Effect of Ethanol on Na^+-P_i Uptake in Opossum Kidney Cells: Role of Membrane Fluidization and Reactive Oxygen Species

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This study was undertaken to examine the effect of ethanol on Na^+-dependent phosphate (Na^+-P_i) uptake in opossum kidney (OK) cells, an established renal proximal tubular cell line. Ethanol inhibited Na^+-dependent component of phosphate uptake in a dose-dependent manner with I_{50} of 8.4%, but it did not affect Na^+-independent component. Similarly, ethanol inhibited Na^+-dependent uptakes of glucose and amino acids (AIB, glycine, alanine, and leucine). Microsomal Na^+-K^+-ATPase activity was not significantly altered when cells were treated with 8% ethanol. Kinetic analysis showed that ethanol increased K_m without a change in V_{max} of Na^+-P_i uptake. Inhibitory effect of n-alcohols on Na^+-P_i uptake was dependent on the length of the hydrocarbon chain, and it resulted from the binding of one molecule of alcohol, as indicated by the Hill coefficient (n) of 0.8-1.04. Catalase significantly prevented the inhibition, but superoxide dismutase and hydroxyl radical scavengers did not alter the ethanol effect. A potent antioxidant DPPD and iron chelators did not prevent the inhibition. Pyrazole, an inhibitor of alcohol dehydrogenase, did not attenuate ethanol-induced inhibition of Na^+-P_i uptake, but it prevented ethanol-induced cell death. These results suggest that ethanol may inhibit Na^+-P_i uptake through a direct action on the carrier protein, although the transport system is affected by alterations in the lipid environment of the membrane.

Key Words: Alcohol, Na-P_i uptake, Antioxidants, Radical scavengers, Renal epithelial cells

INTRODUCTION

Ethanol has been reported to inhibit Na^+-dependent transports of glucose and amino acids in brush-border membrane vesicles prepared from renal proximal tubules (Fernandez et al, 1984; Elgavish & Elgavish, 1985; Parenti et al, 1991). The proposed mechanism of inhibition includes an increase of membrane fluidity (Fernandez et al, 1984), dissipation of the Na^+ gradient (Elgavish & Elgavish, 1985) resulting from an increase in the Na^+ permeability, and a direct interaction of the drug with the carrier proteins (Parenti et al, 1991). Similar mechanisms have been reported in intestinal brush-border membrane vesicles (Tillotson et al, 1981; Hunter et al, 1983). Studies with isolated membrane vesicles could provide an information on the direct effect of drugs on membrane integrity and transport systems.

Changes in membrane fluidity have been demonstrated to affect the activity of various membrane transport systems in epithelial cells (Le et al, 1991). In renal proximal tubular cells, Na^+-phosphate (Na^+-P_i) and Na^+-glucose cotransporters localized in apical membrane domains were shown to be very sensitive to alterations in the physical state of their lipid environment (Friedlander et al, 1988; Le et al, 1991). However, both transport systems are affected differently by changes in membrane fluidity: Na^+-P_i cotransport is stimulated by benzyl alcohol which increases membrane fluidity, whereas Na^+-glucose cotransport is inhibited by the alcohol (Friedlander et al, 1988; Friedlander et al, 1990). Although ethanol also causes an increase in membrane fluidity in various cell types (Chin & Goldstein, 1977; Chin et al, 1979), it is unclear whether it increases phosphate uptake in renal proximal tubular cells and the change is mediated by an alterations in membrane fluidity.

In intact cells, ethanol affects cellular signaling

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pathways (Rubin & Rottenberg, 1983; Zhang et al, 1996). These include generation of reactive oxygen species (ROS) (Bondy & Guo, 1994a; Bondy & Orozco, 1994b), changes in cAMP levels (Friedlander et al, 1988; DePetrillo & Swift, 1992), and alterations in protein kinase C activity (DePetrillo & Liou, 1993). Such changes could be responsible for alterations in Na^+-P_i uptake induced by ethanol in intact cells.

The present study was therefore undertaken to examine whether the Na^+-P_i transport activity depends on the lipid environment and ROS plays a role in the ethanol effect on the Na^+-P_i transport in opossum kidney (OK) cells, an established renal proximal tubular cell line.

METHODS

Cell culture

Opossum kidney (OK) cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained by serial passages in 75 cm^2 culture flasks (Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (Sigma, St. Louis, MO) without antibiotics at 37°C in 95% air-5% CO_2. The medium was supplemented with 10% fetal bovine serum. When the cultures reached confluence, subculture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were seeded on 24-well plate, and the cell monolayers were reached confluence after 4~5 days. The assays were performed for 2~3 days after confluence was reached.

