GLUT4 vesicle trafficking in rat adipocytes after ethanol feeding: regulation by heterotrimeric G-proteins

Laurie A. POIRIER, Nadia RACHDAOUI and Laura E. NAGY¹

Department of Nutrition, Room 201, Case Western Reserve University, 2123 Abington Road, Cleveland, OH 44106-4906, U.S.A.

Long-term ethanol consumption decreases insulin-stimulated glucose uptake in isolated rat adipocytes. Here we investigate the mechanisms for this decrease. Male Wistar rats were fed for 4 weeks with a liquid diet containing 35 % of the calories from ethanol and compared with pair-fed controls. Stimulation of 3-*O*-methylglucose transport in isolated adipocytes by insulin was decreased by 70 % after ethanol feeding. However, stimulation by insulin of the tyrosine phosphorylation of the p85 subunit of phosphoinositide 3-kinase and the phosphorylation of Akt were not affected by ethanol feeding. GLUT4 was mobilized from intracellular light microsomes in response to insulin in both pair-fed and ethanol-fed rats, resulting in 4.3-fold and 3.3-fold increases in GLUT4 associated with plasma membrane in pair-fed and ethanol-fed rats respectively. Surface-accessible GLUT4, assessed by a trypsin cleavage assay or cell-surface labelling with bis-mannose photolabel, was increased 2.3-fold and 1.6-fold

respectively, in pair-fed rats after treatment with insulin. In contrast, insulin did not increase surface-accessible GLUT4 in ethanol-fed rats. Treatment of adipocytes with *R*-phenylisopropyladenosine, an adenosine A₁ receptor agonist, increased the transport of 3-*O*-methylglucose and trypsin-accessible GLUT4, in adipocytes from both pair-fed and ethanol-fed rats. These results demonstrate that whereas the insulin-mediated signalling and translocation of GLUT4 to the plasma membrane is maintained after ethanol feeding, the final fusion of GLUT4 vesicles to the plasma membrane is disrupted, preventing the stimulation of glucose uptake by insulin. Fusion of GLUT4 with the plasma membrane can be stimulated by the activation of adenosine A₁ receptors.

Key words: adenosine, exocytosis, glucose transport, insulin.

INTRODUCTION

Glucose uptake in mammalian cells is mediated by a family of facilitative glucose transporters (GLUT1–6) [1]. GLUT4, the insulin-recruitable isoform, is expressed in adipose and muscle tissue and is the primary mediator of insulin-stimulated glucose uptake in these tissues [2]. In a resting adipocyte, GLUT4 is sequestered from the plasma membrane in an intracellular vesicular compartment. In response to insulin, intracellular GLUT4 vesicles translocate to the plasma membrane, where they dock and fuse, resulting in an increase in GLUT4 accessible at the cell surface [2]. Association of GLUT4 with the plasma membrane, as determined by subcellular fractionation, takes place before increased accessibility of GLUT4 at the cell surface, as measured by the binding of a photoaffinity ligand to GLUT4 [3]. Increased glucose transport occurs only after the final fusion of the GLUT4 vesicle with the plasma membrane, when the GLUT4 protein is accessible at the cell surface [3]. Abnormal expression and translocation of the GLUT4 transporter have been implicated in the development of glucose intolerance associated with type 2 diabetes, streptozotocin-induced diabetes and aging [2].

Although insulin is the predominant signal regulating glucose transport, the surface accessibility of GLUT4 and glucose transport are also modulated by heterotrimeric GTP-binding proteins (G-proteins) [4–6]. Activation of stimulatory G-protein (G_s) decreases glucose transport, whereas activation of inhibitory G-protein (G_i) stimulates glucose transport [4,6]. Vannucci et al. [6] proposed that G_s and G_i influence glucose transport by respectively inhibiting and promoting the extent to which GLUT4 is associated with occluded vesicles at the cell surface. Activation of G_s promotes the formation of an occluded GLUT4 vesicle,

associated with low surface accessibility and glucose transport, whereas activation of G_i promotes the fusion of GLUT4 vesicles at the plasma membrane, increasing surface accessibility and glucose transport [4–6].

Short-term and long-term exposure to ethanol is associated with impaired glucose utilization. Ethanol consumption increases circulating glucose concentrations [7], glucose intolerance and insulin resistance [8,9]; a chronic high level of ethanol consumption is an independent risk factor for the development of type 2 diabetes in some populations [10–12]. However, the mechanisms for this disruption of glucose homeostasis by ethanol are not well understood. We have previously reported that adipocytes from rats fed with ethanol as part of the high-fat Lieber DeCarli diet for 4 weeks have impaired insulin-stimulated glucose uptake [13]. In association with lower glucose uptake, there is no increase in surface-accessible GLUT4 after treatment with insulin in adipocytes from ethanol-fed rats [13]. Ethanol feeding also increases the relative expression of G_sα to G_iα in adipocyte membranes [13]. In the present study we investigated whether this loss in insulin-mediated glucose uptake after ethanol feeding is due to impaired insulin signal transduction and/or trafficking of GLUT4 from its intracellular storage site to docking and fusion at the plasma membrane.

EXPERIMENTAL

Materials

Male Wistar rats (140–150 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN, U.S.A.). Lieber DeCarli ethanol diet was prepared as described previously [14] or from Dyets (Bethlehem, PA, U.S.A.). Antibodies were obtained

Abbreviations used: ATB-BMPA, 2-*N*-4-(1-*az*i-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(*D*-mannos-4-yl)oxy)-2-propylamine; G_s, stimulatory G-protein; G_i, inhibitory G-protein; PI-3K, phosphoinositide 3-kinase; R-PIA, *R*-phenylisopropyladenosine; VAMP-2, vesicle-associated protein 2.

