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ACUTE ETHANOL EXPOSURE INCREASES THE ACTIVITY OF MITOCHONDRIA-ASSOCIATED GLYCOGEN SYNTHASE KINASE-3 BETA (GSK-3B): ROLE IN PHOSPHORYLATION OF MITOCHONDRIAL PROTEINS

Tamara Azarashvili [1], Irina Odinokova [1], Olga Krestinina [1], Yulia Baburina [1], Vera Teplova [1], Arshad Jahangir [2], **Ekhson Holmuhamedov** [2,3*]

[1] Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Institutskaya str., 3, Pushchino, Moscow region, RU-142290, Russia
[2] Center for Integrative Research on Cardiovascular Aging, Aurora Health Care, Milwaukee, WI 53215
[3] Center of Theoretical Problems of Physico-Chemical Pharmacology, Moscow 119991, Russia

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Corresponding Author:
Ekhson Holmuhamedov
Center for Integrative Research on Cardiovascular Aging, Aurora Health Care, Milwaukee, WI 53215; Institute of Theoretical and Experimental Biophysics, Pushchino, RU-142290, Russia

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ABSTRACT

Background: Ethanol oxidation alters mitochondrial metabolism that may be consequence of the closing of mitochondrial voltage dependent anion channels (VDAC) and decrease outer membrane permeability. Phosphorylation of VDAC by cytosolic kinases modulates outer membrane permeability. This study examines how acute ethanol treatment of rats affects the activity of glycogen synthase kinase-3 beta (GSK-3β) associated with liver mitochondria and the level of phosphorylation of several mitochondrial proteins, specifically porins, forming VDAC in the outer membrane of mitochondria.
Results: The level of protein phosphorylation in mitochondria, isolated from ethanol treated animals is differed from that in mitochondria of rats which were not subjected to ethanol treatment. It was found that ethanol treatment increased the activity of GSK-3β activity in isolated mitochondria and increased the rate of incorporation of ^{32}P from [γ-^{32}P] ATP into mitochondrial proteins of 63 kDa, 32 kDa, 25 kDa and 3.5 kDa. Enhanced level of VDAC (32 kDa band) phosphorylation correlated with increased content of phosphotyrosine residues determined in the same 32 kDa band. Additionally, it was found that levels of total GSK-3β kinase as well as phosphorylated forms of this enzyme were decreased in mitochondria isolated from ethanol treated animals as compared with mitochondria from controls. Changes in phosphorylation of mitochondrial porins/VDAC and GSK-3β in ethanol-exposed rats paralleled with decreased Ca^{2+}-retention capacity of isolated mitochondria with accelerated opening of Ca^{2+}-dependent mitochondrial permeability transition pore.
Conclusions: The results of this study conclude that acute ethanol exposure of rats decrease both, the total GSK-3β associated with liver mitochondria and the level of Ser9-phosphorylation of this kinase. It is reported that acute ethanol treatment of animals modulates the activity of mitochondria-associated GSK-3β, enhances phosphorylation of mitochondrial porins/VDAC, probably, due to tyrosine-specific phosphorylation and thus, modulates the permeability of the outer mitochondrial membrane after ethanol exposure.

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INTRODUCTION

Chronic ethanol consumption as well as acute ethanol intoxication significantly affects the normal hepatic function resulting in alteration of mitochondrial functions [1-3]. These alterations include decreased energy production and increased level of reactive oxygen species (ROS) production, enhanced lipid peroxidation and decreased antioxidant levels, damages in both, protein and mitochondrial DNA that make mitochondria a major target for alcohol intoxication [4-7]. Recent demonstrations that communication between mitochondria and intracellular compartments is dependent on the open/closed status of voltage dependent anion channels (VDAC) in the outer mitochondrial membrane [6, 8-12] shaping new vision on the role of the permeability of the outer mitochondrial membrane in the physiology of cells. VDAC, also known as mitochondrial porins, are the pathways by which small ions

and metabolites cross the outer membrane and are thus involved in regulation of the metabolic and energetic functions of the mitochondria [13-14]. VDAC closure was supposed to suppress mitochondrial function, serving as a global regulator, or "governator" of mitochondrial function [11, 15]. It was found that closure of VDAC in the mitochondrial outer membrane after ethanol exposure leads to suppression of mitochondrial metabolite exchange [10, 16]. Thus, regulation of the permeability of VDAC appears to be plausible and vital mechanism in regulation of mitochondrial/cellular metabolism and as an important point for understanding the mechanism of ethanol action.

