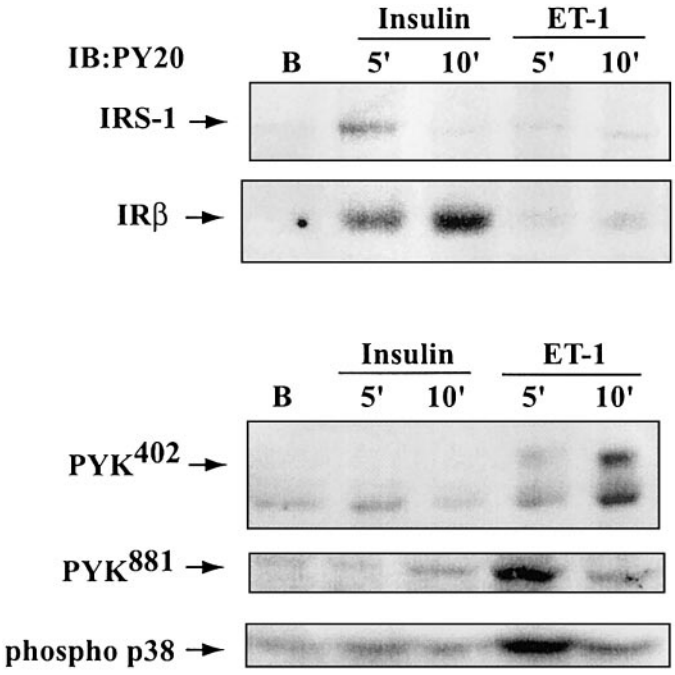

Fig. 5. Insulin- and ET-1-stimulated tyrosine phosphorylation and p38 MAPK activation in rat adipocytes. Adipocytes were isolated from control rats and then stimulated or not with 100 nM insulin or 10 nM ET-1 for 0–15 min. Cells were lysed, and tyrosine phosphorylated proteins were visualized with anti-phosphotyrosine antibody (PY20), antibodies to specific tyrosine phosphorylation sites on proline-rich tyrosine kinase 2 (PYK2), or antibodies to phosphorylated p38. Figure is representative of $n = 3$. IB, immunoblot; IR, insulin receptor; IRS-1, IR substrate-1.

Adipocytes isolated from pair- and ethanol-fed rats were stimulated or not with 10 nM endothelin-1, and tyrosine phosphorylation of PYK2 was measured using phosphospecific antibodies. Tyr^{402} is an autophosphorylation site and a target for interaction with the *src* homology 2 (SH2) domain of Src family kinases. Activated Src, in turn, phosphorylates PYK2-Tyr^{881}, allowing for the association of SH2 domains of adaptor proteins such as Grb2 (4). Phosphorylation of PYK2 at Tyr^{579} and Tyr ^{580} is required for maximal PYK2 activity (4). Endothelin-1 rapidly stimulated the tyrosine phosphorylation of all four tyrosine residues in adipocytes isolated from pair-fed rats (Fig. 6). In contrast, endothelin-1 did not increase the tyrosine phosphorylation of PYK2 at any of the four sites in adipocytes isolated from chronic ethanol-fed rats (Fig. 6). Total quantity of PYK2 was decreased to 64 ± 33% ($n = 5$) in adipocytes from ethanol-fed rats compared with pair-fed rats (Fig. 6).

Activation of endothelin-1 receptor or $G\alpha_{q/11}$ stimulates p38 MAPK in a variety of cell types (10, 37). Furthermore, p38 MAPK activity has been implicated in mediating full activation of glucose transport in response to insulin (2, 26, 27, 30). Here, we show that pretreatment of rat adipocytes with 5 μM SB-203580, an inhibitor of p38 MAPK activation, suppressed endothelin-1-stimulated glucose uptake in adipocytes from pair-fed rats (Fig. 7*A*). In this experiment, chronic ethanol feeding decreased endothelin-1-stimulated glucose uptake compared with pair-fed rats but was not further decreased by pretreatment with SB-203580 (Fig. 7*A*). Endothelin-1 stimulated the phosphorylation of p38 MAPK in adipocytes isolated from pair-fed rats over 5–15 min (Fig. 7*B*). We hypothesized that, if activation of p38 MAPK by endothelin-1 was suppressed after chronic ethanol feeding similarly to the decrease in PYK2 activation, this could also contribute to impaired endothelin-1-stimulated glucose transport. However, chronic ethanol feeding had no effect on either endothelin-1-stimulated phosphorylation of p38 or total p38 expression (Fig. 7*B*).

