Published in final edited form as:

Alcohol Clin Exp Res. 2013 January ; 37(Suppl 1): E40–E51. doi:10.1111/j.1530-0277.2012.01933.x.

ACUTE EFFECT OF ETHANOL ON HEPATIC RETICULAR G6Pase AND Ca²⁺ POOL

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Abstract

Background—Hydrolysis of glucose 6-phosphate via glucose 6-phosphatase enlarges the reticular Ca²⁺ pool of the hepatocyte. Exposure of liver cells to ethanol impairs reticular Ca²⁺ homeostasis. The present study investigated the effect of acute ethanol administration on glucose 6-phosphate supported Ca²⁺ accumulation in liver cells.

Methods—Total microsomes were isolated from rat livers acutely perfused with varying doses of ethanol (0.01%, 0.1%, or 1% v/v) for 8 minutes. Calcium uptake was assessed by ⁴⁵Ca redistribution. Inorganic phosphate (Pi) formation was measured as an indicator of glucose 6-phosphatase hydrolytic activity.

Results—Glucose 6-phosphate-supported Ca²⁺ uptake decreased in a manner directly proportional to the dose of ethanol infused in the liver whereas Ca²⁺ uptake via SERCA pumps was decreased by ~25% only at the highest dose of alcohol administered. The reduced accumulation of Ca²⁺ within the microsomes resulted in a smaller IP₃-induced Ca²⁺ release. Kinetic assessment of IP₃ and passive Ca²⁺ release indicated a faster mobilization in microsomes from ethanol-treated livers, suggesting alcohol-induced alteration of Ca²⁺ releasing mechanisms. Pre-treatment of livers with chloromethiazole or dithio-threitol, but not 4-methyl-pyrazole prevented the inhibitory effect of ethanol on glucose 6-phosphatase activity and Ca²⁺ homeostasis.

Conclusions—Liver glucose 6-phosphatase activity and IP₃-mediated Ca²⁺ release are rapidly inhibited following acute (8 min) exposure to ethanol, thus compromising the ability of the endoplasmic reticulum to dynamically modulate Ca²⁺ homeostasis in the hepatocyte. The protective effect of chloromethiazole and di-thio-threitol suggests that the inhibitory effect of ethanol is mediated through its metabolism via reticular cyP4502E1 and consequent free radicals formation.

Keywords

Ca²⁺ uptake; IP₃; liver; microsomes; glucose 6-phosphatase; chloromethiazole; 4-methyl-pyrazole; EtOH

Introduction

Liver cells actively accumulate cytosolic Ca²⁺ within the endoplasmic reticulum (ER) through specific Ca²⁺-ATPases (SERCA¹ pump, Bennett and Williams, 1992). The accumulated Ca²⁺ can form a complex with various endoluminal proteins including calreticulin and calsequestrin (Worrall et al., 2001), or with inorganic phosphate (Pi)

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Disclosure: Authors have nothing to disclose

generated within the lumen of the endoplasmic reticulum by the hydrolysis of glucose 6-phosphate (G6Pi) via glucose 6-phosphatase (G6Pase, E.C. 3.1.3.9) activity (Benedetti et al., 1986). The reticular Ca^2+Pi complex is not insoluble as the entrapped Ca^{2+} can be released by IP_3 addition (Benedetti et al., 1987), the process being associated with the concomitant release of Pi from the ER (Benedetti et al., 1987; Romani et al., 1988). Experimental evidence indicates that this dynamic accumulation and release of reticular Ca^{2+} is an integral component of various hormonal signaling revolving around PLC activation (e.g. vasopressin, Hoek et al, 1987; Marino et al., 1992), and plays a significant role in maintaining physiological levels of Ca^{2+} within the cytoplasm and the endoplasmic reticulum of the hepatocyte (Benedetti et al., 1986).

Exposure of hepatocytes to ethanol (EtOH) results in an increase in cytosolic Ca^{2+} level (Hoek, 1987), with major implications for gene (Hoek, 1987), and ER stress (Sammels et al., 2010) activation. The impairment of reticular Ca^{2+} homeostasis has been attributed to defects in SERCA pump activity (Mitidieri and de Meis, 1995), endoluminal complexing proteins (Ohlendieck et al., 2003), IP_3 receptor expression or activity (Bokkala et al., 1999), or changes in phospholipid composition and membrane fluidity (Carrasco et al., 2006).

Glucose 6-phosphatase enzymatic complex operates within the endoplasmic reticulum (ER) of liver (Benedetti et al., 1986; Romani et al., 1988), kidney (Fulceri et al., 1990) and pancreatic β -cells (Senesi et al., 2005; Wolf et al., 1988), in which it catalyzes the hydrolysis of G6Pi to glucose and Pi. The main function of this enzyme is to regulate hepatic glycolysis and glucose homeostasis (Banhegy et al., 1997). Structurally, the G6Pase presents nine trans-membrane helices embedded in the ER membrane (Gerin and Schaftingen, 2002; Pan et al., 1998) and an endoluminal catalytic site (Foster and Nordlie, 2002). A specific transport mechanism delivers G6P to the G6Pase hydrolytic site (Pan et al., 1998). Despite extensive studies, the structure and *modus operandi* of this enzymatic complex are still debated. Two models have been proposed. In the substrate-transport model, G6P enters the ER lumen via T1, a specific transporter that is distinct from the hydrolase and represents the rate-limiting factor for the G6Pase activity (Berteloot et al., 1991). The conformational flexibility substrate-transport model, instead, proposes that the G6Pase possesses a hydrophilic substrate binding region at its cytoplasmic site. Binding of G6P to this region will induce a conformation change and the delivery of G6P to the intra-luminal catalytic site. In this model, the substrate-binding site and the hydrolytic site of the G6Pase are two parts of the same protein, and the enzyme is not specific for a particular substrate (Foster and Nordlie, 2002; Gerin and Schaftingen, 2002). In both models, the byproducts of G6P hydrolysis (i.e. glucose and Pi) are released into the cytoplasm via an additional transporter specific for glucose (Banhegy et al., 1997; Berteloot et al., 1991; Foster and Nordlie, 2002; St Denis et al., 1995), and via the glucose 6-phosphate transport mechanism operating as a phosphate-linked antiporter (Chen et al., 2008).