VDAC mediated passages in the outer membrane of mitochondria are regulated through interactions of VDAC forming porins with other proteins (hexokinase, tubulin) or through direct phosphorylation of porins which could lead to the closure of the channels [13, 17-20]. One of the potential regulatory mechanisms of VDAC conductivity could be phosphorylation of VDAC channels. In particular, VDAC can be phosphorylated by the catalytic subunit of cAMP dependent protein kinase (PKA) and PKA-dependent phosphorylation of VDAC is able to reduce VDAC channels conductivity [19, 21]. Moreover, recently it was demonstrated that VDAC can be phosphorylated on tyrosine amino acid residue [22]. Also, glycogen synthase kinase-3β (GSK-3β) and serine threonine kinase Akt (also known as protein kinase B) were shown to be involved in VDAC phosphorylation [23]. All these data indicate that VDAC might regulate permeability of the outer membrane via multi-phosphorylation of VDAC molecules. This idea is supported by experiments demonstrating that phosphorylation of GSK-3β kinase at Ser9 in H9c2 cardiac cells can modulate ethanol-induced permeability of the mitochondrial membranes and the phosphoinositide 3-kinase/serine threonine kinase (PI3K/Akt) signaling pathway is responsible for ethanol-modulated GSK-3β activity [24]. However, there are limited evidences that acute ethanol treatment affects the level of protein phosphorylation in mitochondria it is unknown whether correlation exists between activation of GSK-3β and VDAC phosphorylation [23]. Therefore, in the present study using rats as a model system we studied the effect of acute ethanol exposure (intragastric feeding) on the activity of mitochondria-associated GSK-3β and/or tyrosine kinase/s and whether this activity might be involved in phosphorylation of mitochondrial proteins (in particular, mitochondrial porins forming VDAC).

Methods

Materials and animals

Male Wistar rats, weighing 200–250 g, were housed in a temperature- and light- controlled room (23 ± 2°C; 12 h light/dark cycle), with free access to water and food in accordance with "The Requirements for the Care and Use of Laboratory Animals" approved by Institute of Experimental and Theoretical Biophysics, Russian Academy of Sciences. Primary antibodies were purchased from different sources: anti-GSK-3β ab's from Invitrogen (Carlsbad, CA), anti-phosphoSer9-GSK-3β ab's from Cell Signaling Technology, Inc. (Beverly, MA), anti-VDAC polyclonal ab's from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-phosphotyrosine ab's from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). Enhanced Chemi-Luminescence (ECL) kit was from Thermo Fisher Scientific Inc. (Rockford, IL). Protein Molecular Weight Standards (10-250 kDa) were from Bio-Rad Laboratories (Hercules, CA, USA). Other chemicals and supplies were purchased from Sigma-Aldrich Chemical Corp. (St Louis, MO).

Ethanol treatment

Rats were exposed to ethanol via intra-gastric gavage at 5 g of ethanol per 1 kg of life weight using 70% solution of ethanol in 0.9% NaCl. In control groups, animals were gavaged with equal volume of the saline in which ethanol was substituted with distilled water. Following 4 hours of intra-gastric ethanol feeding animals were anesthetized by intra-peritoneal injection of pentobarbital (50 mg/kg of life weight), and abdominal cavity was surgically opened to access the liver.

Preparation of liver mitochondria

Intact rat liver mitochondria were isolated by differential centrifugation as described [25]. Briefly, animals were subjected to cervical dislocation, liver accessed through abdominal incision and removed into ice-cold isolation buffer. Large and small lobes of the liver were excised and chopped into ~1 mm^3 pieces with scissor to drain the blood. Obtained pieces were rinsed twice with fresh ice-cold isolation buffer (to remove remaining blood/interstitial fluid) and homogenized in ice-cold isolation buffer (containing in mM): 220 mannitol, 70 sucrose, 1 EGTA and 10 HEPES-TRIZMA, pH 7.4, supplemented with 0.3 % bovine serum albumin (BSA). The homogenate was centrifuged at 600 x *g* for 7 min at 4°C, and the supernatant fraction was then centrifuged at 9,000 x *g* for 10 min to sediment mitochondria. The mitochondria were washed twice in the above medium without EGTA and BSA. The final mitochondrial pellet was suspended in the washing medium to yield 60–80 mg protein/ml and kept on ice for further use. Protein content was measured using *DC*™ Protein Assay Kit (Bio-Rad, Hercules USA).