DISCUSSION

Endothelin-1 stimulates GLUT4 translocation to the plasma membrane and increases glucose uptake in 3T3-L1 adipocytes via a mechanism that involves the heterotrimeric G protein $G\alpha_{11}$ (12, 15). Here, we show that endothelin-1 also increases GLUT4 content at the plasma membrane and stimulates glucose uptake in isolated rat adipocytes. We have found that chronic ethanol feeding to rats impairs endothelin-1-stimulated glucose transport in isolated adipocytes. This suppression of endothelin-1-mediated glucose transport was associated with decreased expression of $G\alpha_{11}$ after chronic ethanol feeding as well as impaired ty-

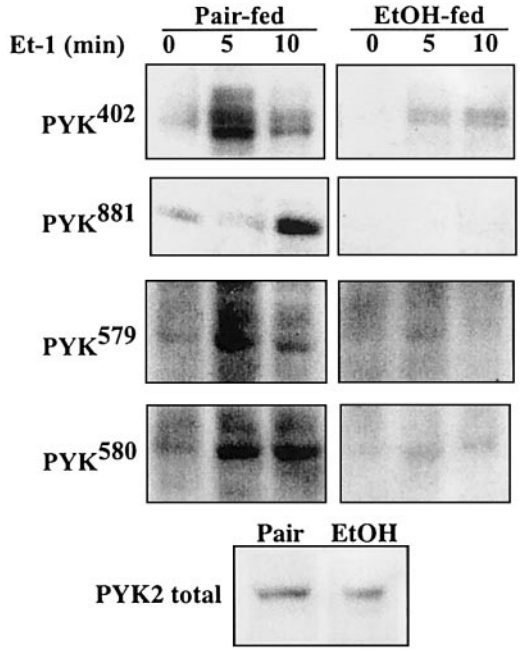

Fig. 6. Chronic EtOH feeding impairs ET-1-stimulated tyrosine phosphorylation of PYK2 in adipocytes. Adipocytes were isolated from pair- and EtOH-fed rats and then stimulated or not with 10 nM ET-1 for 0–10 min and lysed. Lysates were then used for Western blotting with phosphospecific PYK2 antibodies. Figure shows representative Western blots. Means ± SE for fold increase over basal ($n = 4$–6) for pair-fed and EtOH-fed rats, respectively, were PYK^{402}: 1.9 ± 0.07 and 0.84 ± 0.21 at 5 min and 1.9 ± 0.4 and 0.92 ± 0.18 at 10 min; PYK^{881}: 2.50 ± 0.67 and 0.90 ± 0.19 at 5 min and 2.3 ± 0.6 and 1.1 ± 0.2 at 10 min; PYK^{579}: 2.05 ± 0.8 and 1.0 ± 0.33 at 5 min; PYK^{580}: 1.82 ± 0.47 and 1.18 ± 0.42 at 5 min.