Early studies in the seventies have reported changes in G6Pase activity upon prolonged exposure to EtOH (Ishii et al., 1973; Nelson et al., 1970). No study, however, has investigated the occurrence of similar changes in G6Pase activity upon acute (8 min) EtOH administration and the possible repercussions on reticular Ca^{2+} homeostasis. These two aspects have been investigated in the present study. The results reported here indicate that 8 min exposure to EtOH selectively impairs the ability of the G6Pase to generate proper levels of inorganic phosphate within the ER lumen, and results in a decreased accumulation of

¹Abbreviations used in the text

SERCA = sarcoplasmic-endoplasmic reticulum Ca^{2+} pump; G6Pase = glucose 6-phosphatase; G6P = glucose 6-phosphate; G6PT = glucose 6-phosphate transporter; Pi = inorganic phosphate; PPi = pyrophosphate; EtOH = ethanol; 4-MP = 4-methyl-pyrazole; CMZ = chloromethiazole; IP_3 = inositol-1,4,5-trisphosphate; DTT = dithio-threitol; NAC = N-acethyl-cysteine; 2-APB = 2-aminoethoxydiphenyl-borate; HNE = 4-hydroxy-nonenal; ROS = reactive oxygen species.

mobilizable Ca^{2+} within the ER. The protective effect of chloromethiazole and dithiothreitol but not 4-methyl-pyrazole suggests that this defect is consequent to the oxidation of EtOH by cyP4502E1 and associated production of reactive oxygen species.

Materials and Methods

Materials

All chemicals and reagents were from Sigma (St Louis, MO). Lieber-De Carli diet and liquid control diet were from Bio-Serve (Frenchtown, NJ). Antibodies anti-G6Pase catalytic subunit were from Santa Cruz (Santa Cruz, CA). Oxyblot detection kit was from Millipore (Billerica, MA). Antibodies recognizing the HNE/protein adducts were a kind gift from Dr. Szewda (Oklahoma Medical Center, Oklahoma). Radioisotopic ^{45}Ca was from Amersham (Amersham, Piscataway, NJ).

Acute EtOH Exposure and Microsomal Vesicle Isolation

Male Sprague-Dawley rats (180–210 g body weight) were used as organ donors. The animals were anesthetized by i.p. injection of saturated sodium pentobarbital solution (50 mg/bw). Upon attainment of a deep anesthesia, the abdomen was opened, and the portal vein cannulated. The liver was removed, placed on a perfusion platform, and perfused with an oxygenated (95:5 O_2 : CO_2 v/v) medium at a flow rate of 3.5–4 ml/g liver weight at 37°C , as described elsewhere in detail (Tessman and Romani, 1998; Young et al., 2003). The medium composition was as follows (mM): NaCl 120, KCl 3, KH_2PO_4 1.2, NaHCO_3 12, CaCl_2 1.2, MgCl_2 1.2, Hepes 10 and glucose 10 (pH 7.2). This protocol was in agreement with the NIH directives for the use and maintenance of experimental animals, and it was approved by the Institutional Animal Care and Utilization Committee (IACUC) at Case Western Reserve University.

Following 10 min of equilibration, 0.01%, 0.1% or 1% EtOH (v/v) were added directly to the perfusate and infused for 8 min. The protective effect of 4-methyl-pyrazole (4-MP, 50 μM) and chloro-methiazole (CMZ, 100 μM) on alcohol dehydrogenase and cyP4502E1, respectively, was tested by dissolving either agent in the perfusion medium 5 min prior to EtOH addition and maintaining it in the system throughout the alcohol administration period. After 8 min of EtOH administration, the liver was removed, gently blotted on absorbing paper, weighted, and homogenized (10% w/v in 250mM sucrose, 20mM Hepes (pH 7.4 with KOH). Total microsomal vesicles were isolated according to the procedure of Henne and Soling (Henne and Soling, 1986) as previously reported (Romani et al., 1988).

Ca^{2+} accumulation

Total microsomal vesicles were incubated at 37°C in a medium having the following composition: 100mM KCl, 20mM NaCl, 5mM MgCl_2 , 1mM KH_2PO_4 , 10mM MOPS (pH 7.2), in the presence of 1mM ATP plus an ATP-regenerating system (5mM creatine phosphate and 5 μM units/ml creatine phosphokinase), and 20 μM CaCl_2 labeled with 0.1 $\mu\text{Ci/ml}$ $^{45}\text{CaCl}_2$. Ruthenium red (2 μM) was added to the incubation medium as a mitochondrial inhibitor (Benedetti et al., 1987). Microsomal vesicles were added to pre-warmed incubation medium at a final concentration of 0.1mg microsomal protein/ml. The incubation was initiated by addition of ATP plus regenerating system. After 10 min, concentrations of glucose 6-phosphate (G6P) varying between 1mM to 20mM were added to the system, and the incubation continued for additional 45min. Inositol-1,4,5 trisphosphate (IP_3 , 5 μM) was then added to the system and allowed to elicit Ca^{2+} release for 10 min. At the time points indicated in the figures, 0.5ml of the incubation system were withdrawn and filtered under vacuum onto 25mm nitrocellulose filters (0.2 μm pore diameter, Millipore). The filters were quickly washed twice with 5 ml 'ice-cold' 250mM sucrose, and the

radioactivity retained onto the filters determined by liquid β -scintillation counting (Benedetti et al., 1987; Romani et al., 1988) in a Beckman LS 6000. Radioactive ^{45}Ca non-specifically bound to microsomes and filters was assessed in parallel experiments carried out on heat-denature microsomal vesicles in the presence of ATP/regenerating system and G6P, and subtracted point to point. Microsomal Ca^{2+} accumulation was calculated as previously reported (Benedetti et al., 1987). The concentration of Ca^{2+} contaminant present in the incubation system (including G6P) was $9\pm 2\mu\text{M}$ ($n=10$) as measured by atomic absorption spectroscopy (Perkin Elmer 3100, Perkin Elmer, Waltham, MA), and was taken into consideration to calculate the amount of Ca^{2+} accumulated within the microsomes.