Mitochondrial permeability transition pore opening assay

Opening of mitochondrial permeability transition pore (MPTP) in intact mitochondria was induced by sequential addition of equal pulses of Ca^{2+} ions into mitochondria incubated in the aerated buffer (containing in mM): 120 KCl, 2 KH_2PO_4, 10 HEPES, pH 7.4, 5 glutamate and 5 malate as described [26, 27]. The Ca^{2+} retention capacity of mitochondria is monitored from changes in free Ca^{2+} concentration in mitochondrial suspension and is determined as the total amount of Ca^{2+} accumulated into the mitochondria from consecutive Ca^{2+} pulses, resulting in MPT pore opening and release of all accumulated Ca^{2+} from mitochondrial matrix into the incubation medium as monitored by Ca^{2+} selective electrode [28, 29]. The concentration of free Ca^{2+} in suspension of mitochondria was measured using Ca^{2+}-selective electrode (NIKO-ANALIT, Moscow, Russia). The consecutive Ca^{2+} pulses were of 40 μM each (added at 60 s interval) and the maximal Ca^{2+} capacity of mitochondria determined as a total number of pulses sufficient to induce MPT pore opening was expressed as the total amount of Ca^{2+} per mg protein.

Phosphorylation of mitochondrial proteins

Phosphorylation of the mitochondrial proteins was determined by a method described previously [26, 30, 31]. Briefly, aliquots of the intact mitochondria (30 μg of protein) were suspended in the mixture of [γ-^{32}P] ATP and

unlabelled Mg^{2+}-ATP to achieve final concentrations of 2 mM Mg^{2+}, 400 μM ATP and of 5–7 μCi of [γ-^{32}P] ATP in the samples. Experimental buffer contained 1.5 μM of oligomycin, to prevent hydrolysis of added ATP by mitochondrial F_0F_1–ATPase. The samples of mitochondria were incubated for 3 min at room temperature under continuous stirring and the reaction of protein phosphorylation was stopped by addition of 20 μL of Solubilizing Laemmli Sample Buffer (LSB, Sigma-Aldrich, USA) containing 10% glycerol, 15 % SDS, 25% β-mercaptoethanol and 0.35 M Tris, pH 7.8 (with HCl). The samples were then incubated in boiled water bath for 3 min, before processing with SDS–PAGE electrophoresis.

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE)

Polyacrylamide gel electrophoresis under denaturing conditions was carried out using 4% concentrating and 15% separating polyacrylamide gel in a mini vertical unit (Hoefer, USA) by the method of Laemmli [32]. To separate the mitochondrial proteins, the samples of solubilized mitochondria (10 μg of protein per lane) were loaded onto commercial precast 10-well/1 mm gels (Bio-Rad, Hercules, USA). Following electrophoresis, gels were removed from the cast, rinsed with distilled water and fixed using the solution containing 40% methanol and 10% acetic acid, stained with Coomassie R-250 and air-dried between cellophane sheets. Radioactive bands in polyacrylamide gels were visualized by overnight exposure of the dried gels to Kodak X-Omat AR-5 X-ray film (Sigma-Aldrich, St. Louis, MO, USA). For quantitative evaluation of the level of protein phosphorylation, the films were scanned with Bio-Rad densitometer (GS-100 Calibrated Densitometer; Hercules, CA, USA) and changes in density of appropriate radioactive bands were determined from the density of bands in proteins of mitochondria isolated from the liver of control and alcohol-treated animals.

