# Uncovering Protein Kinase Cε as a Central Regulator of Acute Hepatic Steatosis due to Ethanol

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**Abstract:**

Avoiding the occurrence of steatosis could reduce the progression of future stages of alcoholic liver disease. The establishment of steatosis is considered to be a key event in the development of alcoholic liver disease. Although the PKCe enzyme has been connected with hepatic steatosis in non-alcoholic fatty liver disease experimental models, its role in ethanol-induced steatosis is unclear. The current study aims to test the hypothesis that PKCe plays a role in the formation of ethanol-induced steatosis using PKCe knockout mice as well as wildtype mice that have been treated with an antisense oligonucleotide (ASO) to inhibit PKCe expression. The effects of acute ethanol on indicators of hepatic steatosis and insulin signaling were evaluated in both types of mice. Following acute ethanol administration (6 g/kg i.g.), hepatic non-esterified free fatty acids (NEFA) increased significantly and peaked 1 hour after administration. After the increase of NEFA, there was also an increase in diacylglycerol (DAG) concentrations along with concurrent activation of PKCe. Additionally, the administration of acute alcohol altered the expression of multiple insulin-sensitive genes (e.g., G6Pase was upregulated, GK was downregulated) in an apparent manner consistent with disruption of insulin signalling. Furthermore, after acute alcohol consumption, there was a significant increase in hepatic triglyceride (TAG) accumulation. However, in the PKCe knockout and ASO treated animal models, the accumulation of TAG due to ethanol was inhibited. The combination of previous research indicates that an increase in non-esterified fatty acids (NEFA) as a result of metabolic processing of ethanol in the liver leads to increased diacylglycerol (DAG) synthesis via the triacylglycerol pathway. Activation of protein kinase C-epsilon (PKCe) by DAG additionally contributes to the development of hepatic lipids through insulin resistance.

**Keywords:** Protein Kinase C-epsilon; Ethanol; Hepatic steatosis; Insulin resistance; Diacylglycerol; Antisense oligonucleotide; Knockout mice

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## Introduction:

Alcoholic liver disease (ALD) affects millions of people each year and is one of the leading causes of death around the globe. Recent estimates have suggested that over $148 billion was spent on treating patients suffering from ALD in the United States alone between 1985 and 1992 [1]. Although further research is needed to fully understand the pathways leading to ALD, there is currently no FDA-approved drug that will successfully delay or stop this deadly disease's progression. Identifying the molecular mechanisms responsible for ALD will be necessary for developing an effective treatment to prevent or reverse the pathological changes associated with ALD [2].

Hepatic steatosis is recognized as the earliest pathological change associated with ALD. While steatosis was previously regarded as an innocuous pathological condition in relation to ALD, recent evidence has demonstrated that reduction of steatosis may help to slow the progression of ALD [3]. Hepatic steatosis is known for its dire relationship with alcohol metabolism over time. Specifically, metabolism of ethanol (i.e., alcohol) increases the NADH/NAD+ ratio, which inhibits hepatocytes from oxidating fatty acids via β-oxidation [4]. Furthermore, metabolism of ethanol increases the rate of esterification of fatty acids, resulting in increased rates of hepatic triglyceride accumulation from altered fatty acids from ethanol metabolism. However, previous studies suggest that steatosis induced by ethanol may be caused by multiple mechanisms [5]. Precisely, various pharmacological and genetic manipulations (including gene knockouts) have been shown to reduce hepatic steatosis in rodent models of ethanol exposure. For example, multiple animal models (mice) deficient in prooxidants (NADPH oxidase; iNOS) or in LPS-binding (CD14, TLR4, LBP) or signaling molecules, have been documented to exhibit less steatosis as a result of ethanol ingestion, as compared to wild-type (WT) controls. However, there have been no significant influences on ethanol metabolism associated with any of these pharmacological or genetic manipulations shown to influence steatosis in prior studies [6,7]. Therefore, it appears that ethanol metabolism alone does not account for ethanol-induced steatosis. Another

mechanism by which ethanol could induce steatosis is by increasing insulin resistance in the liver. Ethanol consumption—both in the short and long term—has been demonstrated to result in increased hepatic insulin resistance in animal models [8].

