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Ethanol-Induced Mitochondrial Induction of Cell Death-Pathways Explored

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1. Introduction

Alcohol consumption is one of the major source for chronic liver diseases. It is striking that women are more susceptible to the toxic effects of alcohol although alcoholic liver disease (ALD) is common in men (*1*). In recent times, global burden on ALD has prompted researchers to investigate this disease based on age, gender, social status and race. However, in all these conditions and known variable severities of ALD, the basic pathophysiological condition is oxidative stress, which leads to liver damage (*1, 2*). In an overview, ALD leads to hepatocyte death, liver cirrhosis and organ dysfunction through production of reactive oxygen species (ROS), inflammatory cytokines and mitochondrial impairment. ROS are important mediators of apoptosis in liver diseases and are produced in response to paracrine factors such as ethanol (EtOH) (*2*). This chapter focuses on the role of EtOH induced ROS mediated cell death.

Over two decades, several pathways have been proposed in ALD. Recent studies have educated our understanding on these pathways, most of which work as cohort induced by direct/indirect effects of alcohol metabolism and clearance. Majority of cell death pathways (apoptosis, necrosis and the recently described necroptosis) converge at cellular damage associated with excessive production of ROS (superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2)) that results in oxidative stress (*3, 4*). Under pathophysiological conditions, NAD(P)H oxidase, xanthine oxidase (XO) and the mitochondrial respiratory chain are the major sources of ROS. Normally, 5% of the metabolized cellular oxygen is converted into ROS which are effectively detoxified by endogenous antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (Cat). ROS overproduction resulting from acute and chronic exposure to alcohol can exceed the capacity of endogenous antioxidants (*5, 6*). Excessive ROS triggers various cellular signaling pathways leading to cell death in both vascular and epithelial cells. Although ROS is known to elicit liver damage, the signaling pathways operative in alcohol induced ROS overproduction in liver cells remain elusive.

Mitochondrial respiratory chain is the second major source of cellular ROS. However, mitochondria itself is an important target for cellular ROS resulting in mitochondrial dysfunction and permeabilization of outer mitochondrial membrane (OMM) (*7, 8*). In addition, studies have demonstrated that inhibition of mitochondrial electron transport

results in ROS production leading to alteration in mitochondrial morphology and bioenergetics (*9*). Furthermore, OMM permeabilization leads to cytochrome *c* release and mitochondrial dysfunction (*10*).

Multidomain proapoptotic Bcl-2 family proteins are suggested to play a role in $O_2^{\bullet-}$ induced mitochondrial dysfunction (*11, 12*). Studies have shown that chronic EtOH consumption increases the expression of anti-apoptotic Bcl-2 and Bcl-x_L proteins by an interleukin-6-dependent mechanism (*13, 14*). Though, up regulation of proapoptotic Bax protein is observed in patients with ALD, the roles of Bax and Bak in initiating mitochondrial apoptotic events are poorly understood. Our previous studies have shown that $O_2^{\bullet-}$-mediated mitochondrial phase of apoptosis is mainly dependent on Bid but not Bax (*15, 16*).

Enhanced circulation of TNF-α and other cytokines have been reported in both ALD patients and animal models (*17*). In ALD, alcohol-induced $O_2^{\bullet-}$ elicits production of proinflammatory cytokine such as TNF-α which subsequently sensitizes hepatocyte cell death through gangliosides (*18-23*). Interestingly, in hepatocytes, TNF-α binds to either TNFR1 (type1 tumor necrosis factor receptor) or TNFR2 (type 2 tumor necrosis factor receptor) to initiate cell death. TNF-α mediated activation of apoptosis requires two adaptor molecules such as TNF receptor associated death domain protein (TRADD) and Fas – activated death domain protein (FADD). These in turn activate caspase 8 which further proteolytically cleaves downstream caspases and pro apoptotic bcl-2 family protein Bid. The active form of Bid (t-Bid) facilitates OMM permeabilization (*15*). On the other hand, ligation of TNF-α–TNFR1 recruits receptor-interacting protein 1 kinase (RIP1), TNFR death domain serine-theronine kinase 2 (TRAF2) which generates ceramide via activation of sphingomyelinases. Ceramide induces mitochondrial permeability transition pore (MPTP) opening, mitochondrial matrix swelling and membrane permeabilization, in concert with pro-apoptotic Bcl-2 family protein Bad (*24*). Recently our study has shown that TNF-α-induced necroptosis, the alternate form of cell death, requires TNFR adaptor protein FADD and NFκB downstream signaling molecule NEMO. FADD mediates the formation of necrosome consisting of RIP1-RIP3 kinases. The necrosome induced mitochondrial dysfunction in necroptosis requires Bax and Bak (*25*). TNFR1 mediated cell death is an extensively studied model and has been associated in many disease conditions including ALD.