¹ To whom correspondence should be addressed (e-mail len2@po.cwru.edu).

from the following sources: rabbit polyclonal anti-GLUT4 for Western blotting from East Acres Biologicals (Cambridge, MA, U.S.A.) or Biogenesis (Sandon, NH, U.S.A.); those for immunoisolation were a gift from S. Cushman (NIH, Bethesda, MD, U.S.A.); monoclonal antibodies against GLUT4 were a gift from P. Noordstrom; vesicle-associated protein (VAMP)-2 was a gift from A. Klip (Hospital for Sick Children, Toronto, Ontario, Canada); cellubrevin was a gift from R. Jahn; p85 was from Upstate Biotechnologies; anti-phosphotyrosine (PY20) was from Transduction Labs (Lexington, KY, U.S.A.); and anti-(phospho-Akt/protein kinase B) was from New England Biolabs (Beverly, MA, U.S.A.). Goat anti-rabbit or anti-mouse IgG (Fab fragment) coupled to horseradish peroxidase was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). 2-Deoxy[^3H]-glucose and 3-*O*-[^3H]methylglucose were from Amersham (Arlington Heights, IL, U.S.A.). 2-*N*-4-(1-Azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D -mannos-4-yloxy)-2-propylamine-2-[^3H] ([^3H]ATB-BMPA) was from Toronto Research Chemicals (North York, Ontario, Canada). Insulin (regular Iletin) was from Eli Lilly (Indianapolis, IN, U.S.A.). *R*-Phenylisopropyladenosine (R-PIA) was purchased from Research Biochemicals (Natick, MA, U.S.A.).

Animal care and feeding

Rats were housed in individual cages in a room with controlled temperature and humidity in a 12 h light/12 h dark cycle. The rats were acclimatized for 3 days after arrival and provided with free access to Purina rat chow and water. All rats were then allowed free access to liquid diet [15] 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 the calories as ethanol for 2 days and then provided with diet containing 35% of the calories from ethanol for the remainder of the feeding period. Controls were pair-fed with a liquid diet identical with the ethanol diet except that maltose dextrins were isocalorically substituted for ethanol. Pair-fed rats were given the same amount of food as their ethanol pair consumed in the preceding 24 h. Initial body weights were 167 ± 1 and 169 ± 1 g (means $\pm$ S.E.M.); final weights were 293 ± 7 and 280 ± 6 g for ethanol-fed and pair-fed rats ($n = 29$) respectively. Procedures involving animals were approved by the Institutional Animal Care Board at Case Western Reserve University.

Uptake of 2-deoxy[^3H]glucose and 3-*O*-[^3H]methylglucose

After the 4-week feeding period, the rats were anaesthetized by intraperitoneal injection with sodium pentobarbital (0.2 ml/100 g) and epididymal fat pads were removed. Adipocytes were isolated by collagenase digestion as described previously [13], then counted and diluted to 5×10^5 cells/ml in PBS, pH 7.4, containing 1 mM MgCl_2 , 0.68 mM CaCl_2 , 1 mg/ml BSA, 1 mM pyruvic acid and 1 unit/ml adenosine deaminase (incubation buffer). Adipocytes were preincubated with or without R-PIA for 5 min and then treated with or without insulin for 30 min at 37 °C in a shaking water bath (130 rev./min). Non-specific uptake was measured in cells pretreated with 0.3 mM phloretin after 25 min of incubation. After treatment with hormones, uptake of 2-deoxy[1,2- ^3H]glucose (final concentration 2.5 mM, 0.5 μCi per tube) or 3-*O*-[^3H]methylglucose (final concentration 0.1 mM, 0.5 μCi per tube) was measured over 3.5 min for 2-deoxyglucose [13] or 2–8 s for 3-*O*-methylglucose. Transport of 3-*O*-[^3H]methylglucose saturated rapidly after 10–15 s.

Insulin signalling cascade

Isolated adipocytes were treated with or without R-PIA for 5 min and then with or without insulin for 2 min. Cells were lysed for 30 min at 4 °C in lysis buffer [final concentrations: 50 mM Tris/HCl (pH 7.4)/150 mM NaCl/2 mM EGTA/1% (v/v) Nonidet P40/1% (v/v) Triton X-100] containing protease (Complete[®]; Boehringer Mannheim) and phosphatase inhibitors (1 mM Na_3VO_4 , 20 mM sodium pyrophosphate, 100 mM NaF, 1 mM sodium molybdate and 5 mM benzamidine). Lysates were vortex-mixed briefly and centrifuged for 5 s; the fluid below the fat cake was removed with a syringe. A portion of each lysate was adjusted to 0.5 mg/ml protein and incubated overnight at 4 °C with 3.5 μg of anti-phosphotyrosine antibody. Samples were then mixed for 4 h with 50 μl of Protein G-agarose at 4 °C. Immunoprecipitates were collected by centrifugation at 500 *g* for 3 min, washed four times in lysis buffer and solubilized in Laemmli buffer. Lysates and immunoprecipitates were separated by SDS/PAGE and transferred to PVDF for Western blotting.

Isolation of subcellular fractions

Isolated adipocytes (approx. 4×10^6 cells) were incubated with or without insulin in plastic Erlenmeyer flasks for 30 min at 37 °C in incubation buffer with shaking at 130 rev./min. Reactions were terminated with the addition of 2 mM KCN; adipocytes were incubated at room temperature for 5 min. All subsequent handling was performed at 4 °C. Adipocytes were homogenized in 20 mM Tris/HCl (pH 7.4)/1 mM EDTA/255 mM sucrose containing protease inhibitors (homogenizing buffer) with a Wheaton glass homogenizer with the tight-fitting pestle (clearance 0.05 μm) and subcellular fractions were isolated by differential centrifugation [16]. In brief, homogenates were centrifuged at 16000 *g* for 15 min. The pellet was resuspended in 1 ml of homogenizing buffer and layered over a sucrose pad [20 mM Tris/HCl (pH 7.4)/1 mM EDTA/1.12 M sucrose] and centrifuged at 100000 *g* for 70 min. The interface was collected and centrifuged at 48000 *g* for 45 min to isolate plasma membrane. The supernatant (S1 fraction) was centrifuged at 48000 *g* for 20 min (heavy microsomes) and the resulting supernatant (S2 fraction) was either used for the immunoisolation of GLUT4 vesicles or centrifuged at 200000 *g* for 70 min (light microsomes). Protein concentration in the subcellular fractions was measured by using the Bradford method. Subcellular fractions were solubilized in Laemmli sample buffer under reducing conditions and frozen at –80 °C. Enrichment of plasma membrane fractions was monitored with an antibody against the $\alpha 1$ subunit of Na^+, K^+ -ATPase. Protein yields in isolated plasma membrane relative to adipocyte wet weight did not differ between pair-fed ($0.31 \pm 0.08\%$) and ethanol-fed ($0.24 \pm 0.08\%$, $n = 8$ or 9) rats; neither did yields of light microsomes relative to protein in the S1 fraction ($1.03 \pm 0.23\%$ for pair-fed rats and $1.08 \pm 0.27\%$ for ethanol-fed rats).