The development of fat in the liver (hepatosteatosis) has been linked to impaired insulin signaling in the liver, especially in those with non-alcoholic fatty liver disease (NAFLD). Insulin resistance most likely affects hepatic lipid metabolism similarly, but is not as well studied in alcoholic liver disease (ALD). Insulin resistance is a risk factor for developing ALD in humans [9,10]. Recent findings from this group support the link between insulin resistance and hepatic steatosis after ethanol exposure, by showing that the insulin-sensitizing drug metformin blocks ethanol-induced fatty liver [11,12].

One proposed mechanism through which ethanol can induce hepatic insulin resistance/steatosis is through the activation of protein kinase C epsilon (PKCe). Through PKCe's inhibition of the insulin receptor substrate-2 (IRS-2) tyrosine phosphorylation, hepatic insulin signaling is believed to be inhibited [13]. Recent evidence shows that PKCe activation leads to hepatic insulin resistance in experimental models of NAFLD [14]. The activation of this kinase may also contribute to steatosis in humans. In the current study, we investigated in an acute animal model whether PKCe is activated after ethanol exposure, and whether PKCe contributes to the development of ethanol-induced hepatic steatosis.

**Experimental**

**Specimen Grouping and Exposures**

The protocols were approved by the Oriental College Animal Care and Use Committee, and the mice were maintained in a pathogen-free barrier facility, which also had institutional ethics committee approval. Six-week-old C57BL/6J PKCe knockout mice were provided from the Jammu Tawai CSIR laboratory. All mice had been given unrestricted access to both food and tap water prior to testing.

The administration route for the treatment used a single bolus dose of ethanol (6 g/kg) in a 20% saline solution delivered via oral gavage. Two separate types of control, isocaloric or isovolumetric maltose—dextrin, were used. Following the dose of ethanol given, blood ethanol levels were found to be within the range of 250—300 mg/dL; therefore, although animals were ataxic, they did not become unconscious and did not experience fatality due to an overdose of alcohol. Some of the mice were administered PKCε antisense oligonucleotide (ASO); PKCε antisense oligonucleotide sequences were purchased from IDT Technology, Banglore: 5'-CTCGCAGATTTTGATCTTAA-3' and 5'-CATGGTPCCCCCCAACCACC-3' (T or C can be at P). The method for injection of the ASO follows the same methods with small modifications for injection into mice, as described by Samuel, et al. [15]. Mice were administered a dosage of either PKCe ASO or vehicle saline for four consecutive weeks (25 mg/kg by i.p. route with dose administered biweekly). 0–12 hours after ethanol gavage, mice were slaughtered.

Animals were sedated with an i.m. injection of ketamine/xylazine (100/15 mg/kg) and subsequently had their blood collected from the vena cava. The blood was processed into citrated plasma and frozen at -80 °C for further analysis. Plasma insulin levels were determined with an ELISA kit; glucose levels were measured using a glucose assay. Liver tissues were immediately frozen in liquid nitrogen, and additional liver tissues were freeze-fixed in an OCT Cryomount and sectioned/mounted onto microscope slides.

**Western Blotting**

We performed Western Blotting for PKCe as explained earlier [16]. An isolation buffer (50 mM Tris-HCl, pH 7.5, 10 mM EGTA, 50 mM b-mercaptoethanol) with protease inhibitors was used to separate total hepatic protein from tissue. Cell homogenates were centrifuged at 100 g to remove any intact cells; After that, the supernatant was centrifuged for one hour at 4°C at 100,000 g. The supernatant from the centrifugation of the lysate was used as the cytosolic fraction. The pellet was solubilized by suspending it again in the original isolation buffer with 0.1% NP-40 and incubating the pellet on ice for 30 minutes, followed by a brief sonication. Protease, tyrosine phosphatase and serine/threonine phosphatase inhibitor mixtures were added to each of the protein extraction buffers. The corresponding lysates (100 µg protein/gel) were separated using a 10% SDS-polyacrylamide gel. The proteins were then transferred to polyvinylidene difluoride membranes using a semi-dry transferring apparatus. The resulting blots were probed using anti-PKCe antibodies, and the blots were analyzed using the ECL plus kit to visualize the protein bands. Each blot was dyed with Ponceau red to ensure equal amounts of protein on each blot before scanning.

