Increased intracellular localization of brain GLUT-1 transporter in response to ethanol during chick embryogenesis

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In this study, we examined the intracellular localization of GLUT-1 and GLUT-3 transporters in chick embryos exposed to ethanol. The level of GLUT-1 protein is higher in early embryological development and decreases as the organism matures, in contrast to the level of GLUT-3 protein that increases as development proceeds (17). Regulation of the facilitative glucose transport proteins is necessary for maintenance of cellular glucose homeostasis and occurs in response to a variety of physiological stimuli, including various growth factors (4, 6), phorbol esters (22), and serum (13). Increases in GLUT-1 and GLUT-3 transporter expression have been attributed to changes in the rates of transcription (9), translation (43), protein and mRNA turnover (23, 33), and translocation from existing intracellular pools (4, 10, 44).

The increased levels of GLUT-1 and GLUT-3 transporter expression in response to ethanol exposure demonstrate that ethanol exposure alters the regulation of these transporters. The data suggest that ethanol exposure decreases the uptake of glucose, the underlying molecular mechanism has yet to be defined (14, 27, 34, 36). In a tissue as glucose-dependent as the developing brain, even a small aberration in the regulation of glucose transport might result in the defects associated with ethanol exposure. Increased glucose uptake induced by ethanol exposure is associated with increases in the total quantity of both GLUT-1 and GLUT-3 transporters. The data suggest that GLUT-3 expression on the plasma membrane remains resistant to the deleterious effect of ethanol on the embryonic brain. In contrast, GLUT-1 appears to be deregulated by ethanol and the increased localization of GLUT-1 on intracellular membranes, and decreased expression on the plasma membrane...
may be part of the cellular mechanism to maintain normal glucose uptake despite the significant increase in total GLUT-1.

**EXPERIMENTAL PROCEDURES**

Materials. Rabbit anti-rat GLUT-1 antibody was obtained from Charles River PharmServices, Southbridge, MA (formerly East Acres), and rabbit anti-chicken GLUT-3 antibody (41) was a kind gift of Dr. Martyn White (Thomas Jefferson University, Philadelphia, PA). The goat anti-rabbit IgG peroxidase-conjugated antibody was purchased from Sigma (St. Louis, MO), FCS, trypsin, and Dulbecco's modified Eagle's medium with Ham's F-12 were obtained from Sigma. 2-Deoxy-D-[3H]glucose was obtained from New England Nuclear. The kit for performing the bicinchoninic acid (BCA) protein assay was obtained from Pierce Chemical (Rockford, IL). All other reagents used were of analytical grade from commercial sources.

Solutions. Chick Ringer solution (CR) consisted of 123 mM NaCl, 5 mM KCl, 1.6 mM CaCl2, and 5 mM HEPES buffer, pH 7.0. Pilch's homogenization buffer contained 25 mM HEPES, 4 mM EDTA, 25 mM benzamidine, 57 µM phenylmethylsulfonyl fluoride (PMSF), 1 µM leupeptin, and 0.15 µM aprotinin. PBS, pH 7.0, consisted of 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH2PO4, and 8 mM Na2HPO4. Tris-buffered saline (TBS), pH 7.2, contained 0.01 M Tris and 0.15 M NaCl; TBST was TBS containing 0.05% Tween 20. Standard sodium citrate (SSC) (20×) was composed of 3 M NaCl and 0.3 M sodium citrate, pH 7.0.