GLUT4-containing vesicles were immunisolated from the S2 fraction. Magnetic beads were coated with anti-GLUT4 rabbit serum or normal rabbit serum, as previously described [17], and incubated for 16 h at 4 °C with 100 μg of the isolated S2 fraction in homogenizing buffer containing 0.1% BSA. Beads were washed three times for 15 min in homogenizing buffer. Immunisolated vesicles were solubilized in non-reducing sample buffer to prevent the IgG heavy-chain signal from interfering with GLUT4 signals on the Western blots. Proteins were separated by SDS/PAGE [4–18% (w/v) gel] and transferred to PVDF for Western blot analysis.

Trypsin cleavage assay

Isolated adipocytes were incubated with or without R-PIA for 5 min and then treated with or without insulin for 20 min. The reactions were terminated by the addition of 2 mM KCN. Trypsin (1 mg/ml) was added for 20 min and then trypsin inhibitor (2 mg/ml) for a further 5 min at 37 °C, with shaking at 130 rev./min [13,18]. Cells were then washed twice in PBS containing trypsin inhibitor (2 mg/ml) and homogenized in homogenizing buffer (described above). Protein quantity was measured and equal amounts of proteins were separated by SDS/PAGE and transferred to PVDF for Western blot analysis.

Cell surface photolabelling with [³H]ATB-BMPA

Isolated adipocytes were incubated with or without 1 nM insulin for 20 min at 37 °C in a shaking water bath. Adipocytes were then added to 125 μ Ci of [³H]ATB-BMPA in 1 ml of PBS in a 9 cm polystyrene tissue culture dish. Cells were irradiated three times for 20 s in a Rayonet RPR 200 photochemical reactor (RPR 3000 lamps) with gentle agitation between cycles [19]. Adipocytes were then transferred to polystyrene centrifuge tubes and washed three times in PBS with 0.1 % BSA, then homogenized as described above. Plasma-membrane-enriched fractions were isolated as above and proteins were separated by SDS/PAGE. Gels were stained with Coomassie Blue, then destained and sliced into 13 equal pieces. Gel slices were solubilized in 0.5 ml of Scinti-gest tissue solubilizer and then diluted 1:40 in scintillation cocktail for scintillation counting.

Western blotting

PVDF membranes were blocked for 2 h with 5 % (w/v) non-fat dried milk or 3 % (w/v) BSA in Tris-buffered saline (50 mM Tris/150 mM NaCl) containing 0.1 % (v/v) Tween 20 (TBS-T), washed twice with TBS-T and then incubated overnight at 4 °C with antibody. Antibodies were prepared in 1 % (w/v) BSA in TBS-T at dilutions of 1:500 for anti-GLUT4, 1:2000 for anti-cellubrevin, 1:25000 for p85 and 1:1000 for VAMP-2 and anti-(phospho-Akt), and at 0.3 μ g/ml for Na⁺,K⁺-ATPase. Membranes were washed again and probed for 1 h with horseradish-peroxidase-coupled goat anti-rabbit or anti-mouse IgG Fab fragments. Bound antibody was detected with enhanced chemiluminescence reagent. Immunoreactive protein quantity was assessed by scanning densitometry; film exposure times were in the linear range of detectability. Internal standards were included for normalization between blots.

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 five or six rats per dietary treatment. Values reported are means $\pm$ S.E.M. Best-fit curves for time courses of 3-*O*-methylglucose were calculated with Table Curve (Jandel Scientific). Data were analysed 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 Tukey's test or least-square means. Data were log-transformed when necessary to produce a normal distribution.

RESULTS

Stimulation of 2-deoxy[³H]glucose uptake with insulin was lower in adipocytes isolated from ethanol-fed rats than in pair-fed rats (Figure 1A) [13]. To determine whether this decrease was due to lower rates of transport or to decreased intracellular trapping of the 2-deoxyglucose, measurements of 3-*O*-[³H]methylglucose, a non-metabolizable glucose analogue, were performed. Basal rates of 3-*O*-[³H]methylglucose uptake did not differ between ethanol-fed and pair-fed rats (results not shown). Treatment with 4 nM insulin for 30 min increased the transport of 3-*O*-methylglucose in adipocytes from both ethanol-fed and pair-fed rats. However, insulin-stimulated transport after ethanol feeding was 70 % lower than insulin-stimulated transport in pair-fed rats (*P* < 0.03; Figure 1B), indicating that decreased insulin-stimulated glucose

Figure 1 Stimulation by insulin of (A) uptake of 2-deoxyglucose and (B) transport of 3-*O*-methylglucose in adipocytes isolated from ethanol-fed and pair-fed rats

Isolated adipocytes were incubated with or without 4 nM insulin for 30 min. The uptake of 2.5 mM [³H]-2-deoxyglucose was then measured over 5 min (A) and the transport of 0.1 mM 3-*O*-[³H]methylglucose was measured from 2 to 8 s (B). Non-specific uptake was measured in cells preincubated with 0.3 mM phloretin for 5 min. (A) Values are means $\pm$ S.E.M. (*n* = 12) for 2-deoxyglucose. Values with different superscripts are significantly different (*P* < 0.05). Open columns, basal; hatched columns, with insulin. (B) Values are means (*n* = 5 or 6) for the increase in 3-*O*-methylglucose transport after insulin stimulation over basal transport. Rates of transport with different superscripts are significantly different (*P* < 0.05). Symbols: ○, basal, pair-fed; ●, basal, ethanol-fed; ▽, insulin, pair-fed; ▼, insulin, ethanol-fed. Abbreviation: EtOH, ethanol.