Ca^{2+} has been known as an important intracellular second messenger that plays a dual role in cell survival and death. In liver, Ca^{2+} signaling is known to regulate a variety of cellular functions ranging from proliferation to apoptosis. Under pathological conditions, elevation in intracellular calcium ($[Ca^{2+}]_i$) facilitates cell death (*26, 27*) via inositol 1,4,5-triphosphate ($InsP_3$) (*28, 29*) and oxidation of STIM1(*30*). Inositol 1,4,5-triphosphate receptor ($InsP_3R$) mediated $[Ca^{2+}]_i$ changes leads to rapid Ca^{2+} release from ER and the subsequent Ca^{2+} entry through slow-activating plasma membrane store operated channels (SOC) (*31-33*). In hepatocytes, the Type II InsP3 R is known to trigger Ca^{2+} waves that can transmit through intercellular junctions throughout the liver (*34*). ER-mitochondria link and the mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) uptake through uniporter is known to promote $[Ca^{2+}]_m$ overload which subsequently leads to mitochondrial depolarization and increased mROS production (*10, 28, 35, 36*). The aberrant Ca^{2+} homeostasis has been linked with ALD (*37, 38*). Despite the vast knowledge, the actual intricacies on the mechanism of Ca^{2+} induced mitochondrial dysfunction remain largely unexplored. In addition to the functional damage,

the structural damage to the mitochondrion is known to play a very important role in accelerating EtOH induced apoptosis in hepatocytes. In support, a recent study has evidenced the mitochondrial structural changes (fig.1) in an animal model for ALD (39).

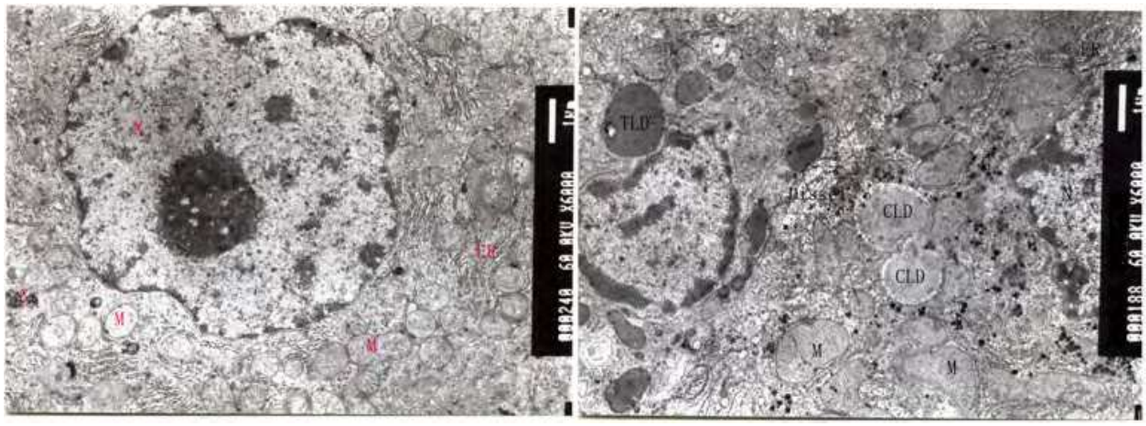

Fig. 1. Mitochondria appearance under electron microscope (EM × 6000); **A**: Mitochondria in normal group; **B**: Mitochondria in model group. M: mitochondria, G: glycogen, N nucleus, ER: endoplasmic reticulum, LD: lipid droplet. The long arrow shows abnormally distributed chromatin in nuclei, the short one is megamitochondrion and the arrow head is U-type mitochondria (Electron micrograph reproduced with permission from © 2007 Yan, M *et al*. Originally published in World J Gastroenterology 2007April 28;13(16): 2352-2356).

2. Role of ethanol in ROS production

Oxidative stress has been implicated to play a major role in ALD. The formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) represent an important cause of oxidative injury associated with free radical formation. ROS is known to damage and degrade lipids, proteins and DNA by which it affects the structure and function of the cell. Using animal models and samples from subjects with ALD, studies have shown the role of ROS in EtOH induced tissue damage (*40, 41*). Modification of mitochondrial proteins by ROS to disulphide, sulphenic, sulphinic and sulphonic residues and RNS to nitration products of tyrosine residues and nitrosation products of thiols have been well documented to occur in membrane and matrix proteins within mitochondria (*42, 43*). This section describes in detail the role of ROS in ALD. Oxygen is foremost common chemical frequently involved in the formation of free radical. Molecular oxygen is oxidized to generate two molecules of water by accepting four electrons and protons at one time. During this process several intermediary state of reactants exist like superoxide ($O_2^{\bullet -}$); peroxide (O_2^{2-}), which normally exists in cells as hydrogen peroxide (H_2O_2); and the hydroxyl radical ($OH^{\bullet}$). Superoxide, peroxide, and the hydroxyl radical are considered the primary free radicals. It has been estimated that only about 3 to 5 percent of the O_2 consumed by the mitochondrial respiratory chain is converted to ROS. Nevertheless, the toxic effects of oxygen in biological systems—such as oxidation of lipids, inactivation of enzymes, nucleic acid mutations and destruction of cell membranes are attributed to the reduction of O_2 to free radicals. The first and foremost effect of alcohol metabolism in the cellular milieu is the loss of NAD^+/NADH ratio that affects mitochondrial respiratory chain and subsequent generation of superoxide anion (*44*). In respect to EtOH induced ROS