Injection and preparation of chicken embryos. Nonincubated fertilized eggs were obtained from the Arbor Acre Arbor Acrechicken flock housed at the Poultry Science Department, North Carolina State University. Every 6–9 mo, the current flock was routinely shipped to market and replaced with a new flock. Therefore, although all experiments used the same strain of chicken, some experiments were done using eggs from different flocks. The eggs were randomly separated into vehicle (Veh) or ethanol (EtOH) groups. The Veh group was injected with 0.2 ml of CR. The EtOH group received 1.35 g/kg of ethanol diluted in CR in a total volume of 0.2 ml, as described previously (27). The selected dose of ethanol was 60% of the maximum ethanol dose that could be tolerated by the Veh and EtOH groups. All eggs were incubated and harvested as described previously by Marette et al. (18) with minor modifications. Briefly, 2 g of tissue per 30 ml of sucrose homogenization buffer (0.32 M sucrose, 10 mM NaHCO3, 1 mM MgCl2, 0.5 mM CaCl2, 5 mM Na3PO4, 57 µM PMSF, 1 µM leupeptin, and 0.15 µM aprotinin) was homogenized by 12 strokes in a Potter-Elvehjem tissue homogenizer. After centrifugation at 1,200 g for 10 min, the supernatant was collected, and the pellet was resuspended in sucrose homogenization buffer. The pellet was rehomogenized and again centrifuged. The second supernatant was combined with the first and centrifuged at 9,000 g for 10 min. The pellet (P3) was discarded, and the supernatant was centrifuged at 190,000 g for 1 h to collect the crude membrane fraction in the pellet. This fraction was resuspended in sucrose homogenization buffer and layered on a discontinuous gradient composed of 25, 30, and 35% sucrose. The gradients were centrifuged at 150,000 g for 16 h, and the bands at the interface of each gradient were harvested, diluted 1:10 with sucrose wash buffer (0.32 M sucrose and 10 mM NaHCO3), and centrifuged at 190,000 g for 1 h to collect the membrane fractions. Marker enzyme analysis indicated that the plasma membrane and the intracellular membranes were enriched in the 25 and 35% sucrose bands, respectively. After protein quantitation by BCA assay, the fractions were snap-frozen and stored at −80°C.

Enzyme marker analysis of membranes. The various membrane fractions were assayed for the activity level of several marker enzymes differentially associated with plasma (PM)
or intracellular membranes (IM) to determine the relative enrichment or depletion of the isolated membrane fractions. The assay for Na−K+ -ATPase activity, an enzyme marker associated with the PM, was performed as described by Schimmel et al. (32). The ouabain-sensitive Na−K+ -ATPase activity was calculated as the difference in ATPase activity in the presence and absence of 2.5 mM ouabain. 5′-Nucleotidase activity (also a PM marker) was measured with a modification of the method of Schimmel et al. Enzyme activity was measured in the presence of 4 µg SDS/20 µg of protein with either 5 mM 5′-AMP or 5 mM 3′-AMP, and the release of Pi from 5′-AMP above that from 3′-AMP was taken as specific 5′-nucleotidase activity. The released Pi levels were quantitated by the Fiske-Subba Row method by use of a kit (part no. 670-A obtained from Sigma (St. Louis, MO). NADPH-cytochrome c reductase activity, an enzyme marker for the light-microsomal fraction, was measured spectrophotometrically at 550 µm, as described by Parry and Pedersen (24). The membrane fraction was pretreated with 10% Tween 80 on ice for 15 min before the assay for NADPH-cytochrome c reductase activity. Thiamine pyrophosphatase (TPPase) activity is associated with the Golgi and endoplasmic reticulum and was assayed in the presence of 1% Triton X-100 by the method of LaFrenz et al. (15), and the released Pi was quantitated as described by Baginski and Zak (2).

RNA isolation and Northern blotting. Total cellular RNA was isolated from the chicken embryo tissue samples harvested on days 4, 5, and 7 by use of Trizol reagent (GIBCO Life Technologies). Twenty-microgram samples were separated on 1.2% agarose-formaldehyde gels, and the bands were transferred overnight to polyvinylidene fluoride (PVDF) membranes (Hybond-N, Amersham, Arlington Heights, IL) by capillary diffusion with 5× SSC buffer. The RNA was cross-linked to the membrane by a 1-min exposure to ultraviolet (UV) light in a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA). All probes were labeled with [α-32P]ATP to high specific activity by the random primer method. The chicken cDNA probes were a kind gift of Dr. Martyn White, Thomas Jefferson University: 1) a 2.0-kb EcoR I/BamH I fragment containing chicken GLUT-1 cDNA (41), 2) a 1.7-kb fragment containing chicken GLUT-3 (42), and 3) a 1.1-kb Pst I fragment containing chicken malic enzyme cDNA (37). The probes were hybridized with the membrane in Hybrisol I (Oncogene, Cambridge, MA) overnight at 48°C, the membrane was then washed twice in a solution of 0.1× SSC/0.1% SDS at room temperature followed by a wash at 50°C. The membranes were then exposed to a PhosphorImager screen, and the RNA bands were quantitated by ImageQuant software (Molecular Dynamics).