**Lipid determinations**

For the detection of hepatocyte lipids, freeze-dried liver sections (10 µm) were stained with oil red O for 10 minutes, and then washed in alcohol before being further stained with hematoxylin for 45 seconds. Homogenization of mouse liver samples for triglyceride and NEFA measurement was achieved using two volumes of phosphate buffered saline in each sample. Lipids were extracted from the tissue using a methanol:chloroform (1:2) solution, then dried using a Speed-Vac, and finally resuspended in 5% fat-free bovine serum albumin. Hepatic triglycerides were quantified as per Bergheim et al. Non-esterified fatty acids were measured colorimetrically using commercial kit. The Bradford assay was carried out on the

homogenate prior to extracting the liver lipids in order to determine the total amount of protein for the extraction. The extraction of hepatic lipids to determine the levels of DAG (Diacylglycerol) in the liver followed Bligh and Dyer's procedure which involves extracting the lipids with a chloroform/methanol/water solution. DAG was quantitated according to Callender et al. [17], where DAG was isolated from phospholipids by silica gel column chromatography using an elution buffer of 65:35:0.7 CHCl3:CH3OH:H2O. Six cm of silica gel was used to fill each elution column and received 10 mL of elution buffer to equilibrate the column prior to use (columns were capped with glass wool after equilibration). The first two milliliters (mL) of elution buffer were removed from the elution column, dried under vacuum, and resuspended in 120 mL of a 9:1 (v/v) solution of CH3OH:CHCl3 containing five mL of 100 mM sodium acetate (CH3COONa) for DAG recovery. All liver extracts received 100 pmol of a known DAG standard (DAG 28:0) to determine the recovery of DAG following separation. The samples yielded by the extraction procedure were analyzed by electrospray ionization mass spectrometry (ESI-MS) on a Micromass ZMD mass spectrometer with a Harvard Apparatus syringe pump with a flow rate of 10 µL/min using positive ionization mode and a 20 - 2000mass-to-charge ratio (m/z). Through the identification of individual DAG species using sodium adducts and their quantification relative to existing standards, selected peaks were identified using mass spectrometry (ESI/MS/MS) by simple disintegration.

**RNA isolation and real-time RT-PCR**

As is typical in this group, certain genes were evaluated for message levels using real-time, reverse transcriptase PCR. Total RNA was extracted from liver tissue samples using a guanidium thiocyanate-based protocol. Total RNA (1 µg) was reverse transcribed (using an AMV reverse transcriptase kit and random primers) following spectrophotometric determination of RNA concentration. Primers were developed using primer 3 (in Table 1). The comparative CT method was used to assess the relative fold changes among tissues and the purity of PCR products was confirmed by gel electrophoresis.

**Table 1: Real-time PCR forward and reverse primers and probes used**

| Gene | Forward primer (5'→3') | Reverse primer (5'→3') | Probe (5'→3') |
|---|---|---|---|
| **Glucose-6-phosphatase** | TGTGGGCTCCACCTATGG | CCGCAAGATGGCGAAAG | Commercial Taq Man assay |
| **Glucose-6-phosphatase (G6pc)** | | | y commonly used (Applied Biosystems) |
| **Glucose-6-phosphatase (G6pc)** | GAAAGCCTTTGGGTTGTTGC | AGAGCGTTGTCCAAACAGAA | SYBR Green assay (no probe) |
| **Glucokinase (Gck)** | TGGATGGAGTTCTCCTTCTTC | CCAGGTCTGTTTCCACATCA | Commercial Taq Man assay available |
| **Glucokinase (Gck)** | GAGATGGATGGAGACAGAGC | TCCAGGTCCTTGAACTTGAT | SYBR Green assay |
| **β-actin (Actb)** | CGACAGGATGCAGAAGGAGAT | CAAGAAAGGGTGTAACGCAACTA | Commercial Taq Man endogenous control available |