Transport experiments

Cells were pretreated with alcohols or H_2O_2 for 60 min at 37°C in Hanks' balanced salt solution (HBSS), washed, and the uptake was measured for 30 min in the uptake medium containing $K_2H^{32}PO_4$, $[^{14}C]$-D-glucose, or $[^{14}C]$-L-amino acids. The composition of the uptake medium was 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM HEPES (pH 7.4). At the end of incubation, the uptake was stopped by drawing off the medium, and the monolayers were washed three times with ice-cold HBSS. The cells were solublized in 1 ml of 0.5% Triton X-100, and an aliquot of the solublized cells was transferred into scintillation vials to determine radio-

activity in a liquid scintillation counter (Packard, Tricarb 2100TR). The uptake of substrates was normalized to cell protein and expressed as pmoles per mg cell protein.

Cell viability

The viability in cultured cells was determined by a trypan blue exclusion assay, since this method was a more sensitive indicator of cell death than LDH release in cultured cells, as demonstrated by Aleo et al (Aleo et al, 1992). Following oxidant stress, the cells were harvested using 0.025% trypsin, incubated with 4% trypan blue solution, and were counted using a hemocytometer under light microscopy. Cells failing to exclude the dye were considered nonviable, and the data were expressed as a percentage of nonviable cells.

Na^+-K^+-ATPase activity measurement

The Na^+-K^+-ATPase activity was measured in the microsomal fraction prepared from OK cells. For the preparation of microsomal fraction, cells were grown to confluence in 100 mm dish, scraped from the dish in 10 mM mannitol and 2 mM Tris/HCl (pH 7.1) at 4°C, and briefly sonicated. Then, the cell lysate was centrifuged for 2 min at 2000 g to remove unbroken cells and the supernatant was saved, centrifuged for 12 min at 15,000 g. Pale-pink layer on top of pellet was removed and resuspended in 10 mM mannitol and 2 mM Tris/HCl (pH 7.1). Microsomal fraction was treated with ethanol for 60 min at 37°C and Na^+-K^+-ATPase was measured.

The ATPase activity was determined by measuring inorganic phosphate (Pi) released by ATP hydrolysis during incubation of microsome with an appropriate medium containing 3 mM ATP (Sigma) as the substrate. The total ATPase activity was determined in the presence of 100 mM Na^+, 20 mM K^+, 3 mM Mg, 2 mM EDTA, and 40 mM imidazole (pH 7.4). The Mg^{2+}-ATPase activity was determined in the absence of K^+ and in the presence of 1 mM ouabain. The difference between the total and the Mg^{2+}-ATPase activities was taken as a measure of the Na^+-K^+-ATPase activity. At the end of a 10-min incubation, the reaction was terminated by the addition of ice-cold 6% perchloric acid. The mixture was then centrifuged at 3,500 g, and Pi in the supernatant fraction was determined by the method of Fiske and
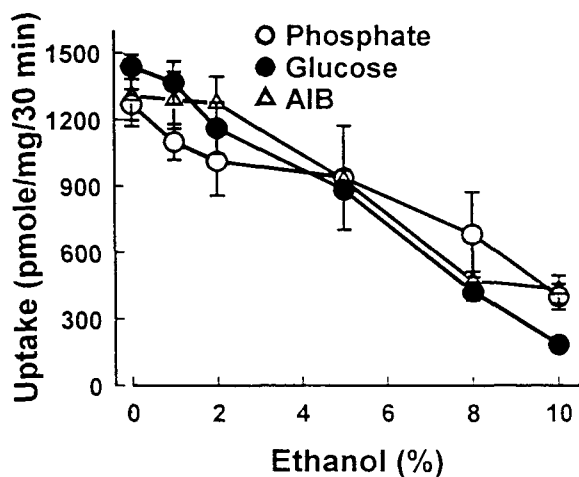
SubbaRow (Fiske & SubbaRow, 1925).

Reagents

K$_2$H^{32}PO$_4$, [^{14}C]-D-glucose, [^{14}C]-L-alanine, [^{14}C]-leucine, [^{14}C]-glycine, and -[^{14}C]-aminoisobutyric acid (AIB), were obtained from Amersham (Arlington Heights, IL). N,N'-diphenyl-p-phenylenediamine (DPPD) and dimethylurea (DMTU) were purchased from Aldrich Chemical (Milwaukee WI). Catalase, superoxide dismutase (SOD), deferoxamine, and phenanthroline were obtained from Sigma (St. Louis, MO). All other chemicals were of the highest commercial grade available.

Statistical analysis

Results were expressed as mean ± SE. Statistical differences between groups were evaluated using Student's t-test. Differences were considered significant at $p < 0.05$.