The effect of acute doses of EtOH on microsomal vesicles was tested by adding 0.01%, 0.1% or 1% EtOH directly to the incubation system, in the absence and in the presence of 4-MP or CMZ. When present, these inhibitory agents were added to the system together with the microsomal vesicles, 2 min prior to the addition of EtOH. Also in this case, Ca^{2+} uptake was initiated by addition of ATP and regenerating system as previously indicated.

Passive Ca^{2+} loading and release

Microsomal vesicles were incubated at the final concentration of 15 mg protein/ml in a cytosol-like medium described above. The vesicles were pre-equilibrated with 1mM CaCl_2 labeled with $20\mu\text{Ci/ml}$ $^{45}\text{CaCl}_2$ for 40 min at 22°C (Giunti et al., 2007). In a separate set of experiments, CaCl_2 was substituted with 1mM sucrose labeled with $10\mu\text{Ci/ml}$ [^{14}C]-sucrose for comparison. At the end of the passive loading period, the microsome suspensions were diluted 100-fold in the same cytosol-like buffer in the absence of cold and labeled CaCl_2 (or sucrose), and incubated therein at 22°C , to determine passive efflux from the vesicles (Giunti et al., 2007). At various time points after the dilution, 0.5 ml of the incubation mixture were withdrawn and filtered under vacuum as described above. The radioactivity retained onto the filters was measured by β -scintillation counting as reported above. For comparison, passive loading and passive Ca^{2+} release experiments were carried out in an ion-free medium containing 250mM sucrose, 20mM Hepes (pH 7.2 with Tris). In separate sets of experiments, 2-aminoethoxydiphenyl-borate (2-APB, $100\mu\text{M}$) was added to the vesicles prior to initiate passive Ca^{2+} loading or passive Ca^{2+} release.

Pi Determination

Glucose 6-phosphatase activity was assayed by measuring the amount of glucose and Pi released as byproducts of G6P hydrolysis. Inorganic phosphate was determined according to Fiske and Subbarow assay (Seddon and Fynn, 1973) at 700 nm in an Agilent 8453 spectrophotometer equipped with a computerized data acquisition program. The basal level of Pi detected at time = 0 min was subtracted from all the subsequent points of hydrolysis to obtain the net amount of G6P hydrolyzed. Glucose production was determined using a glucose oxidase method (Fisher Scientific, Pittsburgh, PA).

Western Blot Analysis

Aliquots of microsomal preparations from livers perfused in vitro with EtOH, or from livers of EtOH-fed and control-diet rats were removed from the incubation system following 2–3 min of equilibration, and rapidly homogenized in a buffer containing (mM): 100 NaCl, 0.01 leupeptin, 1 Na_3VO_4 , 10 Hepes, pH 7.4, in the presence of a cocktail of protease and phosphatase inhibitors (Sigma, St Louis). Following centrifugation ($14,000\text{ g} \times 10\text{ min}$), the membrane fraction was collected and equal amounts of proteins were applied to a 10% acrylamide gels. The proteins were separated by SDS-PAGE and transferred onto a PVDF immobilization membrane (Schleicher & Schuell Inc.). Glucose 6-phosphatase was immunodetected using polyclonal antibodies recognizing its catalytic subunit (Santa Cruz). Blots were routinely stained with Ponceau S before immunostaining to confirm equal

loading of samples and transfer of equivalent amounts of protein. The membranes were extensively washed with PBS, and the primary antibody binding was visualized utilizing peroxidase-conjugated secondary antibody and ECL western blotting detection reagents (GE Healthcare, Piscataway, NJ). Densitometry was performed using Scion Image Program (NIH).

Oxidative modification of proteins

To determine whether exposure to EtOH inhibited Ca^{2+} accumulation via oxidative modification of proteins, 100 μM di-thio-threitol (DTT) or 100 μM N-acetyl-cysteine (NAC) were added to microsomal incubation mixture together with the microsomes, 2 min prior to the addition of EtOH and ATP. As before, the incubation was carried out for 10 min with ATP/regenerating system only and for additional 45 min in the presence of ATP/regenerating system and G6P at 37°C before IP_3 addition. At $t = 0\text{min}$, $t = 20\text{ min}$, and $t = 55\text{ min}$ aliquots of the incubation mixture were withdrawn, and the proteins separated by SDS-PAGE. Western Blot analysis to assess oxidative modification of proteins was then carried out using antibodies recognizing HNE/protein adducts (Lashin et al., 2006) or oxyblot detection kit according to the manufacturer instructions as reported previously (Lashin et al., 2006).

Protein Content

Protein content was measured according to the procedure of Lowry (Lowry et al., 1951), using BSA as standard.

Statistical analysis

The data are reported as mean $\pm$ SEM. Data were first analyzed by one-way ANOVA. Multiple means were then compared by Tukey's multiple comparison test performed with a q value established for statistical significance of $P < 0.05$.

Results

Inhibition of Ca^{2+} Accumulation Following Acute EtOH Exposure

Microsomal vesicles isolated from control livers accumulated $\sim 6\text{ nmol Ca}^{2+}/\text{mg protein}$ within 10 min from the addition of ATP plus regenerating system (Fig. 1A). Calcium accumulation increased further following the addition of glucose 6-phosphate (G6P) in a dose-dependent manner (Fig. 1A). Maximal Ca^{2+} accumulation was attained at $\sim 45\text{ min}$ following G6P addition (i. e. $t = 55\text{ min}$) irrespective of the dose of G6P added, not changing significantly afterwards (Fig. 1A). As 5mM G6P elicited near maximal Ca^{2+} accumulation in this system, this G6P concentration was used for the remainder of the studies reported here. In contrast to the data reported in Fig. 1A, microsomal vesicles isolated from livers perfused with EtOH for 8 min exhibited a significant inhibition of Ca^{2+} accumulation over the same period of time (Fig. 1B). This inhibition was observed at all the concentrations of EtOH tested, ranging from $\sim 20\%$ (0.01%) to $\sim 38\%$ (0.1%) to $\sim 50\%$ (1% EtOH) (Fig. 1B). Doses of EtOH larger than 1% were not used as their infusion in livers resulted in non-specific damage of biological membranes (Tessman and Romani, 1998). Also, this dose is already well above the level attained by the most common modalities of alcohol administration. At the concentration of 0.01% or 0.1% EtOH, the decrease in Ca^{2+} uptake did not depend on an inhibition of SERCA pump activity by EtOH as the amplitude of ATP-supported Ca^{2+} accumulation in these microsomes was comparable to that of control (EtOH-untreated) microsomes both in the short (10 min -Fig. 1C) and long (55min) term (not shown). In contrast, Ca^{2+} uptake in microsomes from livers perfused with 1% EtOH was decreased by $\sim 25\%$ (Fig. 1C). Assessment of G6Pase activity (Table 1) indicated