Western immunoblotting analysis

For immunoblotting, mitochondrial proteins solubilized in LSB were separated under denaturing conditions on SDS-polyacrylamide gels (as described above in SDS-PAGE section) and transferred to nitrocellulose membranes. Precision *Plus Pre-stained Standards* from Bio-Rad Laboratories (Hercules, USA) were used as markers. After overnight blocking in 5% dry milk in TBS containing (in mM): 500 NaCl, 20 Tris-HCl, pH 7.4 and supplemented with 0.01% Tween 20, the membranes were incubated with the appropriate primary antibodies. Anti-GSK-3β (Cat # AHO1312, Invitrogen, Carlsbad, CA, USA) and Anti-GSK-3β Ser9 (Cat # 9336, Cell Signaling Technology Inc., Beverly, MA, USA) were used for blotting of phosphorylated and non-phosphorylated forms of GSK-3*β*. Anti-VDAC1/2/3 (Cat # sc-98708, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-phosphotyrosine (Cat # BML-SA240, Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA) were used for detection of VDAC and its phosphorylation on tyrosine amino acid residues. Briefly, membranes with transferred proteins were incubated for 1 hour under continuous mechanical agitation on belly dancer with primary antibodies and then washed 3 times with 20 mL of TBS (5 min) on belly dancer. Immunoreactivity of appropriate proteins was visualized using appropriate secondary antibodies conjugated with horse radish peroxidase (HP) according to Thermo Scientific Chemiluminescent Western blotting technical guide and protocols (http://www.piercenet.com/files/TR0067-Chemi-Western-guide.pdf).

After 1 hour incubation with a secondary antibodies the membranes were rinsed 3 times with 20 ml of TBS (5 min), transferred into ECL chemiluminescent reagents (Thermo Fisher Scientific Inc., Rockford, IL, USA), incubated 5 min at room temperature and luminescence images were obtained as described [31].

Statistical Analysis

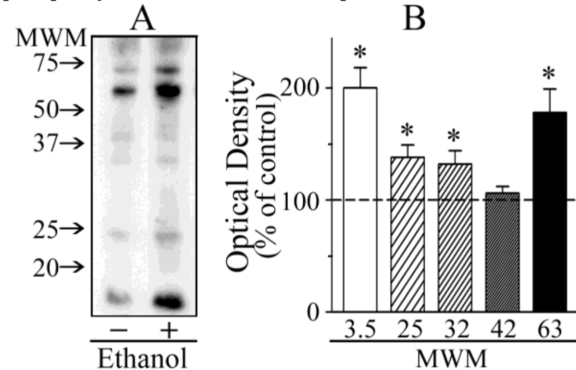
The levels of phosphorylation of appropriate bands were expressed as a means of density of band ± SD. The data were from at least three independent experiments for each condition. Statistical significances of differences were evaluated using Student's t-test and ANOVA with Bonferroni post-hoc comparison. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Acute ethanol exposure increases protein kinase activity in isolated liver mitochondria

Intact liver mitochondria (1 mg/ml) obtained from the livers of control and acute ethanol treated animals were incubated with [γ-$^{32}P_{in}$] labeled ATP for 3 min in buffer containing 2 mM Mg^{2+}, 400 μM ATP, and 1.5 μM oligomycin (as described in MATERIALS AND METHODS). Equal aliquots of mitochondrial suspension and Laemmli Sample Buffer (LSB) were mixed and the proteins were separated using SDS - polyacrylamide gel electrophoresis in Laemmli system as described [31]. Autoradiogram of obtained gel revealed several phosphorylated mitochondrial proteins with molecular masses in range of 63 kDa, 42 kDa, 32 kDa, 25 kDa and 3.5 kDa (Fig. 1A). Phosphorylation of mitochondrial proteins in the presence of [γ-$^{32}P_{in}$] ATP indicates that ethanol treatment of rats increases the activity of mitochondria-associated protein kinase. Acute (4 hour) ethanol exposure increased the activity of mitochondria-associated protein kinase and increased the level of phosphorylation of multiple mitochondrial proteins: phosphorylation of 3.5-kDa and 63-kDa polypeptides was almost doubled; phosphorylation of the 25 kDa and 32 kDa polypeptides were increased moderately by 35-40%, while the extent of phosphorylation of 42 kDa polypeptides was not changed as compared with control mitochondria (Fig. 1B).

Figure 1. Effect of acute ethanol treatment on *in vitro* phosphorylation of mitochondrial proteins. A



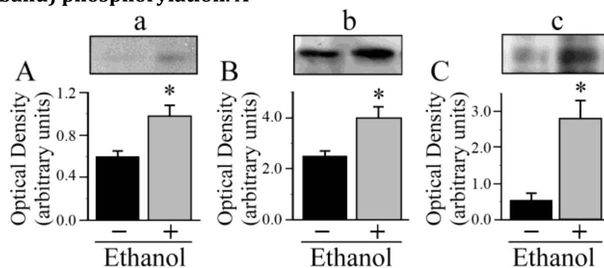
*Representative PAGE autoradiogram of the proteins of isolated mitochondria phosphorylated by ^{32}P-ATP. Left column (**- Ethanol**) shows ^{32}P-labeled proteins of mitochondria obtained from control animals, non-treated with ethanol, and right column (**+ Ethanol**) shows ^{32}P-labeled proteins of mitochondria isolated from ethanol-treated rats. The molecular mass of the marker proteins (**MWM**) is shown on the left side by arrows. **B** - Densitogram of relative levels of protein phosphorylation in mitochondria after 4 hours of ethanol treatment. The level of phosphorylation in control mitochondria was taken as 100% (horizontal **dotted line**). Relative level of protein phosphorylation is expressed as mean ± S.D. from three independent*