RESULTS

Effect of ethanol on Na$^+$-dependent uptakes

Ethanol inhibited uptakes of phosphate, D-glucose, and α-aminoisobutyric acid (AIB) in a dose-dependent manner (Fig. 1). The inhibitory effect of alcohol was similar in the three transport systems. Ethanol
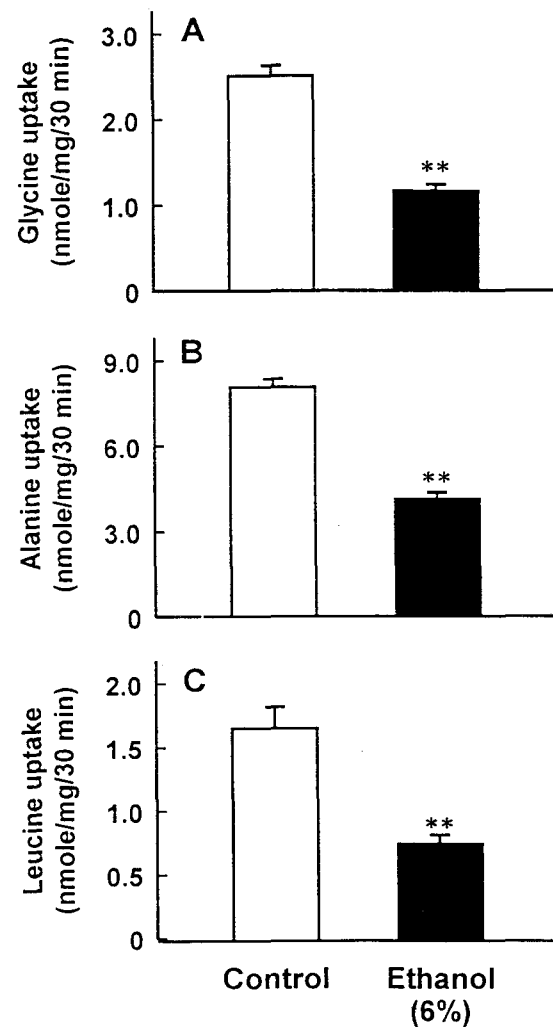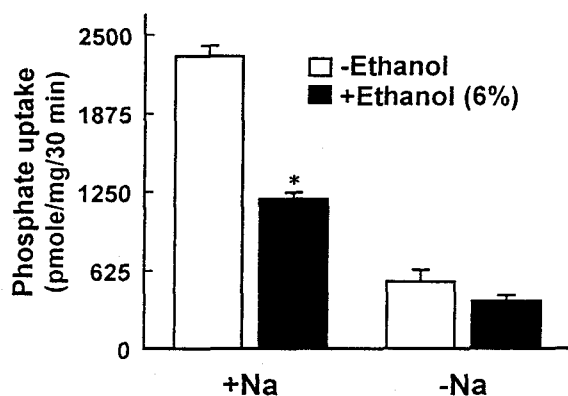

Fig. 1. Effect of ethanol on uptakes of phosphate (Pi), glucose and α-aminoisobutyric acid (AIB) in OK cells. Cells were pretreated with various concentrations of ethanol for 60 min at 37°C in Hanks' balanced salt solution, and the uptake was measured for 30 min in a buffer containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl$_2$, 1.2 mM MgSO$_4$, and 10 mM HEPES (pH 7.4). The substrate concentration was 5 μM K$_2$H^{32}PO$_4$, 50 μM [^{14}C]-D-glucose, and 5 μM [^{14}C]-AIB. Data are mean ± SE of four experiments.

Fig. 2. Effect of ethanol on amino acid uptake in OK cells. Cells were pretreated with 6% ethanol for 60 min at 37°C in Hanks' balanced salt solution, and the uptake was measured for 30 min in a buffer containing [^{14}C]-labeled amino acids, 137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl$_2$, 1.2 mM MgSO$_4$, and 10 mM HEPES (pH 7.4). The substrate concentration was 5 M for glycine and alanine and 0.5 M for leucine. Data are mean ± SE of three experiments. **$p < 0.01$ compared with control.

inhibited uptakes of amino acids, leucine, alanine, and glycine (Fig. 2), but it had no effect on Na^+-independent phosphate uptake (Fig. 3).

Phosphate transport across the apical membrane in renal proximal tubules is driven by the Na^+ gradient maintained by Na^+-K^+-ATPase activity (Murer et al, 1991). Similar mechanism was confirmed in opossum kidney (OK) cells (Caverzasio et al, 1986; Cole et al, 1988). Thus, if ethanol caused a reduction in Na^+-K^+-ATPase activity, the Na^+-P_i uptake should be inhibited. To test the possibility, we examined effect of ethanol on the Na^+-K^+-ATPase activity in microsomes isolated from OK cells. Microsomal Na^+-K^+-ATPase activities in cells treated with 5 and 8% ethanol were 2.09 ± 0.24 and 1.83 ± 0.26 μM Pi/mg/hr, respectively, which were not significantly different from the control (2.18 ± 0.19 μM Pi/mg/hr). These results indicate that ethanol-induced inhibition of Na^+-P_i uptake was not resulted from a reduction in Na^+-pump activity.