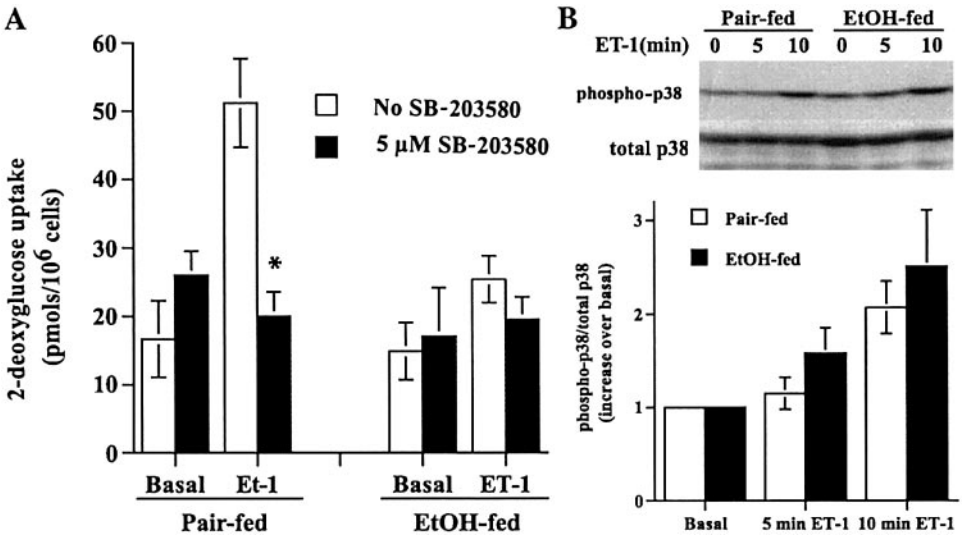

Fig. 7. ET-1 activation of p38 MAPK in isolated rat adipocytes. *A*: adipocytes were isolated from pair- and EtOH-fed rats and pretreated with 5 μM SB-203580 for 15 min and then stimulated or not with 10 nM ET-1 for 30 min. Uptake of 2-deoxy-[^{3}H]glucose was measured over 3 min. Values represent means ± SE; $n = 5–6$. *$P < 0.05$ compared with cells not treated with SB-203580. *B*: chronic EtOH feeding does not impair ET-1-stimulated phosphorylation of p38 MAPK. Adipocytes were isolated from pair- and EtOH-fed rats and then stimulated or not with 10 nM ET-1 for 0–10 min and lysed. Lysates were then used for Western blotting with phosphospecific or total p38 antibodies. Values represent means ± SE; $n = 7$.

rosine phosphorylation of PYK2, a tyrosine kinase required for endothelin-1-stimulated glucose uptake in 3T3-L1 adipocytes (21).

Chronic ethanol feeding also decreases insulin-stimulated glucose uptake (22, 35). However, impaired insulin stimulation of glucose transport is not associated with impaired activation of PI 3-kinase or Akt (22). This suggests that chronic ethanol impairs a PI 3-kinase-independent signaling pathway that contributes to insulin-stimulated glucose transport. One such potential target for chronic ethanol action is the activation of the heterotrimeric G protein $G\alpha_{q/11}$. Activation of $G\alpha_{q/11}$ in response to insulin or endothelin-1 or by overexpression of constitutively active $G\alpha_{11}$ (Q209L) or $G\alpha_q$ (Q209L) increases glucose uptake in 3T3-L1 adipocytes (5, 12, 13, 15, 16). $G\alpha_{q/11}$ also contributes to both endothelin-1- (12) and insulin-mediated glucose transport (13, 15). Several reports have found that the contribution of $G\alpha_{q/11}$ to glucose transport is independent of activation of PI 3-kinase (5, 15, 16, 36), although the involvement of PI 3-kinase remains controversial (12). We have found that chronic ethanol exposure specifically decreases expression of $G\alpha_{11}$ in isolated rat adipocytes. In contrast to the decrease in $G\alpha_{11}$, chronic ethanol feeding increases expression of $G\alpha_s$ (35) and has no effect on $G\alpha_q$ expression. Chronic ethanol exposure regulates expression of heterotrimeric G proteins; the individual G protein family members affected by chronic ethanol are cell type specific (7). Here, we have found that ethanol feeding results in a specific decrease in expression of $G\alpha_{11}$ in adipocytes. Decreased $G\alpha_{11}$ likely contributes to impaired insulin- and endothelin-1-stimulated glucose transport after chronic ethanol exposure.

The downstream elements in the $G\alpha_{q/11}$ pathway leading to glucose uptake in adipocytes are not completely understood. The available data suggest that insulin- or endothelin-1-stimulated $G\alpha_{q/11}$ activation may function via distinct signaling pathways. For example, the tyrosine kinase PYK2 is essential for endothelin-1 stimulation of glucose uptake in 3T3-L1 adipocytes (21). A dominant negative construct of PYK2 (CRNK) inhibits endothelin-1- but not insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (21). In isolated rat adipocytes, endothelin-1, but not insulin, increased tyrosine phosphorylation of PYK2 (Fig. 5), consistent with the response in 3T3-L1 adipocytes (21). However, after chronic ethanol feeding, endothelin-1 no longer increased tyrosine phosphorylation of PYK2 in isolated rat adipocytes. These results suggest that the reduction in $G\alpha_{11}$ expression after chronic ethanol impairs endothelin-1 activation of PYK2, a required intermediate in endothelin-1-stimulated glucose uptake.