**Glucose-6-Phosphatase Activity**

Glucose-6-phosphatase enzyme activity was measured by using glucose-6-phosphate to synthesize phosphate. Livers (100 mg) were homogenized in 500 µl of a 250 mM sucrose–HEPES buffer (adjusted to pH 7.4) using a hand-held homogenizer, and then centrifuged for 10 minutes at 3000 g and 4 °C to separate the supernatant. After the addition of taurocholic acid (25 µl) to the supernatant (100 µl) and incubation on ice for 30 minutes, the sample was tested. The solution was combined with 175 µl of

homogenization buffer after incubation. Combined with 60 µl aliquot was 140 µl of reaction buffer (90 mM Tris–HCl, pH 6.5; 14 mg/ml bovine serum albumin, 28 mM glucose-6-phosphate) and incubated at 37 °C for 20 min. After 0 and 20 min, 140 l of stop solution (15% chilled trichloroacetic acid) was added, and samples were centrifuged at 3000 rcf for 10 min at 4 °C Then 140 µl of supernatant was mixed with phosphate reagent (5% ammonium molybdate tetrahydrate in 4N HCl plus 1% iron (II) sulfate heptahydrate) to determine the total amount of NADH produced from the breakdown of glucose-6-phosphate dehydrogenase. The difference in absorbance for each sample was measured at 650 nm, and the results were corrected for protein concentration.

**Glucokinase Activity**

According to Rossetti et al. [18], the absorbance of NADH produced by glucose-6-phosphate dehydrogenase was used to gauge the activity of glucokinase. In short, 100 mg of livers underwent homogenization in 1 ml of HEPES buffer (50 mM, pH 7.4), which contained 2.5 mM dithioerythritol, 5 mM MgCl2, 1 mM EDTA, and 100 mM KCl. After that, the homogenates were analyzed.

**Statistics Applied**

The findings are presented as means ± SEM (n = 4–6). Parametric and non-parametric data were analyzed for differences among treatment groups using ANOVA with Bonferroni's post hoc test and Mann-Whitney rank sum test, respectively.Prior to the investigation, 'p' of less than 0.05, was chosen as the significance.

**Results Observed**

**Hepatic Lipids got Increased due to Ethanol**

The acute ethanol impact on hepatic triglycerides and fatty acids non-esterified (i.e. NEFA) in wild-type mice can be seen in Figure 1. Hepatic triglyceride levels from the isocaloric maltose-dextrin-fed mice were similar to those of the naïve mice. As previously reported, ethanol increased triglyceride levels incrementally, with the triglyceride level at the 12-hour time point being 20 times higher than in the maltose-dextrin controls [19]. Acute ethanol also increases hepatic NEFA levels but the pattern of response is different from that of hepatic triglyceride levels. Specifically, ethanol caused a 5-fold increase in hepatic NEFA at 1 hr. of exposure, with levels returning to baseline by the 12-hour time point.