Figure 2 Insulin signalling to PI-3K and Akt is not impaired after ethanol feeding

Tyrosine phosphorylation of the p85 subunit of PI-3K (**A**) and serine phosphorylation of Akt (**B**) in response to stimulation with insulin. Isolated adipocytes were stimulated with 0–10 nM insulin for 2 min and tyrosine-phosphorylated proteins were immunoprecipitated with an antibody directed against phosphotyrosine. Lysates and immunoprecipitates were separated by SDS/PAGE and probed with an antibody against phospho-Akt or the p85 subunit of PI-3K respectively. Values are means $\pm$ S.E.M. ($n = 5$ for p85; $n = 6$ for phospho-Akt). Open columns, pair-fed rats; hatched columns, ethanol-fed. Abbreviation: EtOH, ethanol.

uptake after ethanol feeding was not due to impaired phosphorylation or trapping of glucose in the adipocyte.

Stimulation of glucose uptake by insulin requires the activation of phosphoinositide 3-kinase (PI-3K) [20] and Akt/PKB [21]. Activation of PI-3K is associated with tyrosine phosphorylation of the p85 subunit, whereas Akt is activated by serine/threonine phosphorylation [21]. After treatment of isolated adipocytes for 2 min with increasing concentrations of insulin, total cell lysates were prepared and immunoprecipitated with anti-phosphotyrosine antibodies. Immunoprecipitates were then probed for the p85 subunit of PI-3K. The total quantity of p85 in lysates of adipocytes was increased by 25% in adipocytes from ethanol-fed rats (381 ± 20 arbitrary density units) compared with pair-fed rats (300 ± 21 density units; $n = 5$, $P < 0.02$). Insulin dose-dependently increased the proportion of p85 that was immunoprecipitated with anti-phosphotyrosine antibodies (Figure 2); no differences in the extent of phosphorylation of p85 were observed between pair-fed and ethanol-fed groups (Figure 2), even when corrected for the small difference in total quantity of p85 between the groups (results not shown). Similarly, insulin stimulation of Akt phosphorylation was not impaired after ethanol feeding (Figure 2B).

To determine whether ethanol feeding affected the intracellular distribution of GLUT4, subcellular fractions from adipocytes were isolated by differential centrifugation. We have reported previously that total GLUT4 content in adipocytes is decreased by 50% in adipocytes isolated from ethanol-fed rats in comparison with pair-fed rats [13]. Using subcellular fractionation of non-stimulated (basal) adipocytes, we found that this decrease was associated primarily with lower GLUT4 protein in light microsomes (Figure 3, Table 1). Enrichment of the $\alpha 1$ subunit of Na^+/K^+ -ATPase in the plasma membrane fractions did not differ between pair-fed and ethanol-fed rats (34.2 ± 7.8 and 40.9 ± 7.3

Figure 3 Insulin-dependent changes in the distribution of GLUT4 in plasma membrane, light microsomes (LDM) and immunoisolated GLUT4 vesicles

Isolated adipocytes were stimulated with or without 4 nM insulin for 30 min and the distribution of GLUT4 was measured in subcellular fractions. GLUT4-containing vesicles were isolated by incubation with anti-GLUT4 (G4) antibody or normal rabbit serum (N). Representative blots are shown ($n = 15$ for subcellular fractionation; $n = 5$ for immunoisolation). Abbreviations: PF, pair-fed; EF, ethanol-fed.

arbitrary density units respectively; $n = 4$ or 5) and was essentially undetectable in light microsomes from both feeding groups. Ethanol feeding did not affect the GLUT4 content in the plasma membrane or heavy microsomes of non-stimulated cells (Figure 3, Table 1). In contrast with the decrease in light-microsomal GLUT4 content, ethanol feeding did not affect the

Table 1 Subcellular distribution of GLUT4 and v-SNARE proteins (cellubrevin and VAMP-2) in adipocytes from pair- and ethanol-fed rats treated with or without insulin

Values are expressed as percentages of those for basal pair-fed rats within each subcellular fraction (mean $\pm$ S.E.M.). Values in parentheses are arbitrary density units (means $\pm$ S.E.M.) for basal pair-fed rats. * $P < 0.05$ compared with basal; † $P < 0.05$ compared with pair-fed.

Protein	Treatment	Immunoreactive protein quantity (% of pair-fed basal; arbitrary units of density for pair-fed basal)					
		Plasma membranes		Light microsomes		Heavy microsomes	
		Basal	Insulin	Basal	Insulin	Basal	Insulin
GLUT4	Pair-fed	100 (89 $\pm$ 13)	432 $\pm$ 70*	100 (823 $\pm$ 147)	45 $\pm$ 4*	100 (305 $\pm$ 89)	203 $\pm$ 47
	Ethanol-fed	119 $\pm$ 22	319 $\pm$ 46*†	66 $\pm$ 5†	26 $\pm$ 6*†	82 $\pm$ 17	177 $\pm$ 47
Cellubrevin	Pair-fed	100 (108 $\pm$ 46)	123 $\pm$ 28	100 (258 $\pm$ 66)	59 $\pm$ 8*	100 (91 $\pm$ 28)	147 $\pm$ 32
	Ethanol-fed	127 $\pm$ 30	131 $\pm$ 27	92 $\pm$ 11	62 $\pm$ 8*	152 $\pm$ 33	173 $\pm$ 45
VAMP-2	Pair-fed	100 (177 $\pm$ 48)	145 $\pm$ 18	100 (145 $\pm$ 31)	65 $\pm$ 8*	100 (137 $\pm$ 36)	144 $\pm$ 24
	Ethanol-fed	106 $\pm$ 24	129 $\pm$ 25	89 $\pm$ 9	69 $\pm$ 8*	131 $\pm$ 28	156 $\pm$ 33

Table 2 Trypsin-accessible GLUT4 after treatment of adipocytes with insulin and R-PIA

Isolated adipocytes were preincubated with 1 nM R-PIA for 5 min and then treated with or without 1 nM insulin for 20 min. Cell-surface-accessible GLUT4 was measured with a trypsin cleavage assay as described in the Experimental section. Values are means $\pm$ S.E.M. ($n = 5$ or 6), expressed as percentages of basal for each diet group. Values with different superscripts are significantly different ($P < 0.05$).