In contrast to impaired endothelin-1-stimulated PYK2 activation, chronic ethanol feeding had no effect on endothelin-1-stimulated p38 MAPK phosphorylation in isolated rat adipocytes. p38 MAPK is another downstream kinase activated in response to endothelin-1 and insulin (10, 26, 30). Activation of p38 MAPK via G protein-coupled receptors involves activation of $G\alpha_{q/11}$ (19, 37). PYK2 is an intermediate in endothelin-1-mediated activation of p38 MAPK in some, but not all, cell types (28). For example, G protein-coupled receptor activation of MAPK is similar in mouse embryonic fibroblasts from wild-type and *pyk2*$^{-/-}$ mice (1). Because chronic ethanol feeding did not impair endothelin-1-stimulated p38 MAPK activation despite a suppression in PYK2 activation, PYK2 does not appear to be involved in p38 MAPK activation by endothelin-1 in rat adipocytes.

p38 MAPK activity has been implicated in mediating insulin-stimulated glucose transport activity in 3T3-L1 adipocytes, L6 myotubes (26, 30), and skeletal muscle (27). Here, we report that inhibition of p38 MAPK activity by pretreatment with SB-203580 also suppresses endothelin-1-stimulated glucose uptake (Fig. 7*A*). Studies utilizing chemical inhibitors of p38 MAPK suggest that p38 MAPK activation leads to an increase in the catalytic/transport activity of GLUT4 rather than GLUT4 translocation to the plasma membrane (26, 30). After chronic ethanol feeding, GLUT4 content

at the plasma membrane was higher in nonstimulated (basal) adipocytes compared with cells from pair-fed rats. Although endothelin-1 increased GLUT4 at the plasma membrane in adipocytes from pair-fed rats, endothelin-1 did not increase plasma membrane GLUT4 in adipocytes from ethanol-fed rats above baseline. We have previously demonstrated (22, 35) that chronic ethanol feeding impairs the accessibility of GLUT4 at the plasma membrane. Thus, despite the sustained ability of endothelin-1 to activate p38 MAPK, it is unlikely that p38 MAPK could stimulate glucose transport because of a decreased quantity and/or accessibility of GLUT4 at the cell surface after chronic ethanol feeding. Instead, decreased activation of PYK2 after chronic ethanol is more likely an important contributor to impaired endothelin-1-stimulated GLUT4 translocation and glucose uptake. PYK2 is required for the formation of cortical F-actin in response to endothelin-1, a required step for GLUT4 translocation (20, 31, 33). Thus it is possible that impaired PYK2 activation after chronic ethanol may lead to abnormal formation of cortical F-actin and impaired GLUT4 translocation to the plasma membrane.

Chronic ethanol feeding impairs both insulin- and endothelin-1-stimulated glucose transport in rat adipocytes (22, 35). We have found that decreased $G\alpha_{11}$ expression in adipocytes after chronic ethanol feeding may contribute, at least in part, to impaired glucose transport due to a decreased activation of PYK2 tyrosine phosphorylation, a required intermediate in endothelin-1-stimulated glucose uptake. However, the mechanisms by which chronic ethanol feeding lead to decreased $G\alpha_{11}$ expression and impaired transport are not clear. Further experimentation is required to understand the in vivo factors involved in the development of impaired insulin- and endothelin-1-stimulated glucose uptake in adipocytes after chronic ethanol feeding.