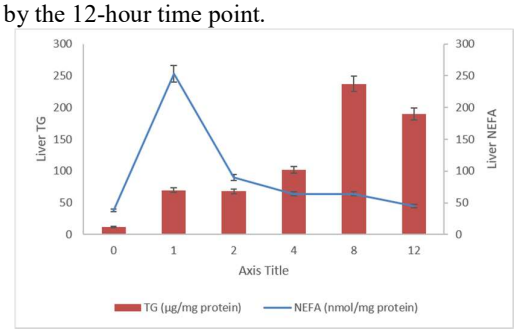



**Figure 1: Hepatic triglycerides (TG) and NEFA levels on acute ethanol exposure**

**Ethanol's impact on PKCe activation and hepatic DAG levels**

DAG are the primary facilitator of PKCe stimulation, as previously stated [20]. Therefore, it was hypothesized that the increase in NEFA resulting from alcohol consumption would also increase the concentration of DAG in the liver, since new DAG is generated from NEFA. Figures 2 and 3 depict the effects of acute ethanol on hepatic DAG concentrations. Figure 2 illustrates the effects of ethanol on some specific DAG species. In particular, DAG 34:2 (m/z 615), DAG 32:0 (m/z 577 [with ether bond at sn1]), and DAG 38:6 (m/z 663) had increased dramatically (1.5-fold), while DAG 32:0 (m/z 577, with ether at sn1) increased the most (over three times higher than controls) compared to all other DAG. Figure 3 contains the summary spectroscopic data obtained from untreated and ethanol-treated animals for these studies. Ethanol caused long-chained DAG to rise significantly over two hours in various species. Thus, ethanol caused an increase in long-chained DAG with no change in short-chained or medium-chained DAG.

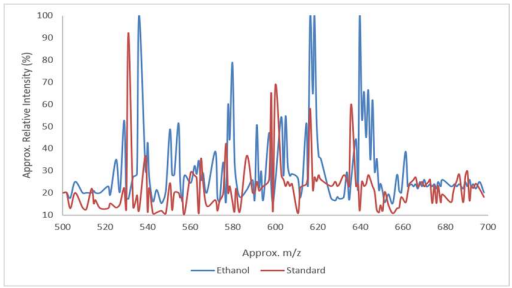



**Figure 2: Mass ionization spectrums showing the effect of ethanol wrt the standard**

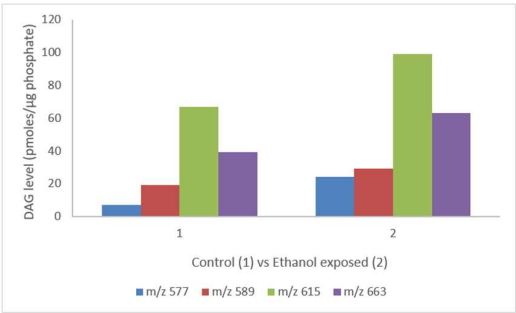



**Figure 3: Summary on the effect of DAG levels**

Ethanol increased the ratio of PKCe in the membrane to cytosol (Figure 4). The greater the ratio, the greater the enzymatic activity. No significant differences were found between maltose dextrin treatment and naïve animals as far as PKCe did not translocate at all to the membrane. After one hour of ethanol treatment, PKCe translocation to the membrane increased by 50%. After ethanol treatment, translocation decreased to basal levels immediately and rebounded after eight hours, until it reached three times higher than the controls 12 hours after treatment.

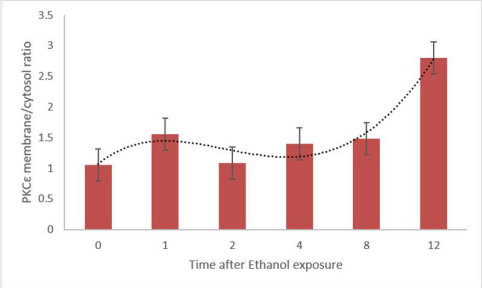
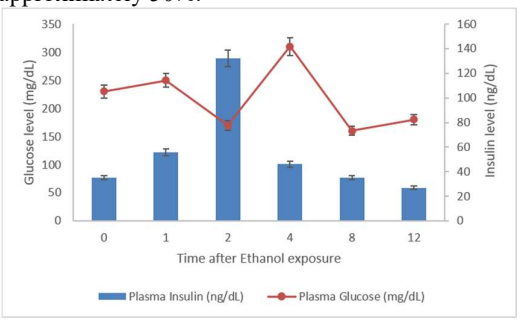