Effect of ethanol on the kinetics of Na^+-P_i uptake

To elucidate the mechanism of the ethanol inhibition, the effect of ethanol on the kinetics of Na^+-P_i uptake was examined. A 15-min uptake was measured as a function of external phosphate concentration in cells pretreated with 6% ethanol for 60 min. The
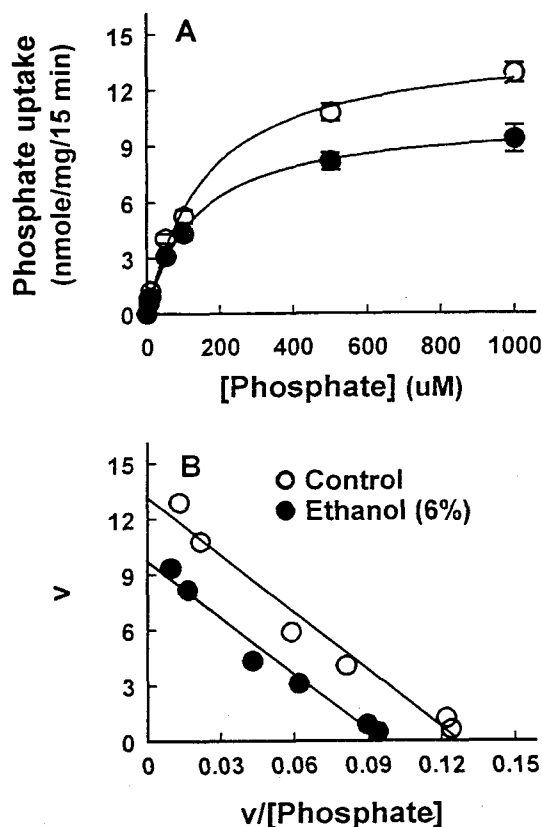
results are summarized in Fig. 4. The uptake data were fitted to the following equation:

$$ v = \frac{V_{max}[S]}{K_m + [S]} + D[S] $$

Where v is the rate of Pi uptake, V_{max} is the maximal rate of Pi uptake, K_m is the Michaelis constant of the

Fig. 4. Concentration dependence of Na^+-Pi uptake in control and ethanol-treated OK cells. Cells were pretreated with 6% ethanol for 60 min at 37°C in Hanks' balanced salt solution, and the uptake was measured for 15 min. A, Total Na^+-Pi uptake in control (○) and ethanol-treated (●) cells. Nonsaturable components were determined by nonlinear regression analyses of the total uptake data in control and ethanol-treated cells, and the values were 6.51 ± 0.39 and 3.64 ± 0.37 nmole/mg protein/15 min/mM, respectively. Data are mean $\pm$ SE of five experiments. B, Hofstee plot of carrier-mediated component of Na^+-Pi uptake, which was calculated from difference between total and nonsaturable uptakes. The regression lines were fitted by least-squares analysis. In this plot, the intercept of the line with Y-axis represents V_{max} and the slope represents $-K_m$.

Fig. 3. Effect of ethanol on Na^+-dependent and independent Pi uptake in OK cells. Cells were pretreated with 6% ethanol for 60 min at 37°C in Hanks' balanced salt solution, and the uptake was measured for 30 min in a buffer containing 5 μM $K_2H^{32}PO_4$, 137 mM NaCl (+Na) or choline chloride (-Na), 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgSO₄, and 10 mM HEPES (pH 7.4). Data are mean $\pm$ SE of three experiments. *p < 0.05 compared with control.

carrier, and D is the coefficient for the nonsaturable component of Pi uptake. Nonsaturable components determined by fitting the total uptake data to the above equation using nonlinear regression analysis were 6.51 and 3.64 nmole/mg/15 min/mM for control and ethanol-treated cells, respectively. Carrier-mediated component was calculated by subtracting the nonsaturable component from the total uptake (Fig. 4A). Analysis of the data in Fig. 4A by Hofstee plot showed that ethanol increased K_m from 0.047 ± 0.05 to 0.077 ± 0.01 mM (p < 0.05), whereas V_{max} of Na^+-P_i uptake remained unchanged (7.04 ± 0.37 vs. 7.27 ± 0.40 nmole/mg/15 min in control, p > 0.5) (Fig. 4B).

Effect of n-aliphatic alcohols on phosphate uptake

The Na^+-P_i uptake was decreased in a dose-dependent manner by various alcohols (Fig. 5A) and the Hill plots of the data are depicted in Fig. 5B. Hill coefficient (n) calculated from the slope of line was 1.04, 0.96, 0.93, 0.98, and 0.80 for ethanol, butanol, pentanol, hexanol, and heptanol, respectively. The Hill coefficient depends on the number of inhibitor molecules producing the inhibition and the degree of cooperativity in their binding. Therefore, the above Hill coefficient values suggest that one molecule may interact with a binding site in the carrier. The alcohol concentration for I_{50}, which is given by the intercept on the X-axis in the plot, was dependent on the length of the hydrocarbon chain of alcohols, varying from 8.4% for ethanol to 1.43, 0.6, 0.056, and 0.02% for butanol, pentanol, hexanol, and heptanol, respectively. In fact, the I_{50} value for the alcohol was inversely related to the partition coefficient (P), as shown in Fig. 6.