Diet	Immunoreactive GLUT4 cleavage product (% of basal; arbitrary units of density for basal in parentheses)			
	Basal	1 nM insulin	1 nM R-PIA	1 nM insulin + 1 nM R-PIA
Pair-fed	100 ^a (192 $\pm$ 62)	232 $\pm$ 49 ^c	164 $\pm$ 24 ^b	235 $\pm$ 66 ^c
Ethanol-fed	100 ^a (228 $\pm$ 69)	90 $\pm$ 13 ^a	176 $\pm$ 37 ^{bc}	168 $\pm$ 30 ^{bc}

quantity or distribution of VAMP-2 or cellubrevin, two proteins involved in GLUT4 trafficking (Table 1). Immunoprecipitated GLUT4 vesicles from ethanol-fed rats exhibited a specific decrease in GLUT4 content (Figure 3), with no differences in the quantities of VAMP-2 (114 $\pm$ 29 and 92 $\pm$ 21 arbitrary density units for pair-fed and ethanol-fed rats respectively; $n = 4$) or cellubrevin [86 $\pm$ 38 and 72 $\pm$ 38 arbitrary density units for pair-fed rats ($n = 6$) and ethanol-fed rats ($n = 3$) respectively].

On treatment with 4 nM insulin for 30 min, the quantity of GLUT4 in isolated plasma membrane fractions increased 4.2 $\pm$ 0.7-fold in adipocytes from pair-fed rats (Table 1). In ethanol-fed animals, GLUT4 content associated with the plasma membrane was increased 3.3 $\pm$ 0.5-fold over basal. This increase in plasma-membrane-associated GLUT4 in response to insulin was approx. 20% ($P < 0.05$) lower in ethanol-fed rats than in pair-fed rats (Table 1). In both feeding groups, GLUT4 content in light microsomes decreased on stimulation with insulin (Figure 3, Table 1); similarly, the quantities of VAMP-2 and cellubrevin in light microsomes decreased in adipocytes from both pair-fed and ethanol-fed rats (Table 1). Whereas GLUT4 content in immunoprecipitated GLUT4 vesicles from pair-fed rats was higher than in ethanol-fed rats before stimulation, after treatment with insulin the GLUT4 content was not different between the two groups (Figure 3), with an 85–95% decrease in GLUT4 in the vesicles. Taken together, these results suggest that the capacity for GLUT4 recruitment from light-microsomal GLUT4 vesicles was not affected by ethanol feeding.

Despite the translocation of GLUT4 from intracellular vesicles to the plasma membrane, plasma-membrane-associated GLUT4

Figure 4 Insulin-stimulated photolabelling of plasma membrane GLUT4 with [³H]ATB-BMPA

Isolated adipocytes were stimulated with or without 1 nM insulin for 20 min, then mixed with 125 μ Ci of [³H]ATB-BMPA and irradiated three times for 20 s. Plasma membranes were isolated and proteins were separated by SDS/PAGE. Gels were sliced and the radioactivity in each slice was measured by scintillation counting. The migration of molecular mass markers (in kDa) is shown at the top. Profiles are representative of results from three pairs of animals. Symbols: $\square$, basal, pair-fed; $\blacksquare$, insulin, pair-fed; $\circ$, basal, ethanol-fed; $\bullet$, insulin, ethanol-fed.

was not accessible at the cell surface after ethanol feeding, either to permeant, as indicated by decreased glucose transport (Figure 1), or to cleavage with trypsin [13] (see Table 2). Cell-surface binding of a bis-mannose photolabel (ATB-BMPA) was also measured. Representative profiles of adipocytes photolabelled with [³H]ATB-BMPA after treatment in the presence and the absence of 1 nM insulin are shown in Figure 4. In adipocytes from pair-fed rats, treatment with 1 nM insulin increased labelling to 157 $\pm$ 27% of basal in the GLUT4 peak on the gel, whereas labelling was not increased by insulin after ethanol feeding (105 $\pm$ 22% of basal; $n = 3$) (Figure 4).

Although translocation of GLUT4 to the plasma membrane is the primary determinant of insulin-stimulated glucose uptake [2], transport via GLUT4 is also modulated by heterotrimeric G-proteins [4,6]. After ethanol feeding, the expression of G_s is

Figure 5 Activation of adenosine A_1 receptors increases transport of 3-O-methylglucose in adipocytes from both ethanol-fed and pair-fed rats

Isolated adipocytes were preincubated with or without 15 nM R-PIA for 5 min and then treated with or without 4 nM insulin for 30 min. Transport of 3-O-[3 H]methylglucose was measured from 2 to 8 s. Values are means ($n = 5$ or 6). Symbols: $\circ$, basal, pair-fed; $\bullet$, basal, ethanol-fed; $\triangle$, R-PIA, pair-fed; $\blacktriangle$, R-PIA, ethanol-fed; $\square$, R-PIA and insulin, pair-fed; $\blacksquare$, R-PIA and insulin, ethanol-fed; ∇ , insulin, pair-fed; $\blacktriangledown$, insulin, ethanol-fed.

increased in adipocytes relative to G_i [13]. We hypothesized that if this relative increase in G_s/G_i expression retained GLUT4 vesicles in the occluded form, thus preventing the complete fusion of GLUT4 vesicles at the plasma membrane, then the stimulation of G_i should shift GLUT4 vesicles from the occluded form and result in increased glucose transport. To test this hypothesis, adipocytes were treated with R-PIA [22], an adenosine A_1 receptor agonist; transport of 3-O-[3 H]methylglucose was measured. PIA (15 nM) increased glucose transport to a greater extent in adipocytes from ethanol-fed rats than from pair-fed rats (Figure 5). Co-stimulation with insulin further increased PIA-activated transport in pair-fed rats but not in adipocytes from ethanol-fed animals (Figure 5).