*experiments, and stars indicate significance with *p < 0.05 vs. control. ANOVA with Bonferroni post hoc comparison with statistical significance calculated separately for the three protein band*

Acute ethanol exposure increases phosphorylation of mitochondrial porins

For further identification of mitochondrial polypeptides with increased phosphorylation level after acute ethanol exposure we used Western blotting technique with target-specific antibodies. As shown in Fig. 2A the incorporation of γ-$^{32}P_i$ into 32 kDa polypeptide band in liver mitochondria was increased in mitochondria from ethanol-treated rats by 1.5 fold as compared to mitochondrial protein from untreated animal. Western blotting of the same membrane with VDAC-specific antibodies (VDAC1/2/3, Santa Cruz Biotechnology, Inc.) demonstrated positive immunoreactivity of 32 kDa polypeptide band with VDAC1/2/3 specific antibodies (Fig. 2B), thus indicating that acute ethanol treatment enhances phosphorylation of mitochondrial porins (VDAC1/2/3. Western blotting of the same membrane with anti-phosphotyrosine specific antibodies (Enzo Life Sciences International, Inc., Plymouth Meeting, PA) demonstrated that acute ethanol-exposure increases level of tyrosine-mediated phosphorylation of 32 kDa polypeptide band in liver mitochondria (Fig. 2C).

Figure 2. Effect of acute ethanol treatment on *in vitro* VDAC (32 kDa band) phosphorylation. A

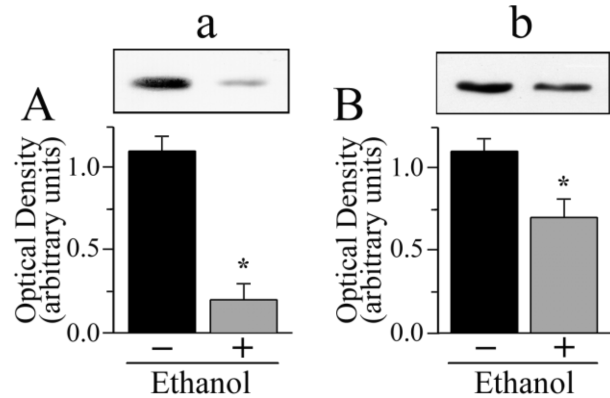


*Representative autoradiogram of Western blot membrane with incorporated ^{32}P into 32 kDa protein obtained from control (**- Ethanol,** left column) and ethanol-treated rats (**+ Ethanol,** right column); graph - densitogram of autoradiogram from (A). **B** - Representative Western blot of VDAC expression in mitochondria from control (**- Ethanol,** left column) and ethanol-treated rats (**+ Ethanol,** right column); graph - densitogram of this Western blot. **C** - Representative Western blot of the same membrane probed with anti-phosphotyrosine antibodies; graph - densitogram of this Western blot. Data for graphs are average from three independent experiments and stars indicate significance with *p < 0.05 vs. control.*

Acute ethanol exposure decreases the amount and phosphorylation of mitochondria-associated GSK-3β

Next we probed whether acute ethanol exposure has an effect on the amount of mitochondria-associated GSK-3β and on the level of phosphorylation of this protein kinase. Western blotting technique and target specific antibodies revealed that acute ethanol exposure of rats dramatically decreases both, the total amount of mitochondria-associated GSK-3β and its phosphorylation at Ser9-selective site (Fig. 3). Membranes with transferred proteins were probed with anti-GSK-3β specific antibodies (to determine the total GSK-3β) and with anti-phosphoserine-9-GSK-3β antibodies (to determine the level of phosphorylation). In rat liver mitochondria isolated from ethanol-treated rats (5 g/kg weight, 4 hours) the level of total GSK-3β was significantly (almost five-fold) decreased as compared with mitochondria obtained from control animals (Fig 3A). On average, acute ethanol exposure of rats decreased mitochondria-associated GSK-3β kinase by ~ 80% (Fig. 3A), and the level of phospho-GSK-3β, detected with Ser9-specific antibodies, was decreased by 40% (Fig. 3B). Probing the same membranes (stripped of GSK-3β and phosphor-Ser9-GSK-3β antibodies) with both, anti-Akt and anti-phospho-Ser473-Akt specific antibodies demonstrated that the content of Akt kinase and/or phosphorylated forms of this kinases associated with isolated mitochondria are below the detection limit of Western blotting used in our conditions (data not shown).