Effects of various drugs on ethanol-induced inhibition of phosphate uptake

The ethanol-induced inhibition of Na^+-P_i uptake was significantly attenuated by catalase, a scavenger of hydrogen peroxide (H_2O_2), but not by SOD, a superoxide scavenger. In hepatocytes and gastric mucosal cells, it has been proposed that iron-catalyzed formation of hydroxyl radicals from hydrogen peroxide may be the main mediator of ethanol-induced damage (Mutoh et al, 1990; Reinke et al, 1990). Thus, we examined effect of hydroxyl radical scavengers DMTU and pyruvate on the ethanol-induced inhibition of Na^+-P_i uptake. As shown in Table 1, these
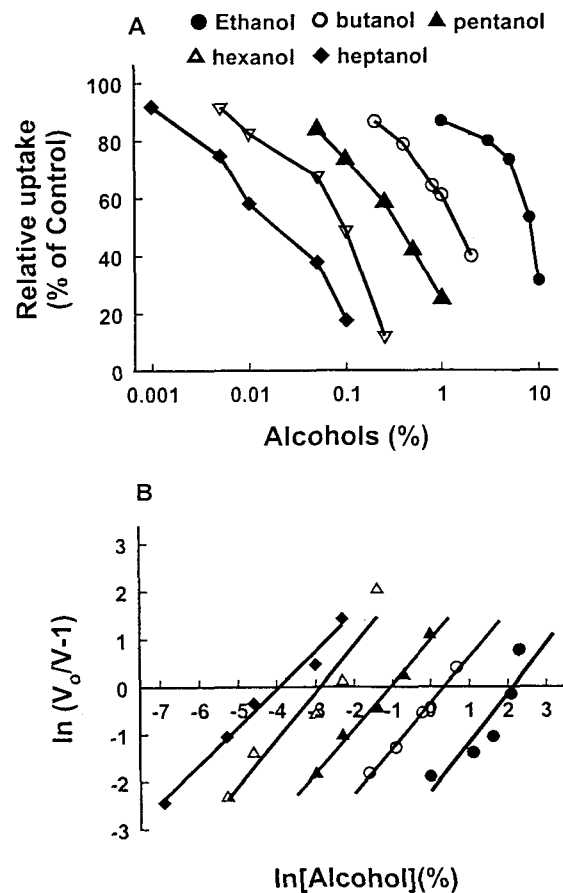
Fig. 5. A, Effect of n-aliphatic alcohols on Na^+-Pi uptake in OK cells. Cells were pretreated with various concentrations of alcohols for 60 min at 37°C in HBSS, and the uptake was measured for 30 min in a buffer containing 5 μM $K_2H^{32}PO_4$, 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM HEPES (pH 7.4). Data are mean $\pm$ SE of 3-4 experiments. B, The data were plotted according to Hill equation; V_0 and V indicate uptakes in the absence and presence of alcohol, respectively. In this plot, the value of the slope represents Hill coefficient, n, and the intercept on the X-axis represents the alcohol concentration for 50% inhibition (I_{50}).

scavengers did not prevent ethanol-induced inhibition of Na^+-P_i uptake. These drugs at 10 mM completely prevented the inhibition of Na^+-P_i uptake induced by 0.3 mM H_2O_2 (854.87 ± 93.47 in H_2O_2 vs. 1488.77 ± 74.57 pmole/mg/30 min in control; 1378.71 ± 111.59 in H_2O_2 plus DMTU; 1539.10 ± 180.93 pmole/mg/30 min in H_2O_2 plus pyruvate). Ethanol effect was also not altered by the presence of thiols GSH and DTT (Table 1). These drugs at concentrations used in this study prevent effectively oxidant-induced renal cell injury (Aruoma et al, 1989; Andreoli & McAteer,
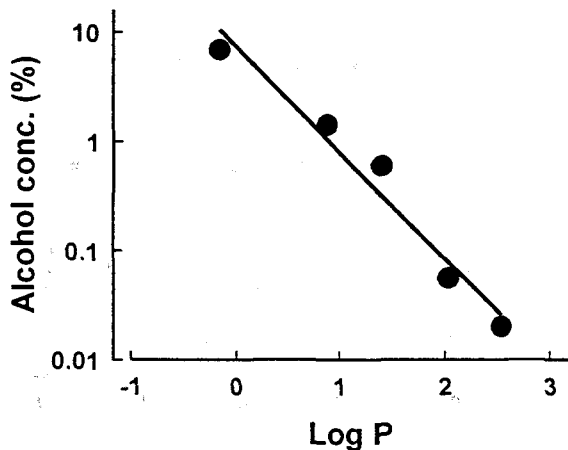
Fig. 6. The log concentrations of alcohols to give 50% inhibition of Na^+-P_i uptake as a function of water-octanol partition coefficients, P, of five n-aliphatic alcohols. in OK cells. Linear regression for the uptake.