Uptake of 2-deoxy-D-[3H]glucose. On the day of assay, embryos were harvested (days 4, 5, or 7 of incubation) and dissociated by trypsinization as described previously (34). Uptake of 2-deoxy-D-[3H]glucose (2-DOG) was performed with minor modifications, as previously described (27). Briefly, cell viability was determined, and 2 × 105 viable cells were resuspended in serum-free medium and added to 200 µl of culture medium containing 50 mM cold 2-DOG in PBS. The cells were centrifuged at 37°C for 5 min. Twenty microliters of 2-DOG (5 µCi/vial) in 0.2 mM or 500 mM cold 2-DOG in PBS were added to replicate vials. The cells were incubated at 37°C and 5% CO2 for 30 min. After a centrifugation at 1,000 g for 5 min at 4°C, the supernatant was decanted and the cell pellet was washed twice with 1.5 ml of ice-cold PBS. The cell pellet was lysed by adding 0.1 ml of 1 N NaOH and incubating the vials at 37°C for 2 h with periodic mixing. Triplicate aliquots were transferred to scintillation vials, 3 ml of 30% Scintisafe (Fisher Scientific) were added, and the vials were counted in a Beckman LS 6500 counter. Specific 2-DOG uptake was calculated as 2-DOG uptake (total) minus 2-DOG uptake (nonspecific). Earlier studies by Pennington et al. (27) demonstrated that cytochalasin B treatment completely blocked 2-DOG transport.

Statistical analysis. Group means and standard errors, as well as post hoc testing of significant differences between means for the various treatments, were calculated using the general linear model procedure of the SAS/PC statistical program (SAS, Cary, NC). Statistically significant differences between group means were determined by one-way ANOVA. The statistical significance of the Western and Northern blot data was performed by Student's t-test using Statgraphics software (Manugistics, Rockville, MD).

RESULTS

Ethanol-induced growth suppression. As shown in Table 1, chicken embryos injected in ovo on day 0 with 1.35 g/kg ethanol (EtOH group) and harvested on days 4 (P < 0.01) and 5 (P < 0.005) had significantly decreased mean total weights vs. both vehicle-injected (Veh) and untreated control groups (Sed). In the EtOH group, the heads of both day 5 and 7 embryos were significantly smaller than those of the control groups. On day 4, the mean head weight of the Veh group constituted 45.6% of the total embryo weight. In the age-matched EtOH group, the head weight decreased to 38.9% of the total weight.

2-DOG uptake by dissociated cells. The uptake of glucose was assayed as 2-DOG uptake in dissociated cells obtained from day 4 embryos and day 5 and day 7 heads. Data are presented in Fig. 1. Ethanol exposure in ovo caused a small but significant increase (P < 0.05) in 2-DOG uptake by cells isolated from day 5 heads compared with Veh controls (21.2 ± 1.7 vs. 18.2 ± 0.8 pmol mg protein−1·10 min−1) and untreated controls (16.7 ± 1.8 pmol mg protein−1·10 min−1). The uptake of 2-DOG by cells derived from day 7 EtOH heads was

Table 1. Ethanol exposure in ovo causes growth suppression of chicken embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>Embryo Age, days</th>
<th>Untreated controls</th>
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<tr>
<td></td>
<td>Head only</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0.049 ± 0.012</td>
<td>0.046 ± 0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10)</td>
<td>(10)</td>
</tr>
<tr>
<td>Vehicle controls</td>
<td>Head only</td>
<td>0.040 ± 0.010</td>
<td>0.047 ± 0.016</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0.046 ± 0.013</td>
<td>0.065 ± 0.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10)</td>
<td>(10)</td>
</tr>
<tr>
<td>EtOH treated</td>
<td>Head only</td>
<td>0.027 ± 0.008</td>
<td>0.028 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0.072 ± 0.011</td>
<td>0.072 ± 0.011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10)</td>
<td>(10)</td>
</tr>
</tbody>
</table>

Notes: *P < 0.01; †P < 0.005 vs. both control groups. NA, not assayed.
also significantly increased (P < 0.025) compared with both control groups.