Figure 3. Effect of ethanol on the level of GSK-3*β* associated with isolated mitochondria. A



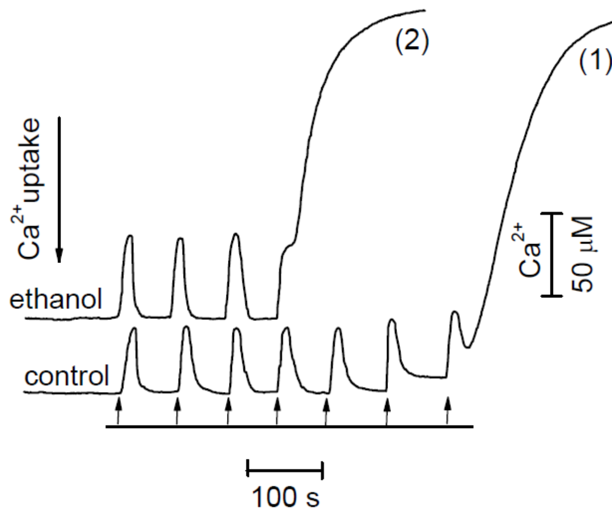
*Representative image of Western blot of GSK-3β in total protein extract obtained from liver mitochondria of control (**- Ethanol,** left column) and ethanol-treated rats (**+ Ethanol,** right column); graph - densitogram of the Western blot. **B** – Representative image of Western blot of phospho-Ser-9 in GSK-3β in total protein extract probed with anti-phosphoSer9- GSK-3β antibody in control (**- Ethanol,** left column) and ethanol-treated rats (**+ Ethanol,** right column); graph - densitogram of the Western blot. Data for graphs are average from three independent experiments and stars indicate significance with *p < 0.05 vs. control.*

Acute ethanol exposure accelerates opening of mitochondrial Ca^{2+}-dependent permeability transition pore

Further we studied the effect of ethanol exposure on the sensitivity of Ca^{2+}- dependent permeability transition pore opening in isolated liver mitochondria. Using well known protocol of step-wise titration of mitochondria with known boluses of Ca^{2+} [26, 27] we measured the threshold of Ca^{2+} loading (Ca^{2+} accumulating capacity) of mitochondria isolated from the liver of control (non-treated) and experimental (ethanol-treated) group of rats. Small boluses Ca^{2+} (x 40 μM each, every 60 s) were added to mitochondrial suspension and time-dependent changes in extramitochondrial (free) Ca^{2+} concentrations were measured using Ca^{2+}-selective electrode. Sequential addition of Ca^{2+} into the suspension of mitochondria isolated from untreated animals followed by rapid and complete accumulation of all added Ca^{2+} into the mitochondrial matrix until the opening of MPT pore (Fig. 4, Control). The total of 7 boluses of Ca^{2+} were needed to induce an opening of MPT pore, and the last (seventh) pulse induced rapid and irreversible loss of all accumulated Ca^{2+} from the matrix, indicating opening of MPT pore (Fig. 4, Control). Similarly, sequential Ca^{2+} boluses induced an opening of MPT pore in mitochondria isolated from the liver of ethanol-treated rats and fourth Ca^{2+} bolus initiated opening of the pore and release of accumulated Ca^{2+} from mitochondria (Fig. 4, Ethanol). On average, Ca^{2+} accumulating capacity of mitochondria decreased from 138.2 ± 46.7 nmol Ca^{2+}/mg protein in control animals to 52.5 ± 3.9 nmol Ca^{2+}/mg protein in mitochondria isolated from ethanol exposed animals (Table 1, n=6). Further we tested the role of VDAC closure on opening of Ca^{2+}-dependent MPT pore. It have been demonstrated that closure of mitochondrial VDAC with erastin and/or