impaired G6P enzymatic hydrolysis. This impairment occurred in an alcohol dose-dependent manner, and was evident at all the concentrations of G6P tested (Table 1), suggesting that the decrease in Ca^{2+} accumulation depended on a defective G6Pase activity. A similar inhibition was observed when glucose rather than Pi production was assessed (Table 2).

Hepatic reticular Ca^{2+} pool enlarged via endoluminal Pi accumulation can be dynamically released upon IP_3 addition (Benedetti et al., 1987; Romani et al., 1988). This release is integral part of the Ca^{2+} signaling triggered by various hormones acting on the hepatocyte (Hoek et al., 1987). As expected, the decreased accumulation of Ca^{2+} within ER/microsomes translated into a reduced (~65%) mobilization of Ca^{2+} upon addition of a maximal dose of IP_3 (Fig. 2A). Evaluation of Ca^{2+} release kinetic supported the notion that at the lowest dose of EtOH utilized here the reduction in Ca^{2+} release mostly depended on the reduced pool of Ca^{2+} accumulated within the microsomal lumen (Fig. 2B). At the two highest doses of EtOH tested (i.e. 0.1% and 1%), instead, an accelerated rate of release was observed (Fig. 2B), suggesting the occurrence of a functional alteration of the IP_3 receptor.

The inhibitory effect of EtOH on Ca^{2+} uptake (Fig. 3A) and G6Pase activity (Fig. 3B) was also observed following *in vitro* addition of 0.1% EtOH to microsomes from both control and 0.1% EtOH-perfused livers. In contrast, *in vitro* addition of EtOH to microsomes from liver perfused with 1% EtOH did not further inhibit G6Pase hydrolysis rate and G6P-supported Ca^{2+} accumulation (not shown).

To test the hypothesis that G6Pase inhibition depended on EtOH-metabolism via cyP4502E1, microsomes were isolated from livers perfused with EtOH in the presence of chloromethiazole (CMZ, 100 μM), or 4-methyl-pyrazole (4-MP, 50 μM) as inhibitors of reticular cyP4502E1 and cytosolic alcohol dehydrogenase, respectively. The results reported in Fig. 4 indicate that pretreatment with CMZ prevented the inhibitory effect of EtOH on Ca^{2+} uptake (Fig. 4A), IP_3 -induced Ca^{2+} release (Fig. 4B) and G6Pase activity (Fig. 4C). A similar protective effect by CMZ was observed in microsomes treated *in vitro* with EtOH (not shown). In contrast, pretreatment of livers with 4-MP did not prevent EtOH-induced inhibition of Ca^{2+} uptake (Fig. 5A), Ca^{2+} release (Fig. 5B), and G6Pase activity (Fig. 5C).

The experiments reported so far indicate that the hydrolytic activity of G6Pase is compromised upon acute exposure of perfused livers or isolated microsomes to EtOH. The G6Pase is an enzymatic complex that requires G6P to enter the endoplasmic reticulum lumen through a specific transport mechanism (T1) separated from the hydrolase or as part of the enzyme, to undergo hydrolysis at the G6Pase catalytic site (Gerin and Schaftingen, 2002). The byproducts glucose and Pi are then released into the cytoplasm via a specific glucose transporter (Foster and Nordlie, 2002) and the operation of G6PT as a Pi-linked antiporter (Chen et al., 2008). Defects in any of these components result in reduced G6P hydrolysis and release of glucose and Pi into the cytoplasm. To determine whether EtOH inhibitory effect occurred at the level of G6Pase catalytic site or at the level of the auxiliary transport mechanisms, G6Pase activity was tested in microsomes isolated from EtOH-perfused livers in the presence of 0.1% taurocholic acid, which permeabilizes the ER/microsomal membrane and allows for G6P delivery to the hydrolytic site of the G6Pase bypassing the entry mechanism (Barfell et al., 2011; Doleh and Romani, 2007). Under these experimental conditions, no major inhibitory effect of EtOH on G6P hydrolysis rate was observed (Table 3). These results, however, do not exclude the possibility that in intact microsomal vesicles EtOH inhibits the transporters releasing glucose or Pi into the cytoplasm, resulting in an accumulation of these products within the lumen and the consequent inhibition of G6Pase hydrolytic activity (Foster and Nordlie, 2002). The G6Pase complex can also hydrolyze pyrophosphate (PPi) to Pi (Foster and Nordlie, 2002), which is then released into the cytoplasm through the same mechanism utilized for Pi extrusion

following G6P hydrolysis. Pyrophosphate, however, is delivered to the ER lumen by a transport mechanism distinct from that used for G6P delivery (Foster and Nordlie, 2002). Replacement of G6P with equivalent doses of PPI over a set of concentrations ranging from 1 to 20mM did not result in detectable differences in hydrolysis rate between microsomes from control livers and from EtOH-perfused livers, irrespective of the dose of EtOH infused (Table 2). Stimulation of Ca^{2+} uptake in microsomes from livers perfused with 0.01% or 0.1% EtOH in the presence 5mM PPI in place of 5mM G6P resulted in an accumulation and IP_3 -induced release that were quite similar (~94%) to those measured in control microsomes. The amount of Ca^{2+} accumulated within microsomes at $t=45$ min, prior to IP_3 addition, and retained therein at $t=55$ min (i.e. 10 min after IP_3 addition) accounted for 40.13 ± 2.80 and 31.67 ± 2.15 nmol Ca^{2+} /mg protein, respectively ($n = 4$, $p > 0.05$), for 0.01% EtOH, and 39.33 ± 2.57 and 31.47 ± 2.04 Ca^{2+} /mg protein, respectively ($n = 4$, $p > 0.05$), for 0.1% EtOH). The Ca^{2+} accumulation and release in microsomes from livers perfused with 1% EtOH, however, remained ~30% depressed, in keeping with an inhibitory effect of EtOH on SRCA pump activity (30.28 ± 1.98 and 22.77 ± 1.54 Ca^{2+} /mg protein at $t = 45$ min and $t = 55$ min, respectively, $n = 4$, $p < 0.05$).