Effect of in ovo ethanol exposure on total glucose transporter proteins. Glucose transporter protein levels were estimated during embryological development to determine whether ethanol-induced changes in transporter proteins were responsible for the increase in glucose uptake seen in ethanol-treated embryos. Western blotting methodology was used to estimate the levels of GLUT-1 and GLUT-3 proteins in total membrane samples of whole embryos on day 4 and in day 5 and day 7 cranial tissue. Representative immunoblots are shown above the bar charts in Fig. 2, A and B. Three proteins were identified in the 45- to 55-kDa range. This result is consistent with the reported range of molecular weights for the GLUT-1 protein, which may vary because of heterogeneous glycosylation (12, 28). As shown in Fig. 2A, in ovo EtOH treatment caused a significant 7- to 10-fold increase (P < 0.001) in the total amount of GLUT-1 protein from day 5 EtOH heads vs. Veh controls. The amount of GLUT-1 protein present in day 7 EtOH heads was increased 2-fold compared with Veh controls (P < 0.005). No significant increase in GLUT-1 was found in day 4 embryos. GLUT-1 in EtOH-treated cranial tissue was increased over controls, and these differences sustained statistical significance in day 5 and day 7 embryos.

The quantitation of the GLUT-3 protein (45 kDa) and a representative immunoblot are presented in Fig. 2B. Total GLUT-3 protein levels in day 4 and day 7 embryos were also increased significantly (P < 0.01 and P < 0.05, respectively) vs. Veh controls.

Effect of ethanol treatment on mRNA levels for GLUT-1 and GLUT-3. The significant increases in glucose transporter protein levels in response to ethanol might most simply be explained by an increase in the amount of GLUT-1 and/or GLUT-3 mRNA. To address this question, Northern blotting methodology was used to estimate the level of GLUT-1 and GLUT-3 mRNA isolated from day 4, day 5, and day 7 embryos. Representative blots are presented in Fig. 3, A and B. Also shown in Fig. 3 is the quantitation of the mRNA bands as a percentage of Veh levels in 2 separate, representative experiments. Ethanol treatment only increased the GLUT-1 mRNA levels 10–20% in all developmental ages tested, and GLUT-3 mRNA levels (3.2-kb band) remained essentially unchanged. There was a slight decrease in GLUT-3 mRNA only in day 7 embryos in the 1.7-kb band. None of these changes was statistically significant. pGAD3 levels, used as a control to correct for unequal loading or a differential transfer of RNA, were the same for all blots after rehybridization of the Northern blot (Fig. 3C). Because EtOH treatment had minimal effects on the glucose transporter mRNA levels, we investigated another mechanism that might explain these findings.

Marker enzyme distribution. In an attempt to reconcile the growth suppression, the small increase in glucose uptake and the large increase in GLUT-1 levels in EtOH embryos, cell subfraction experiments were performed to obtain the PM and IM fractions. Because of the extent of growth suppression and the large increase in total GLUT-1 transporter protein levels observed in the day 5 EtOH embryos, this developmental age was the focus of the membrane subfractionation experiments. As described in Ethanol treatment alters the distribution of GLUT-1 in membrane fractions, and in Fig. 4, sucrose gradients were used to subfractionate total membrane preparations from Sed, Veh, and EtOH embryos into PM and IM fractions. To assess the relative enrichment or depletion of the isolated PM and
IM fractions, various enzyme activities were assayed on the isolated fractions. These data are presented in Table 2. The 5'-nucleotidase and Na⁺-K⁺-ATPase enzyme activities were used as markers for the PM in the brain and other tissue (19, 29). There was at least a 3.0-fold enrichment of these two enzyme markers in the PM fractions compared with the crude fraction and a 2- to 3-fold decrease in the IM. NADPH-cytochrome c reductase is associated with the light-microsomal fractions, and TPAPase activities reside in the Golgi and endoplasmic reticulum (15, 19, 29). TPAPase activity was increased in the IM fraction. There was a relative depletion of NADPH-cytochrome c reductase activity in the PM fraction and a 2-fold increase in activity in the IM fraction. Based on the enrichment of specific marker enzymes in the two fractions (PM and IM), we recovered an average 12.4% of the total PM and 5.5% of the total IM. These are typical recoveries, as is the higher percent recovery of the PM.