phosphorothioate oligonucleotide G3139, known VDAC blockers [33-36], resulted in increased sensitivity of control mitochondria toward Ca^{2+} induced MPT. Treatment of mitochondria with 10 μM of erastin (5 min prior Ca^{2+} boluses) decreased Ca^{2+} accumulating capacity of control mitochondria from 138.2 ± 46.7 nmol Ca^{2+}/mg to 51.8 ± 4.3 nmol Ca^{2+}/mg protein (Table 1). Similarly, in the presence of 5 μM G3139, another VDAC blocker, Ca^{2+} accumulating capacity of control mitochondria also decreased from 138.2 ± 46.7 nmol Ca^{2+}/mg to 54.9 ± 3.5 nmol Ca^{2+}/mg protein. The Ca^{2+} accumulating capacity of control mitochondria in the presence of inhibitors of VDAC was similar to that in mitochondria isolated from ethanol-treated rats with open VDAC.

Figure 4. Effect of ethanol on Ca^{2+}-dependent MPT pore opening and Ca^{2+} retention.



*Isolated mitochondria (1.3 mg protein/ml) were incubated in standard KCl-based medium containing 120 mM KCl, 2 mM KH_2PO_4, 10 mM HEPES (pH 7.4), 5 mM glutamate and 5 mM malate and sequentially loaded with 40 μM pulses (arrows). These two curves reflect changes in extramitochondrial Ca^{2+}- concentration in the suspension of mitochondria obtained from the liver of control (**Control,** lower curve) and ethanol-treated rats (**Ethanol,** top curve). The results represent the typical traces of tree independent experiments.*

DISCUSSION

The liver is the major organ responsible for metabolism of alcohol, with mitochondria playing the central role in the process. The exposure of liver cells to ethanol leads to rapid changes in metabolic state of cells and mitochondrion is a specific target of ethanol toxicity. Ethanol exposure compromises mitochondrial ATP synthesis, increases oxidative stress produces steatosis and suppresses endogenous liver specific functions [1, 3, 37]. Although, the ethanol-induced disruptions of mitochondrial metabolism are well documented, the underlying mechanism(s) of the pathogenesis of alcohol-induced liver disease remains incompletely understood. Strong evidence support the notion that the closure of VDAC is a critical event leading to acute alterations of mitochondrial metabolism after ethanol [16, 35, 38] but the mechanism involved in modulation of VDAC permeability remains unknown. Posttranslational phosphorylation of proteins, including channel forming proteins, is a key element of the complex network of regulatory and signaling pathways [39-41]. Presence of multiple potential phosphorylation of VDAC molecule provide the targets for cytosolic and mitochondria associated protein kinases and this mechanism might be involved in regulation of VDAC permeability. In line with such expectations it was recently demonstrated that VDAC could be phosphorylated by glycogen synthase kinase-3β (GSK-3β), and that this phosphorylation is modulated by Akt-dependent inactivation of GSK-3β [23, 42].

In the present study, it is shown that mitochondria isolated from the liver of acute ethanol treated rats contain altered level of GSK-3β kinase and are characterized by different rate of incorporation of ^{32}P from labeled ATP into mitochondrial proteins of 63, 42, 32, 25 and 3.5 kDa weight. Within these proteins, VDAC was identified in 32 kDa phosphoprotein band by specific anti-VDAC antibody. That indicates involvement of mitochondria associated GSK-3β kinase in VDAC phosphorylation following acute ethanol exposure. Additionally, VDAC phosphorylation promotes its closure and decreases channel conductivity [21, 43]. Thus, ethanol-induced oxidative stress might potentiate activation of the stress kinases in signal transduction from ethanol oxidation to phosphorylation of the porin channels. We demonstrate here that exposure of rats to intra-gastric injections of 70% aqueous ethanol solution (weight of 200 g, at 5g ethanol per kg) strongly decreased the pool of total GSK-3β associated with mitochondria. Alcohol exposure decreased both, association of GSK-3β with mitochondria and phosphorylation of GSK-3β at the amino-terminal serine 9 residue. Taking into consideration, that lowering of level phosphorylation at Ser9 leads to activation of GSK-3β we could suppose that ethanol-induced intoxication results in activation of GSK-3β, in spite of decreased pool of GSK-3β. Earlier, GSK-3β activity was determined in three compartments of the cells (cytosol, nuclear and mitochondria), the lowest pool of GSK-3β was detected in mitochondria, nonetheless the enzyme had the highest activity in this organelle [44]. Lately, examination of subcellular localization of GSK-3β at the electron microscope showed the presence of GSK-3β in outer membrane of mitochondria [45]. Several sites for GSK-3-specific phosphorylation exist in rat mitochondrial VDAC and recently it was shown that GSK-3 β interacts with VDAC in cardiac tissue [46] and could phosphorylated VDAC protein in vitro [23, 42].