If R-PIA were shifting GLUT4 from an occluded vesicle at the plasma membrane to a fully fused state, then the quantity of surface-accessible GLUT4 would increase in response to R-PIA. The relative quantity of surface-accessible GLUT4 was measured with a trypsin cleavage assay [18]. There is a trypsin cleavage site in the first exofacial loop of the GLUT4 protein. If this loop is accessible at the cell surface, trypsin cleavage generates a 35 kDa product that can be detected by Western blotting. Stimulation of adipocytes with 1 nM insulin increased trypsin-accessible GLUT4 2.3-fold in pair-fed rats but had no effect in ethanol-fed rats [13] (Table 2). In contrast, treatment with R-PIA increased surface-accessible GLUT4 in pair-fed rats and ethanol-fed rats by 1.6-fold and 1.8-fold respectively (Table 2). In the presence of both insulin and R-PIA, surface-accessible GLUT4 was further increased beyond that in the presence of R-PIA alone in pair-fed rats but not in ethanol-fed rats (Table 2).

DISCUSSION

Ethanol consumption is associated with abnormal glucose homeostasis in humans and rodents. Chronic ethanol feeding, as part of a high-fat diet, impairs the stimulation of glucose uptake by insulin [13]. Here we have demonstrated that ethanol feeding does not affect the ability of insulin to activate PI-3K or Akt, which are necessary intermediates in the insulin signal cascade required for the stimulation of glucose uptake. Similarly, ethanol feeding did not interfere with the translocation of intracellular

GLUT4 vesicles to the plasma membrane. Importantly, despite the translocation of GLUT4 to the plasma membrane in response to insulin, the accessibility of GLUT4 to the adipocyte surface was not increased after ethanol feeding. Heterotrimeric G-proteins [4,6] regulate the surface-accessibility of GLUT4. GLUT4 at the plasma membrane can shift to an occluded state in which the GLUT4 vesicle is physically associated with the plasma membrane but is inaccessible to permeant or photoaffinity ligands, in response to activation of G_s or to a fully surface-accessible state in response to activation of G_i [4,6]. After ethanol feeding, the expression of G_s is increased relative to G_i in adipocyte membranes [13]. When adipocytes were stimulated with R-PIA, an adenosine A_1 receptor agonist, to activate G_i , rates of glucose uptake in adipocytes from ethanol-fed animals were higher than in pair-fed controls. Similarly, treatment with PIA increased surface-accessible GLUT4 in adipocytes from ethanol-fed rats, suggesting that after ethanol feeding GLUT4 is present in an occluded state at the plasma membrane.

Stimulation of glucose uptake by insulin involves the transduction of insulin-receptor-mediated signals and the translocation of GLUT4 from intracellular vesicles to the plasma membrane. Acute or chronic exposure to ethanol has been shown to impair insulin-mediated signal transduction in a number of systems *in vitro*. Brief exposure to ethanol decreases the phosphorylation of insulin receptor substrate 1 in response to stimulation by insulin or by insulin-like growth factor 1 [23–25]. Ethanol also decreases the phosphorylation of downstream targets of insulin stimulation, p36 and neuronal thread proteins, in cultured hepatocarcinoma and neuronal cells respectively [23,25]. Chronic treatment of cultured human trophoblasts with ethanol also impairs insulin-stimulated amino acid uptake at a post-receptor site [26]. However, much less is known about the effects of ethanol exposure *in vivo* on insulin-dependent signal transduction. Chronic feeding of ethanol to rats decreases insulin receptor binding to hepatocytes by 25–40% [14,27], possibly owing to ethanol-induced alterations in hepatic protein trafficking [14]. Ethanol feeding also decreases the phosphorylation of insulin receptor substrate 1 during liver regeneration [28], although it is not clear whether this decrease is due to lower concentrations of circulating ligands for receptor tyrosine kinase or is an effect of ethanol on the receptor tyrosine kinase signalling cascade. In the present investigation we did not observe any differences in the ability of physiological concentrations of insulin to activate the tyrosine phosphorylation of the p85 subunit of PI-3K or the serine phosphorylation of Akt (Figure 2), two downstream events in the insulin signalling cascade required for stimulation of glucose transport. Because the sensitivity of these downstream signalling intermediates to insulin activation was maintained after ethanol feeding, it is unlikely that the loss of insulin-stimulated glucose transport is due to impairments in the insulin-signalling cascade.

Consistent with the sustained activation of PI-3K after ethanol feeding is the observation that GLUT4 was translocated from its intracellular vesicular compartment to the plasma membrane in response to insulin treatment in animals chronically exposed to ethanol. Whereas intracellular GLUT4 vesicles contained a decreased quantity of GLUT4 after ethanol feeding, the quantity of VAMP-2 and cellubrevin, two vesicular proteins hypothesized to be involved in the docking of the GLUT4 vesicle to the plasma membrane [29], was not affected by ethanol feeding. The quantity of GLUT4 in plasma-membrane-enriched fractions was increased 3.3-fold in response to insulin in adipocytes from ethanol-fed rats, compared with 4.3-fold in pair-fed rats. GLUT4 was essentially depleted from intracellular vesicles after treatment with insulin in adipocytes from ethanol-fed rats (Figure 3).

Therefore the difference in maximal quantity of GLUT4 at the plasma membrane after stimulation with insulin probably reflects the decrease in quantity of GLUT4 in light microsomes that is available for translocation after ethanol feeding, rather than an impaired translocation response.

While GLUT4 effectively translocated to the plasma membrane after ethanol feeding, GLUT4 was not accessible to cleavage by trypsin, a measure of surface accessibility [18], or to binding of the bis-mannose photolabel ATB-BMPA [19]; neither did it mediate the transport of glucose analogues. The lack of surface accessibility is consistent with the localization of GLUT4 in an occluded vesicle. Work over the past decade has suggested that heterotrimeric G-proteins can regulate the accessibility of GLUT4 to the extracellular space. Activation of G_s promotes the formation of an occluded vesicle, decreasing glucose transport, whereas activation of G_i promotes the fusion of GLUT4 vesicles at the plasma membrane, increasing glucose transport [4–6]. Ethanol feeding increases the relative quantity of G_s to G_i in adipocytes [13]. We hypothesized that this imbalance in the quantity of the two heterotrimeric G-proteins that can modulate the final fusion of GLUT4 vesicles might contribute to the inability of insulin to stimulate glucose uptake, despite the translocation of GLUT4 to the plasma membrane. Activation of G_i with R-PIA increased glucose transport in adipocytes from ethanol-fed animals; the addition of 4 nM insulin had no further stimulatory effect. In pair-fed rats, PIA on its own increased glucose transport above basal; the addition of insulin further stimulated glucose transport but only to rates observed in the presence of insulin alone. Stimulation of glucose transport by PIA was paralleled by increases in the surface accessibility of GLUT4, as measured by the trypsin cleavage assay. Although insulin alone did not increase the surface accessibility of GLUT4 after ethanol feeding (Table 2), treatment with R-PIA increased trypsin-accessible GLUT4 in ethanol-fed rats. These results suggest that activation of G_i via the adenosine A_1 receptor stimulated the final fusion of the GLUT4 vesicle with the plasma membrane, restoring glucose transport.