Based on marker enzyme analysis, the PM and IM fractions were defined as the 25 and 35% sucrose fractions, respectively. Additively, these results supported a differential enrichment/depletion of the expected enzyme activities in the isolated PM and IM fractions, indicating that the membrane fractions used in the experiments yet to be described are relatively enriched for PM or IM.

Ethanol treatment alters the distribution of GLUT-1 in membrane fractions. Immunoblotting analysis was used to determine the amount of GLUT-1 and GLUT-3 proteins in each of the membrane fractions. A representative blot is shown in Fig. 4A. The total amount of GLUT-1 protein in day 5 cranial tissue in the EtOH group (obtained by summing together the amount of GLUT-1 protein present in the membrane fractions

Fig. 2. Effect of ethanol and embryo age on glucose transporter proteins. Total membranes were prepared from chick embryonic tissue (day 4 whole embryos, day 5 and day 7 cranial tissue) as described in EXPERIMENTAL PROCEDURES. Proteins were resolved by SDS-PAGE and analyzed by Western blotting. Blots show distribution of GLUT-1 (45- and 55-kDa bands, A) and GLUT-3 (B) glucose transporters in day 4, day 5, and day 7 embryos, with 30 µg of protein loaded in each lane. V, vehicle controls; E, EtOH treated. Histograms summarize quantitation of densitometrically scanned immunoblots for glucose transporters performed in 5 experiments. Values are arbitrary units (means ± SE). *P < 0.05; **P < 0.01; ***P < 0.001 vs. Veh control.
from a given treatment group) increased to 151% of controls. These results agree with the increase in GLUT-1 protein in the total membrane preparation of EtOH-treated embryos. As shown in Fig. 4A, the amount of GLUT-1 protein in the EtOH PM was 30% less than that found in the Veh or Sed group (P < 0.05), and the amount of GLUT-1 in the IM fraction was 60% greater in the EtOH group than in either of the control groups (P < 0.05). The results are also expressed as arbitrary units ± SE in the legend for Fig. 4. The mean ratio of GLUT-1 protein in the PM-to-IM ratio (PM/IM) was 2.02 ± 0.42 in the EtOH group vs. 12.45 ± 0.63 in the Veh group. The exposure to ethanol in ovo significantly decreased the amount of GLUT-1 protein in the PM and increased the amount in the IM in day 5 embryos.

GLUT-3 transporter protein levels in the PM remain unchanged. In contrast to the decreased amount of GLUT-1 protein found on the PM, the amount of GLUT-3 transporter on the PM of EtOH-treated embryos was unchanged vs. Veh controls. These data and a representative blot are presented in Fig. 4B. In the EtOH-treated embryos, the total quantity of GLUT-3 transporter present increased to 131% of controls. Greater than 90% of the GLUT-3 protein in the three treatment groups was found on the PM, and less than 10% was on the IM. However, the amount of GLUT-3 on
Fig. 4. In ovo ethanol exposure causes an increased localization of GLUT-1 protein on intracellular membranes (IM). Cranial tissue was harvested from day 5 chick embryos, and subcellular fractions were isolated by discontinuous sucrose gradients, as described in EXPERIMENTAL PROCEDURES. Proteins were resolved by SDS-PAGE and analyzed by immunoblotting for GLUT-1 (A) and GLUT-3 (B) transporter proteins. Representative blots are shown. Protein (10 µg) was loaded in each lane. CM, crude membrane, the whole membrane fraction immediately before subfractionation on sucrose gradients; PM, plasma membrane; PEL, pellet from sucrose gradient fractionation. Histogram summarizes quantitation of densitometrically scanned immunoblots performed in 3 different experiments. Results are expressed as % of total GLUT-1 or GLUT-3 protein (obtained by dividing amount of transporter protein in each fraction by total amount of that transporter in sample). Values are means ± SE for GLUT-1 (A): untreated (UnRx), PM 120,555 ± 7,453 and IM 18,227 ± 1,108; Veh, PM 137,148 ± 15,960 and IM 13,896 ± 684; EtOH, PM 207,073 ± 12,854 and IM 70,365 ± 4,617; and for GLUT-3 (B): UnRx, PM 8,226 ± 756 and IM 462 ± 411; Veh, PM 12,834 ± 2,545 and IM 838 ± 411; EtOH, PM 16,822 ± 3,100 and IM 416 ± 76. *P < 0.05 vs. control.