**Figure 4: Effect on activation of PKCe by Ethanol**

**Ethanol's effects on insulin-sensitivity genes expression**

Both acute and chronic exposure to ethanol has been shown to result in insulin resistance in rats' livers; this is widely recognised (see Introduction). In Onishi et al. [21], rats were exposed to bolus doses of ethanol and then assessed, via the degree of phosphorylation of both the insulin receptor (IR) and IRS-1 and -2, as well as euglycaemic, hyper-insulinaemic clamping, for the development of insulin resistance within 2 hours. The effect of ethanol on surrogate markers of insulin resistance & signalling was assessed to confirm these earlier observations under the current study conditions. As seen in Figure 5a, the effect of ethanol on plasma glucose and insulin was assessed. Two hours after the administration of ethanol, plasma insulin levels had increased by approximately 300%, but began to return to baseline levels over the next 12 hours. Plasma glucose levels showed no significant decline in response to the increase in plasma insulin. In fact, 4 hours following the administration of ethanol, serum glucose levels had increased by approximately 50%.




**a**

**b**

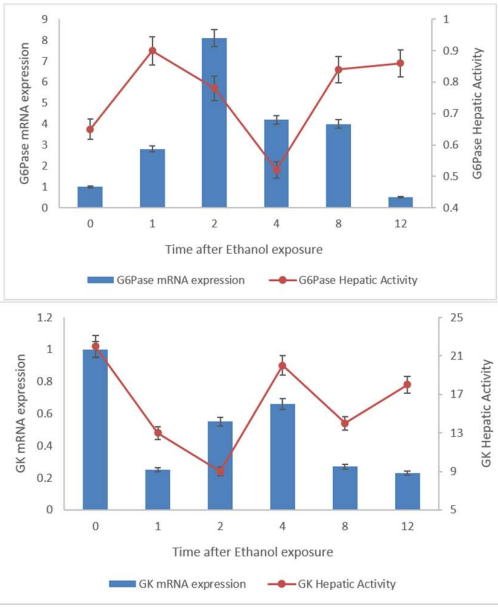



**Figure 5: Ethanol induces alterantion in - (a) Insulin and glucose concentrations in plasma (b, c) on the expression and also activity of G6Pase**

**c**

**and GK genes**

Both insulin and glucose levels in the serum were determined as well as how ethanol affects insulin's ability to regulate gene expression and activity. Specifically, the effect of ethanol on the two important insulin-regulated genes' (mRNA and protein) activities was investigated. Messenger RNA levels of G6Pase (downregulated by insulin) were increased dramatically by ethanol (8-fold) at two hours of exposure (Figure 5b), with expression gradually returning to basal after that, with mRNA levels being no different than control after 12 hours of ethanol. The effect of ethanol on hepatic G6Pase activity was also significantly higher after bolus ethanol than control, but ethanol had less impact on hepatic G6Pase enzyme activity than on its respective gene's mRNA expression. Ethanol decreased GK (activated by insulin) mRNA levels five-fold throughout the study (Figure 5c), with an additive effect from repeated bolus ethanol; there was a corresponding decrease in GK enzyme activity as well. The effect on the protein function was established to be only a portion of what the effect on mRNA expression was. Furthermore, the protein function had returned to its pre-ethanol treatment levels within four hours of the completion of the ethanol treatment.

**Reduction of Steatosis Mediated by Ethanol by PKCe Elimination or Knockdown**

In the previous studies, PKCe has been shown to induce steatosis in NAFLD models, as previously

mentioned in Figure 4. The current study demonstrated that ethanol also induced PKCe. These findings provided a foundation to determine whether "knocking-down" (ASOs or genetic knockout) PKCe would attenuate ethanol-induced steatosis as measured by oil red O staining and hepatic triglyceride quantification (Figure 6) in an ethanol-induced model. Ethanol-exposed mice had a steady-state PKCe mRNA level of 23.3 ± 4.7% lower than those PKCe mRNA levels of mice that were administered ASOs. In the present study, ethanol had enhanced oil red O staining in the liver with macrovessicular and microvessicular steatosis noted at the 12-hour time point in the liver (consistent with findings noted in other studies using this same animal model). The effect of ethanol on lipid accumulation in the liver of PKCe ASO animals was attenuated when compared to ethanol-treated animals.