Interestingly, in both pair-fed and ethanol-fed rats, increased GLUT4 surface accessibility and glucose transport in response to insulin and PIA seemed to be blunted relative to the availability of GLUT4 at the plasma membrane. For example, in pair-fed rats, the surface accessibility of GLUT4 increased only 2.3-fold and 1.6-fold as measured by trypsin cleavage and bis-mannose photolabelling respectively after stimulation with insulin, in comparison with the more than 4-fold increase in GLUT4 associated with plasma-membrane-enriched fractions after stimulation with insulin. Similarly, even though insulin increased GLUT4 recruitment to the plasma membrane in ethanol-fed rats, the PIA-mediated increase in surface accessibility and transport was not further enhanced by the presence of insulin. It is possible that only a portion of total plasma membrane GLUT4 is subject to regulation by heterotrimeric G-proteins. Alternatively, additional regulatory factors might be involved in limiting glucose transport [30,31] in the adipocytes used in the present study. In particular, the high fat content in the liquid diet provided to both pair-fed and ethanol-fed animals [13] might decrease the transport capacity or catalytic activity of GLUT4. For example, high-fat diets decrease insulin-stimulated glucose transport without decreasing GLUT4 translocation to the plasma membrane [32]. The mechanism for this effect of high-fat diets on limiting glucose transport is not known. However, because the high-fat diet was fed in equal amounts to pair-fed and ethanol-fed groups, it is unlikely that the high fat content of the diet contributed to the differential loss of glucose transport after ethanol feeding.

Changes in the activity of G_s and G_i *in vivo* have been associated with changes in the sensitivity of glucose transport to insulin. For example, desensitization of β -adrenergic receptors by chronic treatment with isoprenaline (isoproterenol) increases the sensitivity of glucose transport to insulin [33]. In contrast, transgenic mice with a deficiency of $G_{i\alpha_2}$ exhibit insulin resistance and impaired insulin-stimulated glucose transport in adipocytes [34], whereas the expression of a constitutively active form of $G_{i\alpha_2}$ has insulinomimetic effects in mice [35,36]. Taken together with these previous studies *in vivo*, our results suggest that ethanol-induced increases in the quantity of G_s relative to $G_{i\alpha}$ in adipocytes [13] contribute to the decreased ability of insulin to stimulate glucose transport in adipocytes.

We thank Julia Frenkel and Peggy Muhlenkamp for their expert technical assistance, and Dr Ronald Przybylski for generously sharing his Rayonet RPR 200 photoreactor. The monoclonal antibody against the α_1 subunit of Na^+, K^+ -ATPase ($\alpha 6F$), developed by D. M. Fambrough, was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, U.S.A., under contract NO1-HD-7-3263. This work was supported in part by United States Public Health Service Grant RO1 AA11876.

REFERENCES

- Mueckler, M. (1994) Facilitative glucose transporters. *Eur. J. Biochem.* **219**, 713–725
- Stephens, J. M. and Pilch, P. F. (1995) The metabolic regulation and vesicular transport of GLUT4, the major insulin-responsive glucose transporter. *Endocrine Rev.* **16**, 529–546
- Satoh, S., Nishimura, H., Clark, A. E., Kozka, I. J., Vannucci, S. J., Simpson, I. A., Quon, M. J., Cushman, S. W. and Holman, G. D. (1993) Use of bismannose photolabel to elucidate insulin-regulated GLUT4 subcellular trafficking kinetics in rat adipose cells. *J. Biol. Chem.* **268**, 17820–17829
- Ferrara, C. M. and Cushman, S. W. (1999) GLUT4 trafficking in insulin-stimulated rat adipose cells: evidence that heterotrimeric GTP-binding proteins regulate the fusion of docked GLUT4-containing vesicles. *Biochem. J.* **343**, 571–577
- Londos, C. (1988) Receptor G_s and G_i complexes in rat adipocyte plasma membranes: regulators of cAMP-related and cAMP-independent processes. In *Adenosine Receptors* (Cooper, D. M. F. and Londos, C., eds.), pp. 75–86. Alan R. Liss, New York
- Vannucci, S. J., Nishimura, H., Satoh, S., Cushman, S. W., Holman, G. D. and Simpson, I. A. (1992) Cell surface accessibility of GLUT4 glucose transporters in insulin-stimulated rat adipose cells. *Biochem. J.* **288**, 325–330
- Fowman, D. T. (1988) The effect of ethanol and its metabolites on carbohydrate, protein and lipid metabolism. *Ann. Clin. Lab. Sci.* **18**, 181–189
- Shelmet, J. J., Reichard, G. A., Skutches, C. L., Hoeldtke, R. D., Owen, O. E. and Boden, G. (1988) Ethanol causes acute inhibition of carbohydrate, fat, and protein oxidation and insulin resistance. *J. Clin. Invest.* **81**, 1137–1145
- Yki-Jarvinen, H. and Nikkila, E. A. (1985) Ethanol decreases glucose utilization in healthy man. *J. Clin. Endocrinol. Metab.* **61**, 941–945
- Holbrook, T. L., Barrett-Connor, E. and Wingard, D. L. (1990) A prospective population-based study of alcohol use and non-insulin-dependent diabetes mellitus. *Am. J. Epidemiol.* **132**, 902–909
- Tsumura, K., Hayashi, T., Suematsu, C., Endo, G., Fujii, S. and Okada, K. (1999) Daily alcohol consumption and the risk of type 2 diabetes in Japanese men – the Osaka Health Survey. *Diab. Care* **22**, 1431–1437
- Wei, M., Kampert, J. B., Gibbons, L. W., Blair, S. N. and Mitchell, T. L. (2000) Alcohol intake and incidence of type 2 diabetes in men. *Diab. Care* **23**, 18–22
- Wilkes, J. J., DeForrest, L. L. and Nagy, L. E. (1996) Chronic ethanol feeding in a high-fat diet decreases insulin-stimulated glucose transport in rat adipocytes. *Am. J. Physiol.* **271**, E477–E484
- Tuma, D. J., Casey, C. A. and Sorrell, M. F. (1991) Chronic ethanol-induced impairments in receptor-mediated endocytosis of insulin in rat hepatocytes. *Alcoholism: Clin. Exp. Res.* **15**, 808–813
- Lieber, C. S. and DeCarli, L. M. (1982) The feeding of alcohol in liquid diets: two decades of application and 1982 update. *Alcoholism: Clin. Exp. Res.* **6**, 523–531
- Simpson, E. A., Yver, D. R., Hissin, P. J., Wardzala, L. J., Darnielli, E., Salans, L. B. and Cushman, S. W. (1983) Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cell: Characterization of subcellular fractions. *Biochim. Biophys. Acta* **763**, 393–407
- Yu, B., Poirier, L. A. and Nagy, L. E. (1999) Mobilization of GLUT4 from intracellular vesicles by insulin and K^+ depolarization in cultured H9c2 myotubes. *Am. J. Physiol.* **277**, E259–E267