Table 2. Marker enzyme distribution

<table>
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<tr>
<th>Treatment</th>
<th>Membrane Fraction</th>
<th>Na+–K+-ATPase*</th>
<th>TPPase*</th>
<th>5'-Nucleotidase*</th>
<th>NADPH-Cytochrome c Reductase†</th>
</tr>
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<tbody>
<tr>
<td>Untreated controls</td>
<td>CM</td>
<td>2.5 ± 0.83</td>
<td>23.4 ± 3.9</td>
<td>2.63 ± 0.021</td>
<td>5.80 ± 0.02</td>
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<td></td>
<td>PM</td>
<td>14.5 ± 0.57</td>
<td>26.9 ± 1.06</td>
<td>7.92 ± 0.46</td>
<td>5.57 ± 0.31</td>
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<tr>
<td></td>
<td>IM</td>
<td>8.5 ± 1.83</td>
<td>44.7 ± 2.1</td>
<td>0.62 ± 0.18</td>
<td>7.36 ± 0.31</td>
</tr>
<tr>
<td>Vehicle controls</td>
<td>CM</td>
<td>2.6 ± 0.06</td>
<td>26.1 ± 1.4</td>
<td>2.00 ± 0.08</td>
<td>5.57 ± 0.94</td>
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<tr>
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<td>PM</td>
<td>15.3 ± 0.28</td>
<td>28.8 ± 1.7</td>
<td>8.8 ± 0.92</td>
<td>4.46 ± 0.45</td>
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<td>IM</td>
<td>7.1 ± 0.28</td>
<td>45.1 ± 0.21</td>
<td>0.41 ± 0.29</td>
<td>7.58 ± 1.26</td>
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<tr>
<td>EtOH treated</td>
<td>CM</td>
<td>0.96 ± 0.42</td>
<td>22.8 ± 0.15</td>
<td>2.3 ± 0.28</td>
<td>6.01 ± 0.31</td>
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<tr>
<td></td>
<td>PM</td>
<td>14.6 ± 0.35</td>
<td>31.3 ± 0.35</td>
<td>6.5 ± 0.18</td>
<td>5.74 ± 0.075</td>
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<tr>
<td></td>
<td>IM</td>
<td>5.5 ± 0.35</td>
<td>41.5 ± 1.7</td>
<td>0.78 ± 0.14</td>
<td>7.36 ± 0.94</td>
</tr>
</tbody>
</table>

Crude cell membranes (CM) obtained from day 5 chick embryonic brains were subfractionated on sucrose gradients into plasma (PM) and intracellular membrane (IM) fractions as described in EXPERIMENTAL PROCEDURES. Fractions were assayed for enzyme activities to ascertain the relative enrichment/depletion. Values are means ± SE of 3 separate experiments. Na+–K+-ATPase and 5'-nucleotidase are enzyme markers for the PM. Thiamine pyrophosphatase (TPPase) and NADPH-cytochrome c reductase are enzyme markers for IM. Specific activities are in *µmol Pi·mg protein⁻¹·h⁻¹; †nmol cytochrome c·mg protein⁻¹·h⁻¹.
the IM was significantly decreased in the EtOH-treated cranial tissue. PM/IM in the EtOH-treated group was $36.9 \pm 4.3$ vs. $16.9 \pm 3.3$ in the Veh controls. As shown in the legend of Fig. 4, this shift in the ratio reflects a small, nonsignificant increase of GLUT-3 on the PM and a significant decrease on the IM.