The decrease in Ca^{2+} accumulation and the increased rate of IP_3 -induced Ca^{2+} release following 1% EtOH infusion could depend – at least in part - on an increased leakiness of the ER membrane and in particular the translocon pore (Giunti et al., 2007) to Ca^{2+} , the process being more evident under conditions in which reticular Ca^{2+} uptake is enlarged by Pi generation within the ER/microsome lumen (Figs. 1 and 2). Measurement of Ca^{2+} release from microsomes passively loaded with 1mM CaCl_2 labeled with ^{45}Ca according to Giunti et al. (2007) indicated that both passive Ca^{2+} loading capacity and passive Ca^{2+} release were unaffected following EtOH exposure (data not shown).

Ethanol metabolism by cyP4502E1 is associated with the formation of free radicals which, in turn, could affect sulphydryl groups of ER protein including the IP_3 receptor (Schuller et al., 1985). Pretreatment of perfused livers with 100 μM dithiothreitol (DTT) prior to EtOH infusion protected both Ca^{2+} accumulation (Fig. 6A) and release by IP_3 (Fig. 6B), and G6Pase activity (Fig. 6C). Similar results were obtained in microsomes isolated from livers pretreated with 100 μM NAC (not shown). The presence of ROS-modified carbonyls (Fig. 7A) and HNE/protein adducts (Fig. 7B) in microsomes from livers exposed to EtOH supported the hypothesis that the observed defect in G6P-mediated Ca^{2+} accumulation depended – at least in part - on the formation of free radicals within the ER/microsomes. The formation of HNE adducts (Fig. 7B) and ROS-modified carbonyl (not shown) were significantly attenuated by pre-treatment with 100 μM CMZ (Figs. 7B and 7C). Western Blot assessment of G6Pase expression (Fig. 7D) indicated the presence of equivalent amounts of the protein catalytic subunit in all microsome preparations irrespective of the treatment (i.e. EtOH or sham perfusion).

Discussion

Cytosolic free Ca^{2+} level has been reported to become elevated following EtOH administration to hepatocytes (Gasbarrini et al., 1996; Hoek et al., 1987). This increase has been attributed to a combination of factors including 1) the inability of the endoplasmic reticulum of the hepatocyte to properly accumulate and/or retain Ca^{2+} within its lumen due to defective SERCA pump activity, IP_3 release, or ER phospholipid composition and membrane permeability (Schuller et al., 1985), and 2) an altered operation of Na^+ -dependent mechanism(s) in the plasma membrane, whereby the initial Na^+ overload promotes an increased exchange of Na^+ for Ca^{2+} via the Na/Ca exchanger (Carini et al., 2000). Irrespective of the mechanism, the elevated cytosolic free Ca^{2+} level would translate into an

accumulation of the cation within the mitochondrion, with consequent bioenergetics uncoupling (Burke et al., 1977), and/or activation of ER stress (Sammels et al., 2010).

The intraluminal location and operation of the glucose 6-phosphatase complex has been associated with the ability of the enzyme to increase Ca^{2+} sequestration with the ER and enlarge the luminal Ca^{2+} pool within hepatocytes (Benedetti et al., 1987; Romani et al., 1988), kidney microsomes (Fulceri et al., 1990), and pancreatic β -cells (Wolf et al., 1989) through the generation of intrareticular inorganic phosphates (Pi) that complex Ca^{2+} ions transported by the SERCA pump. Far from being insoluble, the Ca^*Pi complex is in a dynamic equilibrium as the addition of IP_3 elicits Ca^{2+} release from microsomal vesicles (Benedetti et al., 1987, Romani et al., 1988) or intact ER of digitonin-permeabilized hepatocytes (Benedetti et al., 1986), in association with Pi (Romani et al., 1988), resulting in a mobilization of Ca^{2+} that is directly proportional to the sequestered amount.

Chronic ethanol administration has been reported to enhance the activity of hepatic glucose 6-phosphatase (Ishii et al., 1973; Nelson et al., 1970). To the best of our knowledge, no study prior to this report has investigated whether acute ethanol administration has a similar effect on the enzyme activity and its repercussion on reticular Ca^{2+} homeostasis.

Effect of Ethanol on Reticular Ca^{2+} Cycling

Acute administration of ethanol up to a concentration of 0.1% (~16mM) does not affect ATP-supported Ca^{2+} accumulation via SERCA pump in liver microsomes. SERCA pump activity is instead impaired by 25% following 1% (~160mM) ETOH infusion into a liver for 8 min. Because 1% EtOH is well above the pharmacological level achieved by the most common modalities of EtOH administration, the discussion will predominantly emphasize the effects of the lower doses of EtOH utilized (i.e. 0.01% and 0.1%).

Noteworthy, the damaging effect of EtOH is fully prevented by chloromethiazole (CMZ), an inhibitor of cyP4502E1, or by dithio-threitol (DTT), a non specific scavenger for SH groups, but not by 4-methyl-pyrazole (4-MP), an inhibitor of cytosolic alcohol dehydrogenase.