DISCLOSURE

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REFERENCES

1. **Andreev J, Galisteo ML, Kranenburg O, Logan SK, Chiu ES, Okigaki M, Cary LA, Moolenaar WH, and Schlessinger J.** Src and Pyk2 mediate G-protein-coupled receptor activation of epidermal growth factor receptor (EGFR) but are not required for coupling to the mitogen-activated protein (MAP) kinase signaling cascade. *J Biol Chem* 276: 20130–20135, 2001.
2. **Barros LF, Young M, Saklatvala J, and Baldwin SA.** Evidence of two mechanisms for the activation of the glucose transporter GLUT1 by anisomycin: p38 (MAP kinase) activation and protein synthesis inhibition in mammalian cells. *J Physiol* 504: 517–525, 1997.
3. **Baumann CA, Ribon V, Kanzaki M, Thurmond DC, Mora S, Shigematsu S, Bickel PE, Pessin JE, and Saltiel AR.** CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature* 407: 202–207, 2000.
4. **Blaukat A, Ivankovic-Dikic I, Gronroos E, Dolfi F, Tokiwa G, Vuori K, and Dikic I.** Adaptor proteins Grb2 and Crk couple Pyk2 with activation of specific mitogen-activated protein kinase cascades. *J Biol Chem* 274: 14893–14901, 1999.
5. **Bose A, Cherniack AD, Langille SE, Nicoloro SM, Buxton JM, Park JG, Chawla A, and Czech MP.** G(alpha)11 signaling through ARF6 regulates F-actin mobilization and GLUT4 glucose transporter translocation to the plasma membrane. *Mol Cell Biol* 21: 5262–5275, 2001.
6. **Chiang SH, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, Macara IG, Pessin JE, and Saltiel AR.** Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. *Nature* 410: 944–948, 2001.
7. **Diamond I and Gordon AS.** Cellular and molecular neuroscience of alcoholism. *Physiol Rev* 77: 1–20, 1997.
8. **Fowman DT.** The effect of ethanol and its metabolites on carbohydrate, protein and lipid metabolism. *Ann Clin Lab Sci* 18: 181–189, 1988.
9. **Holbrook TL, Barrett-Connor E, and Wingard DL.** A prospective population-based study of alcohol use and non-insulin-dependent diabetes mellitus. *Am J Epidemiol* 132: 902–909, 1990.
10. **Husain S and Abdel-Latif AA.** Endothelin-1 activates p38 mitogen-activated protein kinase and cytosolic phospholipase A2 in cat iris sphincter smooth muscle cells. *Biochem J* 342: 87–96, 1999.
11. **Imamura T, Huang J, Dalle S, Ugi S, Usui I, Luttrell LM, Miller WE, Lefkowitz RJ, and Olefsky JM.** Beta-arrestin-mediated recruitment of the Src family kinase Yes mediates endothelin-1-stimulated glucose transport. *J Biol Chem* 276: 43663–43667, 2001.
12. **Imamura T, Ishibashi K, Dalle S, Ugi S, and Olefsky JM.** Endothelin-1-induced GLUT4 translocation is mediated via Galpha(q/11) protein and phosphatidylinositol 3-kinase in 3T3-L1 adipocytes. *J Biol Chem* 274: 33691–33695, 1999.
13. **Imamura T, Vollenweider P, Egawa K, Clodi M, Ishibashi K, Nakashima N, Ugi S, Adams JW, Brown JH, and Olefsky JM.** G alpha-q/11 protein plays a key role in insulin-induced glucose transport in 3T3-L1 adipocytes. *Mol Cell Biol* 19: 6765–6774, 1999.
14. **Isakoff SJ, Taha C, Rose E, Marcusohn J, Klip A, and Skolnik EY.** The inability of phosphatidylinositol 3-kinase activation to stimulate GLUT4 translocation indicates additional signaling pathways are required for insulin-stimulated glucose uptake. *Proc Natl Acad Sci USA* 92: 10247–10251, 1995.
15. **Kanzaki M, Watson RT, Artemyev NO, and Pessin JE.** The trimeric GTP-binding protein (Gq/G11) α subunit is required for insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes. *J Biol Chem* 275: 7167–7175, 2000.
16. **Kishi K, Hayashi H, Wang L, Kamohara S, Tamaoka K, Shimizu T, Ushikubi F, Narumiya S, and Ebina Y.** Gq-coupled receptors transmit the signal for GLUT4 translocation via an insulin-independent pathway. *J Biol Chem* 271: 26561–26568, 1996.
17. **Le Marchand-Brustel Y, Tanti JF, Cormont M, Ricort JM, Gremeaux T, and Grillo S.** From insulin receptor signalling to Glut 4 translocation abnormalities in obesity and insulin resistance. *J Recept Signal Transduct Res* 19: 217–228, 1999.
18. **Lieber CS and DeCarli LM.** The feeding of alcohol in liquid diets: two decades of application and 1982 update. *Alcohol Clin Exp Res* 6: 523–531, 1982.
19. **Nagao M, Yamauchi J, Kaziro Y, and Itoh H.** Involvement of protein kinase C and Src family tyrosine kinase in Galphaq/11-induced activation of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase. *J Biol Chem* 273: 22892–22898, 1998.
20. **Omata W, Shibata H, Li L, Takata K, and Kojima I.** Actin filaments play a critical role in insulin-induced exocytotic recruitment but not in endocytosis of GLUT4 in isolated rat adipocytes. *Biochem J* 346: 321–328, 2000.
21. **Park JG, Bose A, Leszyk J, and Czech MP.** PYK2 as a mediator of endothelin-1/G alpha 11 signaling to GLUT4 glucose transporters. *J Biol Chem* 276: 47751–47754, 2001.
22. **Poirier LA, Rachdaoui N, and Nagy LE.** GLUT4 vesicle trafficking in rat adipocytes after ethanol feeding: regulation by heterotrimeric G-proteins. *Biochem J* 354: 323–330, 2001.
23. **Saltiel AR and Kahn CR.** Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414: 799–806, 2001.
24. **Sethi JK and Hotamisligil GS.** The role of TNF alpha in adipocyte metabolism. *Semin Cell Dev Biol* 10: 19–29, 1999.