**DISCUSSION**

Various investigators have searched for a unifying hypothesis to explain ethanol-induced fetal growth suppression and motor, sensory, and neural defects (26, 34, 37). Because glucose is the primary source of fuel energy for the brain during development (17, 21), the studies reported here focused on the uptake/transport of glucose by fetal brain tissue during early chicken development. This report is the first to show ethanol-induced changes in the subcellular distribution of the GLUT-1 transporter protein in an embryological chick model. Although the fundamental relationships between glucose uptake, glucose transporter protein, and mRNA and the EtOH-induced changes have been investigated previously, the results remain ambiguous. This result could be due to the use of model systems in which the ethanol was subject to metabolism (whole animal models) (35, 37), and/or the effect on embryological development could not be measured (cultured cells) (44).

The data presented here also include the first report of the relationship among glucose uptake, GLUT-1 and GLUT-3 transporter protein levels, and transporter mRNA as induced by in ovo ethanol exposure of the fetal brain during early embryogenesis. These findings are summarized in Table 3. Because the placental function and/or the nutritional state of the mother can affect the fetus (1), the chicken developmental model was selected because it allows the study of the direct effect of ethanol on the developing embryo without placental or maternal influences. Additionally, the studies presented here used embryos before day 9 of gestation to avoid the complication of ethanol metabolism by alcohol dehydrogenase that appears developmentally in the chick at that time (45), thus ruling out the complicating effect of acetaldehyde on glycoprotein secretion (40) and ensuring that essentially constant levels of ethanol are maintained in ovo.

In ovo ethanol exposure resulted in a significant degree of overall growth suppression at all three embryological ages tested (days 4, 5, and 7), supporting our previous studies performed in day 5 chicken embryos (7, 27). Growth suppression of the fetus/embryo by ethanol is well documented in human fetuses (8), whole rat embryos cultured in vitro (37), and brains from rat pups exposed in utero (35). In the data presented here, the ethanol-induced growth suppression of cranial tissue on days 5 and 7 was also significant.

The increases in GLUT-1 levels (total membranes) and in glucose uptake concur with those of Singh et al. (36), who reported that the brains of adult rats chronically fed an ethanol diet had small but significant increases in glucose transport and in GLUT-1 transporter number but decreased mRNA levels. The increased glucose uptake in cranial tissue also confirms an earlier report from this laboratory describing an increase in glucose uptake by cultured mixed fibroblasts obtained from day 5 chicken embryos exposed to ethanol in ovo (34).

However, in an earlier study (27), our group had found that in ovo ethanol exposure resulted in decreased basal glucose uptake in cells isolated from day 5 chicken embryos. Those studies used cells dissociated from whole embryos, whereas the cells used in the current study were obtained only from cranial tissue. The report of Eckstein et al. (7) from this laboratory found a slight increase in glucose uptake in dissociated cells and a small but significant decrease in the 45-kDa band of the GLUT-1 protein in heated whole lysates of day 5 chick embryonic cranial tissue (7). Haspel et al. (11) have reported that heating of GLUT-1 proteins can cause the formation of aggregates and may alter the density and/or number of bands observed. Unheated proteins derived from total membrane preparations were used in the experiments reported here, and these methodological changes may explain the observed differences.

Interestingly, ethanol treatment appears to have altered transporter distribution, with marked accumulation of GLUT-1 in the IM and decreased expression on the PM. In contrast, GLUT-3 was significantly decreased on the IM and unchanged on the PM. There is evidence from the literature to suggest that ethanol treatment can disturb protein processing and trafficking (38, 40). The findings might suggest that the total glucose transport potential of the PM (GLUT-1 + GLUT-3) in fetal chicken brain exposed to ethanol in ovo has decreased. Alternatively, the small significant increase in glucose transport observed in day 5 brains might result from modulation of the intrinsic activity of the remaining transporters.

<table>
<thead>
<tr>
<th>Experimental Parameter</th>
<th>Changes in EtOH-Treated Embryos Relative to Vehicle Controls</th>
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<tbody>
<tr>
<td></td>
<td>Day 4</td>
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<tr>
<td><strong>Growth suppression</strong></td>
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<tr>
<td>Head weight</td>
<td>NA</td>
</tr>
<tr>
<td>Total weight</td>
<td>50%</td>
</tr>
<tr>
<td>2-DOG uptake</td>
<td>NS</td>
</tr>
<tr>
<td>GLUT-1</td>
<td></td>
</tr>
<tr>
<td>Total membrane protein</td>
<td></td>
</tr>
<tr>
<td>(45 kDa)</td>
<td>NS</td>
</tr>
<tr>
<td>PM protein</td>
<td>NA</td>
</tr>
<tr>
<td>IM protein</td>
<td>NA</td>
</tr>
<tr>
<td>mRNA</td>
<td>NS</td>
</tr>
<tr>
<td>GLUT-3</td>
<td></td>
</tr>
<tr>
<td>Total membrane protein</td>
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<tr>
<td>(45 kDa)</td>
<td>2-fold</td>
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<tr>
<td>IM protein</td>
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</tr>
<tr>
<td>mRNA</td>
<td>NS</td>
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</table>