While the addition of 0.01% EtOH affects the amplitude of IP_3 -induced Ca^{2+} release essentially by limiting the enlargement of the microsomal (reticular) Ca^{2+} pool through an effect on G6Pase activity (see next Section), the addition of 0.1% EtOH is already sufficient to impact the kinetic of Ca^{2+} release upon IP_3 addition, and result in a faster rate of IP_3 -induced Ca^{2+} release despite the smaller level of Ca^{2+} accumulated within the microsomal vesicles. Also in this case, pretreatment with CMZ or DTT restored Ca^{2+} release to an extent comparable to that observed in normal microsomes. In terms of IP_3 -induced Ca^{2+} release, the results reported here show a slower kinetics as compared to those previously reported by Benedetti et al. (1986). In that study, however, the authors used purified rough and smooth endoplasmic reticulum subpopulations (Benedetti et al., 1986) while for our study we use total microsomes. Hence, we cannot exclude that the purification step undertaken in the former study may have resulted in more pure membranes (or in the loss of auxiliary components) and an accelerated IP_3 release under seemingly similar experimental conditions.

Taken together, these results suggest that the impairing effect of EtOH at the two highest doses tested in the study is mainly consequent to its oxidation through cyP4502E1 and the concomitant formation of free radicals, which damage SH groups in SERCA pump (Voss et al., 2008) and IP_3 -receptor (Kraus-Friedmann, 1990). Western Blot analysis did not indicate modification of either SERCA pump or IP_3 -receptor by HNE. As our antibodies specifically recognize adduct formation between proteins and HNE, we cannot exclude that SERCA pumps and/or IP_3 receptor are modified by acetaldehyde or reactive oxygen species

(generated by EtOH metabolism via cyP4502E1) or lipid peroxidation products other than HNE (Niemela et al., 1998). More ad hoc studies need to be performed to clarify the mechanism(s) damaging SERCA pump and IP₃ receptor following acute EtOH administration.

Effect of Ethanol on G6Pase activity

In contrast to the enhancement of glucose 6-phosphatase activity by chronic ethanol administration (Ishii et al, 1973; Nelson et al., 1970), acute ethanol administration for 8 min markedly inhibits the enzyme hydrolytic activity in a dose-dependent manner. In fact, the inhibition of G6Pase hydrolytic activity can already be observed after administration of 0.01% EtOH (~1.6), and increases progressively following addition of higher doses of EtOH. This is at variance of the effect of EtOH on Ca²⁺ uptake and release mentioned above, which only occurs at concentrations of EtOH higher than 0.01%.

The G6Pase inhibition results in a reduced Pi generation, and consequently a reduced Ca²⁺ sequestration within the microsome (ER) lumen. Administration of CMZ or DTT, but not 4-MP, also protects G6Pase activity from EtOH damaging effects. The administration of CMZ or DTT, in fact, increases intraluminal production of Pi and enlarges the reticular Ca²⁺ pool, with consequent enhancement of IP₃-induced Ca²⁺ release.

In addition, our data suggest that EtOH effect occurs at the point of G6P entry. Permeabilization of the microsomal membrane by taurocholic acid, which bypasses the point of entry and results in proper substrate delivery to the G6Pase catalytic site, abolishes the inhibitory effect of EtOH on G6P hydrolysis. Unfortunately, the membrane permeabilization by taurocholic acid prevented us from assessing the effect of normal G6P hydrolysis on Ca²⁺ uptake in the presence of EtOH treatment. However, the results obtained in the presence of PPi suggest that irrespective of the source Pi generation within the microsome (ER) lumen can enlarge the reticular Ca²⁺ pool provided that the SERCA pump and the IP₃ channel are not affected by acute EtOH administration. Whether the effect of EtOH on G6P entry mechanism occurs directly at the G6P binding site or at the protein/phospholipid inter-phase is topic for future studies. It also remains to be determined as to whether the increase in G6Pase activity observed following chronic EtOH administration is the result of an increased protein expression for compensatory reasons or the consequence of changes in G6Pase regulation within the ER.

Conclusions

For the first time, the results reported here: 1) indicate the occurrence of a specific damage of hepatic G6Pase following acute alcohol administration, and 2) highlight the consequence of such a damage for the ability of hepatic endoplasmic reticulum to sequester and mobilize its Ca²⁺ store.

Based upon these results, a complex scenario can be envisioned whereby acute administration of low doses of EtOH is already sufficient to dys-regulate Ca²⁺ uptake and cycling within the hepatocyte by impairing the hydrolytic activity of G6Pase and the production of inorganic phosphate within the ER lumen. This dys-regulation occurs to a greater extent following the acute administration of a high dose of EtOH, as the alcohol not only inhibits the G6Pase activity to a larger extent but also impairs the operation of SERCA pump and IP₃-receptor, further compromising Ca²⁺ homeostasis within the hepatocyte. This dys-regulation can be more significant under conditions of hormonal activation of PLC, and can have severe and far reaching implication for cell bioenergetics (i.e. mitochondrial uncoupling by Ca²⁺) and gene activation.

Acknowledgments

The authors want to thank Dr. William Schilling for helpful comments and criticisms. The study was supported by NIAAA-11593 to AR.

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Fig. 1A

Fig. 1B

Fig. 1C

Figure 1. Ca^{2+} accumulation in microsomes isolated from control livers or from livers perfused with varying doses of EtOH for 8 min

Microsomes from control livers or from livers perfused with 0.01%, 0.1% or 1% EtOH for 8 min were isolated and incubated for calcium uptake as described under Material and Methods. Calcium accumulation was initiated by adding 1mM ATP (plus regenerating system, see Materials and Methods) at time 0. Different concentrations of glucose 6-phosphate were added after withdrawal of time = 10 min (Fig. 1A). Calcium uptake was monitored over 60 min as the accumulation of ^{45}Ca within microsomes. Data are reported as net ^{45}Ca accumulation (subtracted point-by-point of passive $^{45}\text{Ca}^{2+}$ binding to microsomes and filter). The effect of the different doses of EtOH on microsomal Ca^{2+} accumulation is reported in Fig. 1B. Figure 1C reports net Ca^{2+} accumulation supported by ATP (plus regenerating system) during initial 10 min on an expanded scale. Data in all panels are means $\pm$ S.E. of 4 different preparations each tested in duplicate for each experimental condition. Fig. 1A: *Statistically significant vs. corresponding values in Basal and 'ATP only' samples. Fig. 1B: all the time points of Ca^{2+} uptake following G6P addition are statistically significant vs. the corresponding values in the 'no EtOH' sample. Labeling is omitted for simplicity. Fig. 1C: *Statistically significant vs. corresponding values in all other samples.