Table 1. Effects of radical scavengers and thiols on ethanol-induced inhibition of phosphate uptake in OK cells

Treatment conditions	Phosphate uptake (pmole/mg/30 min)
Control	1472.76 ± 79.62
+ Ethanol (6%)	847.80 ± 59.00*
+ Catalase (800 units/ml)	1109.00 ± 60.96*#
+ SOD (300 units/ml)	979.62 ± 127.86*
+ DMTU (20 mM)	906.98 ± 65.71*
+ Pyruvate (10 mM)	818.18 ± 58.49*
+ GSH (2 mM)	857.38 ± 43.29*
+ DTT (2 mM)	822.13 ± 37.93*
+ DPPD (10 μM)	807.50 ± 77.07*

Cells were pretreated with ethanol for 60 min in Hanks' balanced salt solution with or without each drug and the uptake was measured for 30 min in a buffer (5 μM $K_2H^{32}PO_4$, 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM HEPES, pH 7.4) without ethanol and drugs. Data are mean ± SE of 4-5 experiments. *$p < 0.05$ compared with control. #$p < 0.05$ compared with ethanol alone. SOD, superoxide dismutase; DMTU, dimethylurea; GSH, reduced glutathione; DTT, dithiothreitol; DPPD, N,N'-diphenyl-p-phenylenediamine.

1990; Kim & Kim, 1996). Furthermore, a potent antioxidant DPPD did not affect the inhibition of Na^+-Pi uptake induced by ethanol (Table 1), suggesting that
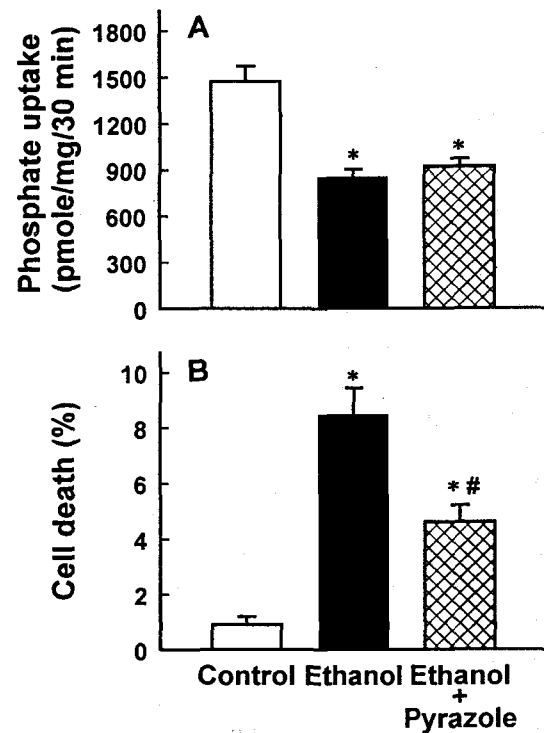

Fig. 7. Effect of pyrazole on the inhibition of Na^+-P_i uptake and cell death induced by ethanol in OK cells. Cells were pretreated with 6% ethanol for 60 min at 37°C in HBSS with or without 2 mM pyrazole, and the uptake and cell death were measured. Data are mean ± SE of five experiments. **$p < 0.01$ compared with control. #$p < 0.05$ compared with ethanol alone.

lipid peroxidation is not involved in the ethanol-induced inhibition of Na^+-P_i uptake.

To further confirm if iron-catalyzed metabolites of hydrogen peroxides are involved in the ethanol inhibition of Na^+-P_i uptake, effects of iron chelators were examined. As shown in Table 2, the ethanol effect was not altered by the pretreatment of iron chelators. When the effect of iron chelator on oxidant-induced inhibition of Na^+-P_i uptake was examined as positive control, the H_2O_2-induced inhibition was significantly prevented by the iron chelators.

Pyrazole, an inhibitor of alcohol dehydrogenase, did not affect the ethanol effect on phosphate uptake, whereas it significantly attenuated ethanol-induced cell death (Fig. 7). This indicated that reduced phosphate uptake induced by ethanol is attributed to a direct action of ethanol rather than its metabolites, but ethanol-induced cell death may be resulted from its metabolite such as acetaldehyde.