EtOH, ethanol; 2-DOG, 2-deoxy-o-[3H]glucose; NA, not assayed; NS, nonsignificant change vs. vehicle controls.
One potential conclusion from these findings is to assign GLUT-3 a role as the primary glucose transporter in ethanol-exposed cranial tissue. However, because the individual affinities of chicken GLUT-1 and GLUT-3 for 2-DOG are not described, we are unable to determine which, if either, is the primary transporter responsible for the small significant increase in 2-DOG uptake. The increased 2-DOG uptake is associated with increases in the total quantity of both transporters. If ethanol exposure perturbs GLUT-1 regulation and not GLUT-3, possibly by upregulating GLUT-1 synthesis, then the increased localization of GLUT-1 to the IM, and not to the PM, may be a successful attempt to maintain normal glucose uptake. Krauss et al. (14) reported that GLUT-3, in contrast to GLUT-1, is resistant to ethanol suppression. They also concluded that the suppressive effect of ethanol is directly on transport and not on glucose metabolism. Alternatively, the decrease in the PM could reflect an accelerated internalization of the protein.

The discrepancy between the 7- to 10-fold increase in total GLUT-1 and the lack of comparable changes in GLUT-1 PM and IM is most probably explained by the disparate losses in the different membrane compartments. We found 1–10% of total GLUT-1 in the fraction enriched in mitochondria but devoid of PM or IM based on enzyme marker analysis. GLUT-1 and GLUT-3 have been found to be associated with mitochondria (16). Based on the enrichment of specific marker enzymes in the two fractions, we recovered an average 12.4% of the total PM and 5.5% of the total IM. These are typical recoveries, as is the higher percent recovery of the PM.

Additionally, the studies utilizing total membrane proteins were conducted temporally earlier than the membrane subfractionation experiments. In the ensuing time gap, the chicken flock was shipped to market and replaced by a new one of the same strain, and the 55-kDa isoform was found to be absent in the new flock. This phenomenon seems to be associated only with the 55-kDa form, because we see a consistent ethanol-induced effect on the 45-kDa form of GLUT-1 in all the flocks we have tested.

One limitation of this study is the inability to identify which CNS cell population(s), glial, endothelial and/or others, have been affected by ethanol exposure. Furthermore, the cellular distribution of both GLUT-1 forms and GLUT-3 in the chicken brain remains unknown. Even though there is no blood-brain barrier at this developmental age of the chicken, ethanol may have a deleterious effect on the cells of the developing vasculature, possibly resulting in a negative impact on the nutrition of the immature brain (31). Although GLUT-3 may be functionally resistant to ethanol suppression, and GLUT-1 expression may be deregulated by the exposure, we are unable to assign these effects to a particular cell type.

In summary, in ovo ethanol exposure caused significant growth suppression during early chick development. This growth suppression is associated with a small but significant increase in glucose uptake, a dramatic increase in the total amount of GLUT-1, and a smaller increase in GLUT-3 protein. As opposed to the pattern in controls, an abnormal increased localization of GLUT-1 occurs on the IM in concert with a decrease on the PM. In contrast, GLUT-3 expression on the PM remains unchanged and is decreased on the IM. Ethanol may have deregulated the ability of the cell in the developing brain to control the location and, perhaps, the quantity of GLUT-1. The ethanol-induced overall increase in GLUT-1 may reflect a perturbation of GLUT-1 that does not result in an abnormal increase in glucose uptake because of the increased IM localization and reduced transporter on the PM. The data presented here also reveal the importance of examining the subcellular distribution of glucose transporters.

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