Fig. 2A

Fig. 2B

Figure 2. Ca^{2+} accumulation and release in microsomes isolated from livers perfused with varying doses of EtOH for 8 min

Microsomes were incubated for Ca^{2+} uptake as described in the legend to Figure 1 and under Material and Methods in detail. Calcium release was initiated by addition of $5\mu\text{M}$ inositol 1,4,5-trisphosphate (IP_3) following attainment of maximal Ca^{2+} uptake, and followed for 10 additional min (Fig. 2A). Data are reported as net ^{45}Ca accumulation (subtracted point-by-point of passive $^{45}\text{Ca}^{2+}$ binding to microsomes and filter). Figure 2B reports Log₁₀ Ca^{2+} release using corresponding Ca^{2+} accumulation level at time = 55 min (i.e. before IP_3 addition) as 100%. Data are means $\pm$ S.E. of 4 different preparations each tested in duplicate for each experimental condition. Fig. 2A: all the time points of Ca^{2+} uptake following G6P addition are statistically significant vs. the corresponding values in the 'Control' sample. Labeling is omitted for simplicity. Fig. 2B: *Statistically significant vs. corresponding values in 'Control' and 0.01% EtOH sample.

Fig. 3A

Fig. 3B

Figure 3. Ca^{2+} accumulation and Pi generation in microsomes exposed to 0.1% EtOH during the incubation time

Microsomes from control livers and from livers perfused with 0.1% EtOH were incubated for Ca^{2+} uptake as described in the legend to Figure 1 and under Material and Methods in detail. Aliquots of microsomes from both experimental groups were exposed to 0.1% EtOH (v/v) added to the incubation system at time = 0 min, together with ATP (plus regenerating system) mixture. Data in Fig. 3A are reported as net ^{45}Ca accumulation (subtracted point-by-point of passive $^{45}\text{Ca}^{2+}$ binding to microsomes and filter). Data in Fig. 3B report Pi generation following addition of 5mM G6P to the incubation system at time 10 min (corresponding to time = 0 min for the Pi generation). Data are means $\pm$ S.E. of 3 different preparations each tested in duplicate for each experimental condition. *Statistically significant vs. corresponding values in Control and EtOH samples, respectively.

Figure 4. Ca^{2+} accumulation and release, and Pi generation in microsomes isolated from livers pretreated with chloromethiazole and perfused with varying doses of EtOH for 8 min Livers were perfused with 100 μM chloromethiazole (CMZ) for 5 min prior to the administration of the indicated doses of EtOH. Chloromethiazole was maintained throughout EtOH administration (8 additional min). Microsomes were isolated and incubated for Ca^{2+} uptake as described in the legend to Figure 1 and under Material and Methods in detail. Calcium release was initiated by addition of 5 μM inositol 1,4,5-trisphosphate (IP_3) following attainment of maximal Ca^{2+} uptake, and followed for 10 additional min. Data in Fig. 4A are reported as net ^{45}Ca accumulation (subtracted point-by-point of passive $^{45}\text{Ca}^{2+}$ binding to microsomes and filter). Figure 4B reports Log_{10} Ca^{2+} release using corresponding Ca^{2+} accumulation level at time = 55 min (i.e. before IP_3 addition) as 100%. Data in Fig. 4C report Pi generation following addition of 5mM G6P to the incubation system at time 10 min (corresponding to time = 0 min for the Pi generation). Data are means $\pm$ S.E. of 5 different preparations each tested in duplicate for each experimental condition. Fig. 4C: *Statistically significant vs. corresponding values in all other samples.

Figure 5. Ca²⁺ accumulation and release, and Pi generation in microsomes isolated from livers pretreated with 4-methyl-pyrazole and perfused with varying doses of EtOH for 8 min Livers were perfused with 50μM 4-methyl-pyrazole (4-MP) for 5 min prior to the administration of the indicated doses of EtOH. The alcohol dehydrogenase inhibitor 4-methyl-pyrazole was maintained throughout EtOH administration (8 additional min). Microsomes were isolated and incubated for Ca²⁺ uptake as described in the legend to Figure 1 and under Material and Methods in detail. Calcium release was initiated by addition of 5μM inositol 1,4,5-trisphosphate (IP₃) following attainment of maximal Ca²⁺ uptake, and followed for 10 additional min. Data in Fig. 5A are reported as net ⁴⁵Ca accumulation (subtracted point-by-point of passive ⁴⁵Ca²⁺ binding to microsomes and filter). Figure 5B reports Log₁₀ Ca²⁺ release using corresponding Ca²⁺ accumulation level at time = 55 min (i.e. before IP₃ addition) as 100%. Data in Fig. 5C report Pi generation following addition of 5mM G6P to the incubation system at time 10 min (corresponding to time = 0 min for the Pi generation). Data are means±S.E. of 5 different preparations each tested in duplicate for each experimental condition. Fig. 5A: all the time points of Ca²⁺ uptake following G6P addition are statistically significant vs. the corresponding values in the 'Control-4MP' sample. Labeling is omitted for simplicity. Fig. 5B: *Statistically significant vs. corresponding values in the 'Control-4MP' sample. Fig. 5C: *Statistically significant vs. corresponding values in the 'Control-4MP' sample.

Figure 6. Ca^{2+} accumulation and release, and Pi generation in microsomes isolated from livers pretreated with dithiothreitol and perfused with varying doses of EtOH for 8 min

Livers were perfused with $100\mu\text{M}$ dithio-threitol (DTT) for 5 min prior to the administration of the indicated doses of EtOH. Dithiothreitol was maintained throughout EtOH administration (8 additional min). Microsomes were isolated and incubated for Ca^{2+} uptake as described in the legend to Figure 1 and under Material and Methods in detail. Calcium release was initiated by addition of $5\mu\text{M}$ inositol 1,4,5-trisphosphate (IP_3) following attainment of maximal Ca^{2+} uptake, and followed for 10 additional min. Data in Fig. 6A are reported as net ^{45}Ca accumulation (subtracted point-by-point of passive $^{45}\text{Ca}^{2+}$ binding to microsomes and filter). Figure 6B reports Log_{10} Ca^{2+} release using corresponding Ca^{2+} accumulation level at time = 55 min (i.e. before IP_3 addition) as 100%. Data in Fig. 6C report Pi generation following addition of 5mM G6P to the incubation system at time 10 min (corresponding to time = 0 min for the Pi generation). Data are means $\pm$ S.E. of 5 different preparations each tested in duplicate for each experimental condition.