Table 2. Effects of iron chelators on inhibition of phosphate uptake induced by ethanol and H$_2$O$_2$ in OK cells

Pretreatment (30 min)	Treatment (60 min)	Phosphate uptake (pmole/mg/30 min)
No drug	Control	1487.77 ± 45.12
No drug	Ethanol (6%)	765.52 ± 45.42*
DFX (1 mM)	Ethanol + DFX (1 mM)	658.65 ± 64.34*
PTL (1 mM)	Ethanol + PTL (1 mM)	741.72 ± 27.38*
DFX (10 mM)	DFX (1 mM)	1572.57 ± 139.55
DFX (10 mM)	Ethanol + DFX (1 mM)	672.03 ± 122.50*
No drug	H$_2$O$_2$ (0.3 mM)	892.51 ± 22.42*
DFX (1 mM)	H$_2$O$_2$ + DFX (1 mM)	1389.00 ± 90.40
PTL (1 mM)	H$_2$O$_2$ + PTL (1 mM)	1194.36 ± 46.16

Cells were pretreated with deferoxamine (DFX) or phenanthroline (PTL) for 30 min and treated with ethanol or H$_2$O$_2$ for 60 min in Hanks' balanced salt solution (HBSS) with or without iron chelators and the uptake was determined for 30 min in a buffer (5 μM K$_2$H^{32}PO$_4$, 137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl$_2$, 1.2 mM MgSO$_4$, and 10 mM HEPES, pH 7.4) containing no ethanol and iron chelators. Data are mean ± SE of 3 experiments. *p < 0.05 compared with control.

DISCUSSION

A decrease in the Na$^+$-P$_i$ uptake by benzyl alcohol treatment was demonstrated in intact renal proximal tubular cells (Friedlander et al, 1988; Friedlander et al, 1990). Na$^+$-dependent transport systems located at the apical membrane of renal proximal tubules are affected differently by benzyl alcohol: the alcohol stimulates the Na$^+$-P$_i$ uptake, inhibits the Na$^+$-glucose uptake, and produces no affect on the Na$^+$-alanine uptake (Friedlander et al, 1988; Friedlander et al, 1990). Local anesthetics such as benzyl alcohol have been known to affect membrane transport function by membrane fluidization (Le et al, 1991). Since ethanol also causes an increase in membrane fluidity in various cell types (Chin & Goldstein, 1977; Ives & Verkman, 1985), the effect of ethanol on Na$^+$-coupled uptakes may also be associated with membrane fluidization, as with benzyl alcohol. However, the present study demonstrated that ethanol inhibits Na$^+$-dependent uptakes of P$_i$, glucose and various amino acids. In addition, ethanol did not stimulate Na$^+$-P$_i$ uptake at the range of concentration examined. Thus, the results on Na$^+$-dependent uptakes of phosphate and alanine are completely different from those of benzyl alcohol.

The major determinant of the strength of alcohol as an inhibitor of Na$^+$-P$_i$ transport appeared to be the lipid solubility, as indicated by an inverse relationship between the I$_{50}$ and partition coefficient (Fig. 6). We also observed similar results with p-aminohippurate

uptake in renal cortical slices (Kim et al, 1985). These results are consistent with those of Fernandez et al (1984) who observed a similar correlation between the I$_{80}$ (alcohol concentration reducing D-glucose uptake by 80%) and partition coefficient in renal brush-border membrane vesicles. These authors stated that the fluidity modified by perturbation of hydrophobic bonds of the membrane modulates the Na$^+$-coupled D-glucose uptake. Although alcohols increase membrane fluidity, it is not clear whether alcohols exert their effects through alterations in the fluidity. Levine et al (1991) observed that changes in Na$^+$-P$_i$ transport did not correlate with changes in membrane fluidity in renal brush-border membrane. Tillotson et al (1981) also reported that ethanol inhibits the Na$^+$-dependent glucose uptake without alterations in membrane fluidity in intestinal brush-border membranes. In the present study, stimulation of Na$^+$-P$_i$ uptake was not observed at concentrations of alcohols tested, which is different from those of other studies in which a moderate fluidization by benzyl alcohol stimulated the Na$^+$-P$_i$ transport in both isolated brush-border membranes and cultured renal cells (Friedlander et al, 1988; Friedlander et al, 1990). Thus, it is likely that effect of ethanol-induced fluidization on Na$^+$-P$_i$ uptake in OK cells is different from that of benzyl alcohol. Effects of fluidity on uptakes of Na$^+$-P$_i$ and Na$^+$-glucose are controversial under various conditions that induce alterations in the fluidity in renal brush-border membrane. Na$^+$-P$_i$ uptake is stimulated by benzyl alcohol (Yusufi et al, 1989; Friedlander et

al, 1990) or low-phosphate diet (Yusufi et al, 1989), but it is decreased by gentamicin treatment *in vivo* which induces an increase in membrane fluidity (Levi & Cronin, 1990b). Na^+-glucose uptake is inhibited by benzyl alcohol (Carriere & Le, 1986) and renal ischemia (Linas et al, 1987), whereas it is not affected by gentamicin (Levi & Cronin, 1990b).