- 18 Czech, M. P. and Buxton, J. M. (1993) Insulin action on the internalization of the GLUT4 glucose transporter in isolated rat adipocytes. *J. Biol. Chem.* **268**, 9187–9190
- 19 Holman, G. D., Kozka, I. J., Clark, A. E., Flower, C. J., Saltis, J., Habberfield, A. D., Simpson, I. A. and Cushman, S. W. (1990) Cell surface labeling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. *J. Biol. Chem.* **265**, 18172–18179
- 20 Kummer, J. L., Calera, M. R. and Pilch, P. F. (1998) Separation of IRS-1 and PI3-kinase from GLUT4 vesicles in rat skeletal muscle. *Biochem. Biophys. Res. Commun.* **246**, 282–286
- 21 Taha, C. and Klip, A. (1999) The insulin signalling pathway. *J. Membrane Biol.* **169**, 1–12
- 22 Gatch, M. B., Wallis, C. J. and Lal, H. (1999) The effects of adenosine ligands R-PIA and CPT on ethanol withdrawal. *Alcohol* **19**, 9–14
- 23 Bhavani, K., de la Monte, S., Brown, N. V., Xu, Y. Y., Sasaki, Y. and Wands, J. R. (1995) Effect of ethanol on p36 protein kinase substrate and insulin receptor substrate 1 expression and tyrosyl phosphorylation in human hepatocellular carcinoma cells. *Alcoholism: Clin. Exp. Res.* **19**, 441–446
- 24 Resnicoff, M., Sell, C., Ambrose, D., Baserga, R. and Rubin, R. (1993) Ethanol inhibits the autophosphorylation of the insulin-like growth factor (IGF-1) and IGF-1-mediated proliferation of 3T3 cells. *J. Biol. Chem.* **268**, 21777–21782
- 25 Xu, Y. Y., Bhavani, K., Wands, J. R. and de la Monte, S. M. (1995) Ethanol inhibits insulin receptor substrate-1 tyrosine phosphorylation and insulin-stimulated neuronal thread protein gene expression. *Biochem. J.* **310**, 125–132
- 26 Karl, P. I. and Fisher, S. E. (1994) Chronic ethanol exposure inhibits insulin and IGF-1 stimulated amino acid uptake in cultured human placental trophoblasts. *Alcoholism: Clin. Exp. Res.* **18**, 942–947
- 27 Patel, B. C., D'Arville, C., Iwahashi, M. and Simon, F. R. (1991) Impairment of hepatic insulin receptors during chronic ethanol administration. *Am. J. Physiol.* **261**, G199–G205
- 28 Sasaki, Y. and Wands, J. R. (1994) Ethanol impairs insulin receptor substrate-1 mediated signal transduction during rat liver regeneration. *Biochem. Biophys. Res. Commun.* **199**, 403–409
- 29 Rea, S. and James, D. E. (1997) Moving GLUT4: the biogenesis and trafficking of GLUT4 storage vesicles. *Diabetes* **46**, 1667–1677
- 30 Sweeney, G., Somwar, R., Ramlal, T., Volchuk, A., Ueyama, A. and Klip, A. (1999) 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
- 31 Zierler, K. (1998) Does insulin-induced increase in the amount of plasma membrane GLUTs quantitatively account for insulin-induced increase in glucose uptake? *Diabetologia* **41**, 724–730
- 32 Rosholt, M. N., King, P. A. and Horton, E. S. (1994) High-fat diet reduces glucose transporter responses to both insulin and exercise. *Am. J. Physiol.* **266**, R95–R101
- 33 Green, A., Carroll, R. M. and Dobias, S. B. (1996) Desensitization of beta-adrenergic receptors in adipocytes causes increased insulin sensitivity of glucose transport. *Am. J. Physiol.* **271**, E271–E276
- 34 Moxham, C. M. and Malbon, C. C. (1996) Insulin action impaired by deficiency of the G-protein subunit G_{α_2} . *Nature (London)* **379**, 840–844
- 35 Guo, J. H., Wang, H. Y. and Malbon, C. C. (1998) Conditional, tissue-specific expression of Q205L G_{α_i2} in vivo mimics insulin activation of c-Jun N-terminal kinase and p38 kinase. *J. Biol. Chem.* **273**, 16487–16493
- 36 Zhang, X.-L., Guo, J. H., Wang, H.-Y. and Malbon, C. C. (1998) Expression of constitutively activated G_{α_2} in vivo ameliorates streptozotocin-induced diabetes. *J. Biol. Chem.* **273**, 23649–23651

Received 28 June 2000/6 November 2000; accepted 12 December 2000