Figure 7. Modification of microsomal protein by ROS and HNE

Microsomes were isolated from EtOH perfused livers as described in detail under Material and Methods. Non-specific modification of protein carbonyls by reactive oxygen species (ROS) assessed by Oxyblot staining according to the manufacturer protocol is reported in Figure 7A. One typical experiment out of three is reported. Formation of HNE/protein adducts is distinct microsomes preparations for the various experimental conditions, both in the absence and in the presence of chloromethiazole (CMZ, 100 μ M) is reported in Fig. 7B. Densitometry determination of HNE adducts is reported in Figure 7C ($n = 7$ for each experimental condition, each tested in duplicate). *Statistically significant vs. the Control and CMZ-treated samples. Fig. 7D reports Western Blot analysis of G6Pase catalytic subunit (2 distinct microsomes preparations out of 4 for each experimental condition are reported).

Table 1

Glucose 6-Phosphatase Activity in Liver Microsomes Following Perfusion with Different Doses of EtOH.

	15 min	30 min	% Difference at 30 min vs. corresponding Control value
<u>Control MS</u>			
1 mM G6P	0.09±0.004	0.19±0.03	100
2 mM G6P	0.12±0.01	0.26±0.02	100
5 mM G6P	0.35±0.03	0.74±0.06	100
10 mM G6P	0.55±0.05	1.17±0.07	100
20 mM G6P	0.93±0.08	1.96±0.05	100
<u>0.01% EtOH</u>			
1 mM G6P	0.07±0.003 *	0.15±0.02	78
2 mM G6P	0.11±0.01	0.24±0.03	92
5 mM G6P	0.29±0.02	0.62±0.06	82
10 mM G6P	0.47±0.04	1.03±0.06	85
20 mM G6P	0.88±0.07	1.87±0.05	95
<u>0.1% EtOH</u>			
1 mM G6P	0.06±0.003 *	0.13±0.02	67
2 mM G6P	0.09±0.02	0.19±0.02	75
5 mM G6P	0.23±0.03 *	0.49±0.03 *	66
10 mM G6P	0.39±0.06	0.83±0.04 *	71
20 mM G6P	0.74±0.05	1.57±0.07 *	79
<u>1% EtOH</u>			
1 mM G6P	0.05±0.004 *	0.11±0.03	56
2 mM G6P	0.07±0.01 *	0.15±0.02 *	58
5 mM G6P	0.16±0.02 *	0.34±0.04 *	46
10 mM G6P	0.31±0.06 *	0.66±0.05 *	57
20 mM G6P	0.64±0.06 *	1.36±0.07 *	69

Values are $\mu\text{mol Pi/mg protein}$ generated at the reported time points. Data are means $\pm$ S.E. of 8 different preparations for each experimental condition, each tested in duplicate.

* Statistically significant versus the corresponding control value

Table 2

Glucose 6-Phosphatase Activity in Liver Microsomes Following Perfusion with Different Doses of EtOH.

	30 min	% Difference at 30 min vs. corresponding Control value
<u>Control MS</u>		
1 mM G6P	0.21±0.03	100
2 mM G6P	0.25±0.03	100
5 mM G6P	0.75±0.05	100
10 mM G6P	1.22±0.06	100
20 mM G6P	1.96±0.07	100
<u>0.01% EtOH</u>		
1 mM G6P	0.17±0.02	81
2 mM G6P	0.22±0.04	88
5 mM G6P	0.62±0.03	82
10 mM G6P	1.00±0.05 *	82
20 mM G6P	1.82±0.08	93
<u>0.1% EtOH</u>		
1 mM G6P	0.13±0.03	62
2 mM G6P	0.20±0.02	80
5 mM G6P	0.49±0.04 *	66
10 mM G6P	0.81±0.05 *	67
20 mM G6P	1.63±0.06 *	83
<u>1% EtOH</u>		
1 mM G6P	0.11±0.03	54
2 mM G6P	0.17±0.02	66
5 mM G6P	0.34±0.04 *	45
10 mM G6P	0.66±0.07 *	55
20 mM G6P	1.45±0.09 *	73

Values are μmol glucose/mg protein generated at the reported time points. Data are means $\pm$ S.E. of 8 different preparations for each experimental condition, each tested in duplicate.

* Statistically significant versus the corresponding control value

Table 3

Glucose 6-Phosphatase Activity in Liver Microsomes Following Perfusion with 1% EtOH and in vitro treatment with 0.1% taurocholic acid and G6Pi or 5mM pyrophosphate to replace G6Pi

	30 min	% Difference at 30 min vs. corresponding Control value (Table 1)
<u>Taurocholic acid</u>		
<u>Control MS</u>		
1 mM G6P	0.31±0.03	100
5 mM G6P	1.03±0.08	105
20 mM G6P	1.35±0.04	98
<u>1% EtOH</u>		
1 mM G6P	0.33±0.05	106
5 mM G6P	1.02±0.06	104
20 mM G6P	1.36±0.07	99
<u>Pyrophosphate (PPi)</u>		
<u>Control MS</u>		
1 mM PPi	0.29±0.02	94
5 mM PPi	1.04±0.03	106
20 mM PPi	1.41±0.04	102
<u>1% EtOH</u>		
1 mM PPi	0.27±0.04	87
5 mM PPi	0.92±0.06	94
20 mM PPi	1.29±0.07	94

Values are $\mu\text{mol Pi/mg protein}$ generated at the reported time points. Data are means + S.E. of 5 different preparations for each experimental condition, each tested in duplicate.