25. **Shelmet JJ, Reichard GA, Skutches CL, Hoeldtke RD, Owen OE, and Boden G.** Ethanol causes acute inhibition of carbohydrate, fat, and protein oxidation and insulin resistance. *J Clin Invest* 81: 1137–1145, 1988.
26. **Somwar R, Kim DY, Sweeney G, Huang C, Niu W, Lador C, Ramlal T, and Klip A.** GLUT4 translocation precedes the stimulation of glucose uptake by insulin in muscle cells: potential activation of GLUT4 via p38 mitogen-activated protein kinase. *Biochem J* 359: 639–649, 2001.
27. **Somwar R, Perreault M, Kapur S, Taha C, Sweeney G, Ramlal T, Kim DY, Keen J, Cote CH, Klip A, and Marette A.** Activation of p38 mitogen-activated protein kinase alpha and beta by insulin and contraction in rat skeletal muscle: potential role in the stimulation of glucose transport. *Diabetes* 49: 1794–1800, 2000.
28. **Sorokin A, Kozlowski P, Graves L, and Philip A.** Protein-tyrosine kinase Pyk2 mediates endothelin-induced p38 MAPK activation in glomerular mesangial cells. *J Biol Chem* 276: 21521–21528, 2001.
29. **Stephens JM and Pilch PF.** The metabolic regulation and vesicular transport of GLUT4, the major insulin-responsive glucose transporter. *Endocr Rev* 16: 529–546, 1995.
30. **Sweeney G, Somwar R, Ramlal T, Volchuk A, Ueyama A, and Klip A.** An inhibitor of p38 mitogen-activated protein kinase prevents insulin-stimulated glucose transport but not glucose transporter translocation in 3T3-L1 adipocytes and L6 myotubes. *J Biol Chem* 274: 10071–10078, 1999.
31. **Tsakiridis T, Vranic M, and Klip A.** Disassembly of the actin network inhibits insulin-dependent stimulation of glucose transport and prevents recruitment of glucose transporters to the plasma membrane. *J Biol Chem* 269: 29934–29942, 1994.
32. **Tsumura K, Hayashi T, Suematsu C, Endo G, Fujii S, and Okada K.** Daily alcohol consumption and the risk of type 2 diabetes in Japanese men—the Osaka Health Survey. *Diabetes Care* 22: 1431–1437, 1999.
33. **Wang QH, Bilan PJ, Tsakiridis T, Hinek A, and Klip A.** Actin filaments participate in the relocalization of phosphatidylinositol 3-kinase to glucose transporter-containing compartments and in the stimulation of glucose uptake in 3T3-L1 adipocytes. *Biochem J* 331: 917–928, 1998.
34. **Wei M, Kampert JB, Gibbons LW, Blair SN, and Mitchell TL.** Alcohol intake and incidence of type 2 diabetes in men. *Diabetes Care* 23: 18–22, 2000.
35. **Wilkes JJ, DeForrest LL, and Nagy LE.** Chronic ethanol feeding in a high-fat diet decreases insulin-stimulated glucose transport in rat adipocytes. *Am J Physiol Endocrinol Metab* 271: E477–E484, 1996.
36. **Wu-Wong JR, Berg CE, Wang J, Chiou WJ, and Fissel B.** Endothelin stimulates glucose uptake and GLUT4 translocation via activation of endothelin ETA receptor in 3T3-L1 adipocytes. *J Biol Chem* 274: 8103–8110, 1999.
37. **Yamauchi J, Nagao M, Kaziro Y, and Itoh H.** Activation of p38 mitogen-activated protein kinase by signaling through G protein-coupled receptors. Involvement of Gbetagamma and Galphaq/11 subunits. *J Biol Chem* 272: 27771–27777, 1997.
38. **Yki-Jarvinen H and Nikkila EA.** Ethanol decreases glucose utilization in healthy man. *J Clin Endocrinol Metab* 61: 941–945, 1985.