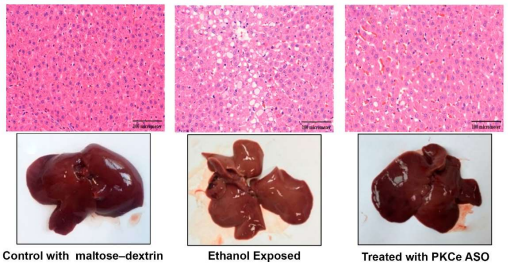



**Figure 6: PKCe ASO Effect on steatosis by ethanol**

Overall, the impact of PKCe ASO treatment on liver steatosis caused by ethanol may differ more greatly for macro (large) versus micro (smaller) lipid droplets. More complete regression of ethanol-induced triglyceride elevation (50% decrease) was also observed when PKCe knockout mice received ASO treatment.

**PKCe ASO Impact on ethanol altered insulin-responsive genes expression**

Data demonstrating that PKCe plays a role in mediating the stimulatory effects of insulin on lipid metabolism in NAFLD via PKCe has been previously presented. The relationship of PKCe activity to ethanol-induced insulin response gene expression changes due to ethanol (i.e., corresponding to G6P amount with respect to GK) was further inferred to be due to PKCe downregulation in mice treated with ASO. The ethanol-induced increase in G6P was decreased twofold by pretreatment with ASO, while there was no effect of ASO on GK downregulation (Figure 7).

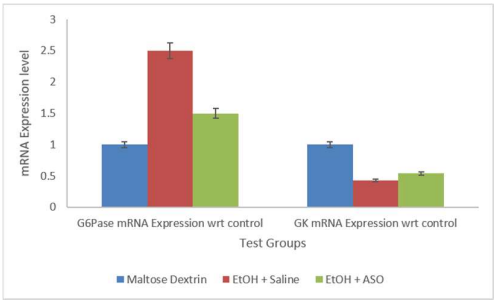



**Figure 7: PKCe ASO impact on expression of G6Pase and GK genes**

**Overall Discussion**

Steatosis is an important component of AIDS pathogenesis as steatosis can provide predictive information regarding the future development of advanced disease (cirrhosis, fibrosis). In this regard, steatosis may have potential value in protecting patients who have developed steatosis against developing late complications of ALD. Consequently, understanding the reasons for fat build-up in this acute model could help identify potential treatments for ALD and find new pathways that cause steatosis from alcohol. PKCe has previously been linked to hepatic steatosis in animal models with NAFLD. In this study, we investigated the relationship between PKCe and ethanol-induced steatosis. The data collected provide evidence that the activation of PKCe can be causally linked to the formation of ethanol-induced steatosis in the acute model of ethanol exposure. The data presented in this section demonstrate that ethanol triggers a rise in PKCe's activity, which coincides with the increase in insulin resistance that occurs because of ethanol's effects. Additionally, inhibiting PKCe protects against the development of both acute ethanol-induced steatosis and the increase in resistance to insulin.

**Mechanism behind fatty liver by PKCe**

PKCe may play a role in the development of fatty liver disease through its interference with the insulin signaling pathway in the liver. Previous studies have shown that both acute and chronic ethanol consumption lead to hepatic insulin resistance in both rats and other species. One way that ethanol consumption can lead to insulin resistance is through the effects of ethanol on the levels of insulin and glucose in the circulating bloodstream and on the expression of insulin responsive genes (such as GK and G6Pase). This resistance to normal levels of insulin in the body is measured by levels of hyperinsulinemia in the bloodstream.