Alteration of phosphorylation status of GSK-3β in mitochondria isolated after ethanol exposure was revealed in parallel with increased level of $^{32}P_i$ incorporation into another mitochondrial proteins (63, 42, 32, 25 and 3.5 kDa), in particular, in VDAC. Recently, both GSK-3β and Akt were shown to be involved in VDAC phosphorylation of heart mitochondria, where at first VDAC was phosphorylated by Akt and then by GSK-3β [23]. It was also reported that VDAC acts as early sensor of lipid toxicity and GSK-3-mediated VDAC phosphorylation status controls outer mitochondrial membrane permeabilization in hepatosteatosis [47]. In our experiments, anti-Akt antibodies (against both phosphorylated and non-phosphorylated Akt) were not able to recognize any form of Akt (not shown). However, we revealed increased content of phosphotyrosine residues in 32 kDa where VDAC molecules are located. That allowed us to suppose that VDAC might be pre-phosphorylated by tyrosine kinase at first and after that could be phosphorylated by GSK-3β. Interestingly, that GSK-3β could be autophosphorylated at Tyr216 that activate GSK-3β [48]. Thus, acute ethanol treatment of rats lead to activation of tyrosine kinase and

of GSK-3β in mitochondria resulting in increased phosphorylation and closure of VDAC.

Permeability of the outer mitochondrial membrane considered as the key factor of ethanol-induced dysfunction of mitochondria. Earlier, ethanol exposure of cultured hepatocytes was found to decrease mitochondrial outer membrane permeability by inhibition (closing) of VDAC [10, 16, 38]. Being the major protein of the outer membrane, which forms the channel at the interface of the mitochondria and cytosol, VDAC determines the permeability of the outer membrane to hydrophilic metabolites like ATP, ADP and respiratory substrates [8, 9]. It is known that both ethanol and the phosphorylation of VDAC [21, 43] promote VDAC closure. Moreover, VDAC closure increases calcium ion flux [33, 34], that is why increased phosphorylation status of VDAC might lead to induction of PTP opening. Moreover, it was demonstrated that microinjection of anti-VDAC antibody into the cytosol of hepatocytes was able to prevent the ethanol-induced apoptosis, suggesting that the participation of VDAC and opening of the VDAC channel is essential for the induction of apoptosis [50, 51]. In the present study, we demonstrated alteration of phosphorylation status both VDAC and GSK-3β in mitochondria isolated from ethanol-treated rats with decreased calcium capacity (lowering of threshold calcium concentrations in almost 3 times) and acceleration of PTP opening. These observations are in line with our earlier observations that VDAC closure sensitizes mitochondria toward Ca^{2+}-induced MPT [35]. This is the first study to demonstrate that ethanol treatment modulates the level of phosphorylation of VDAC in vivo, possibly, by both a tyrosine kinase and GSK-3β, which provides circumstantial evidence for accelerated MPT pore opening in mitochondria isolated from acute ethanol exposed animals. A better understanding of the role of mitochondria in alcohol-induced tissue injury may provide new avenues for diagnostic and therapeutic interventions.

CONCLUSIONS

The results of this study conclude that acute ethanol exposure of rats decrease both, the total GSK-3β associated with liver mitochondria and the level of Ser9-phosphorylation of this kinase. It is reported that acute ethanol treatment of animals modulates the activity of mitochondria-associated GSK-3β, enhances phosphorylation of mitochondrial porins/VDAC, probably, due to tyrosine-specific phosphorylation and thus, modulates the permeability of the outer mitochondrial membrane after ethanol exposure.

ABBREVIATIONS

GSK-3β, Glycogen synthase kinase-3β; LSB, Laemmli Sample Buffer; MPT, mitochondrial permeability transition; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TBS, Tris Buffer Solution; VDAC, voltage dependent anion channels;

COMPETING OF INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

IO, OK, YuB, and VT carried out experimental work and analyzed data. TA, EH and VT designed the study, coordinated the experiments, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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