The alternative mechanism is that ethanol impairs Na^+-P_i uptake by interacting directly with essential carrier protein components of the membrane rather than the membrane lipid. This hypothesis is favored by the observation that inhibition of luciferase enzyme by local anesthetics is correlated with lipid solubility, even though no lipid is present (Franks & Lieb, 1984). Ethanol has also been reported to inhibit Na^+-glucose cotransport by a direct interaction with transporter in renal brush-border membrane vesicles (Parenti et al, 1991). A direct inhibition of the translocation step of choline carrier by n-alkanols including ethanol has been clearly demonstrated in human erythrocytes (Deves & Krupka, 1990). The present study also showed that ethanol significantly increased K_m without any effect on V_{max} of Na^+-P_i uptake (Fig. 4), suggesting that the mechanism involves competitive inhibition of substrate binding on the carrier. These results may indicate that the inhibition of Na^+-P_i uptake results from a direct effect of ethanol on carrier protein. Modulation of Na^+-P_i transport by changes in membrane fluidity is always manifested by an increase or decrease in V_{max} of Na^+-P_i transport without changing K_m in intact renal cells and isolated renal brush-border membrane vesicles (Friedlander et al, 1988; Levi et al, 1990a). Thus, it is unlikely that ethanol inhibition of Na^+-P_i uptake is due to changes in membrane fluidity. Our data are consistent with results of amino acid uptake by rat liver cells (Rosa & Rubin, 1980).

It has been proposed that ROS may be involved in the pathogenesis of ethanol-induced cell injury in stomach (Mutoh et al, 1990) and liver (Reinke et al, 1990; Bondy & Guo, 1994a). However, whether ROS is involved in ethanol-induced alterations of membrane transport function in renal tubular cells has not been determined. In the present study, we assessed the role of ROS in ethanol-induced inhibition of Na^+-P_i uptake indirectly by using compounds that are capable of scavenging ROS. Catalase, a H_2O_2 scavenger, significantly prevented the ethanol effect. However, a superoxide scavenger SOD and a hydroxyl radical scavenger DMTU did not affect the ethanol

effect. These results suggest that H_2O_2 may be involved in the ethanol-induced inhibition of Na^+-P_i uptake. H_2O_2 can be converted to hydroxyl radical through the Fenton reaction in the presence of iron, which is highly toxic species that induces lipid peroxidation and cell injury (Koppenol, 1993). Therefore, the toxicity of H_2O_2 is dependent on the existence of iron and prevented by pretreatment with iron chelators such as deferoxamine (Rush et al, 1985; Starke & Farber, 1985). Indeed, Mutoh et al (1990) observed in cultured gastric mucosal cells that ethanol-induced cell damage is attenuated by an iron chelator deferoxamine and a hydroxyl radical scavenger dimethylsulfonic acid, indicating that hydroxyl radical is critical in the ethanol-induced damage. However, in the present study, hydroxyl radical scavengers DMTU and pyruvate did not alter the ethanol-induced inhibition of Na^+-P_i uptake. Similarly, the ethanol effect was not attenuated by pretreatment with iron chelators (Table 2). These results are consistent with those of Kvietys et al (1990) who did not observe the involvement of ROS in the ethanol-induced damage in rat gastric mucosal cells.

Since Na^+-coupled solute uptake is dependent on the maintenance of Na^+ gradient, an inhibition of Na^+-pump activity would retard phosphate uptake. However, the present study showed that ethanol at 8% concentration did not produce a significant inhibition of Na^+-K^+-ATPase activity in microsomes isolated from OK cells.

The present study showed that the ethanol effect on the Na^+-P_i uptake is not affected by addition of 5 mM GSH or 2 mM DTT, suggesting that thiol groups are not related to ethanol-induced inhibition of Na^+-P_i uptake. If H_2O_2 is involved in ethanol effect, the inhibition of Na^+-P_i uptake may be resulted from lipid peroxidation. However, the present study showed that a potent antioxidant DPPD did not attenuate the ethanol-induced inhibition of Na^+-P_i uptake. Therefore, it is likely that the H_2O_2 generated by ethanol mediates the inhibition of Na^+-P_i uptake by lipid peroxidation-independent mechanisms. H_2O_2 may cause modification of carrier protein for Na^+-P_i uptake. Such an effect might be responsible for ethanol-induced decrease in the affinity of substrate as suggested by an increase in K_m.

The inhibition of Na^+-P_i uptake could be due to acetaldehyde, a product of ethanol metabolism, rather than a direct effect of ethanol. The acetaldehyde inhibits Na^+-dependent glucose uptake with higher po-

tency than ethanol in intestinal brush-border membrane (Tillotson et al, 1981). To test the possibility, we examined the effect of ethanol in the presence of 2 mM pyrazole, a potent inhibitor of alcohol dehydrogenase. Pyrazole did not prevent the ethanol-induced inhibition of Na$^+$-P$_i$ uptake, whereas it significantly attenuated the ethanol-induced cell death, suggesting that the inhibition of Na$^+$-P$_i$ uptake was not due to cell death.

In conclusion, ethanol inhibits Na$^+$-dependent uptakes of Pi, glucose, and various amino acids in OK cells. Ethanol inhibition may result from modifications of substrate binding site of transporter protein by a mechanism dependent on H$_2$O$_2$.

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