After administering acute ethanol, insulin plasma levels increased 3-fold (3X) (Figure 5a); while there was no measurable change in plasma glucose levels, which supports earlier findings from studies using euglycemic clamps that insulin was unable to reduce plasma glucose levels under these same conditions (Figure 5b) and that ethanol might produce a pattern of insulin-responsive gene expression that would

suggest insulin resistance. For example, GK is up-regulated by insulin and down-regulated by insulin during periods of complete insulin sensitivity. Thus, insulin's function allows cells to shift their glucose metabolism from synthesizing glucose to consuming glucose. Ethanol, however, reverses the pattern of gene expression produced by insulin by increasing G6Pase expression and decreasing GK expression (Figure 5c). These collective findings provide support for the postulation that ethanol is clinically producing insulin resistance of hepatic origin in vivo.

The impact of ethanol on insulin-dependent genes was also assessed in those mice that received the PKCe ASO (Figure 7). Interestingly, whereas the ASO almost completely blocked the increased G6Pase gene expression that was caused by the ethanol treatment, it did not block the decreased GK gene expression that occurred with ethanol treatment. These studies support the hypothesis that certain mechanisms or aspects of insulin resistance are mediated through PKCe, while other mechanisms or aspects of insulin resistance are not. It is not surprising, therefore, that PKCe may inhibit the pathway resulting from insulin-mediated downregulation of G6Pase via its action on two separate pathways as a result of having distinct downstream signal pathways for the actions of these two genes due to the action of insulin. In addition, these results suggest that the alterations of hepatic insulin signaling induced by this acute model of ethanol exposure may also be partially attributed to the metabolic activities of PKCe.

**Pathway of PKCe Activation by Ethanol**

A well-established metabolic effect of ethanol metabolism is to produce an increase in the NADH to NAD+ ionised ratio. This increased concentration of the pyridine nucleotide results in decreased b-oxidation of free fatty acids, which leads to the accumulation of NEFA. Hence, the substantial and immediate increase in hepatic NEFA observed in this model (Figure 1) can likely be attributed in part to the enhanced availability of NEFA through increased b-oxidation of free fatty acids. However, there are many ways to generate DAG, including de novo synthesis through triacylglycerol synthesis. Ethanol metabolism should produce additional metabolites conducive to the production of DAG as well as increase the availability of these metabolites (e.g., a-glycerophosphate, dihydroxyacetone phosphate, etc.). The rising ratio of NADH to NAD+ ionised ratio prevents the activation of Glyceraldehyde phosphate dehydrogenase and the activation of Glycerol-3-phosphate dehydrogenase. This is predicted to lead to the build-up of dihydroxyacetone phosphate and α-glycerophosphate, both favouring the production of diacylglycerol (DAG) from NEFA.

The inability of the PKCe blocker to completely inhibit the development of fatty liver during early exposure to ethanol supports the view that other mechanisms, aside from PKCe, may contribute to the induction of steatosis by ethanol. Therefore, it is also possible that ethanol-induced steatosis is the combined result of several PKC isoforms functioning together with PKCe. Ethanol-induced fatty liver could also arise from substances that cause insulin resistance through mechanisms that do not involve PKC. Lastly, the residual steatosis that is not inhibited by the PKCe ASO may result from ethanol metabolism-related redox inhibition of β-oxidation of free fatty acids. It is clear that there exist multiple potential pathways, including PKCe, that may be working cooperatively to produce fatty liver as a result of ethanol consumption.

**Conclusions**

The main goal of this study was to investigate and characterize the role of PKCe during the early events of liver injury caused by ethanol. The results presented in the following chapters suggest a possible mechanism for both the activation of PKCe and its involvement in the development of ethanol-induced liver damage (steatosis). More specifically, ethanol increases the synthesis of DAG (via an increase in NEFA levels). PKCe is also allosterically activated by DAG, which negatively impacts insulin signaling, leading to hepatic steatosis. Overall, the results of this study support the hypothesis that PKCe is the mediator of ethanol-induced hepatic steatosis. PKCe therefore may represent a potential therapeutic target to halt or prevent the progression of ALD.

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