production, our laboratory has demonstrated that EtOH induced mROS production lead to mitochondrial morphology changes and functional alterations (Fig. 2). Briefly, (1) Acute delivery of EtOH (50mM) resulted in mitochondrial fragmentation (filamentous to globular morphology - fig.2A). (2) EtOH-fragmented mitochondria exhibit exaggerated $O_2^{\bullet-}$ production (fig.2B &C). (3) EtOH treatment induced elevated mROS, altered mitochondrial Ca^{2+} handling and mitochondrial dysfunction (fig.2D&E). (4) $O_2^{\bullet-}$ induced mitochondrial membrane potential ($\Delta\Psi_m$) loss and cytochrome *c* release was abrogated by the antiapoptotic Bcl-2 protein Bcl-xL and (5) Bax/Bak double knockout cells are resistant to $O_2^{\bullet-}$-mediated $\Delta\Psi_m$ loss and cytochrome *c* release, however, Bak but not Bax is essential for $O_2^{\bullet-}$-induced $\Delta\Psi_m$ loss and cytochrome *c* release (fig 3A-D).

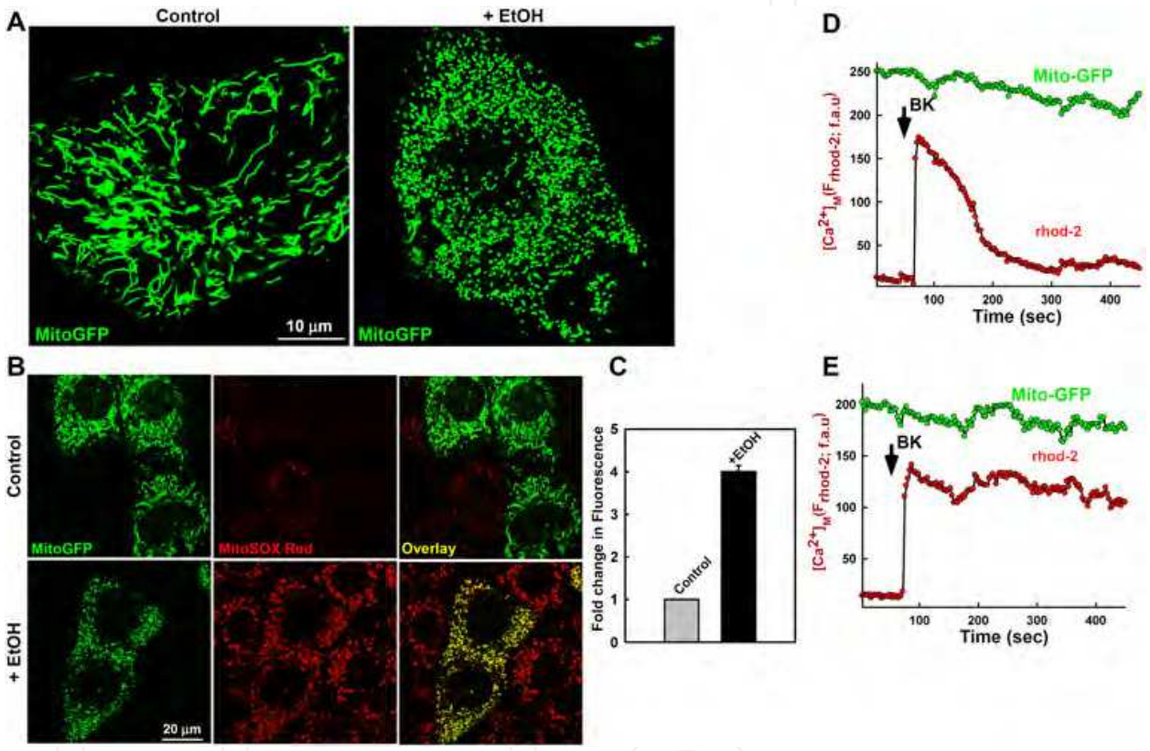

Fig. 2. EtOH augments alterations of mitochondrial morphology, $O_2^{\bullet-}$ production, and mitochondrial Ca^{2+} uptake in live cells. (A) Mito-eGFP (enhanced GFP)-expressing vascular endothelial cells (left panel) were exposed to 50 mM EtOH for 30 h (right panel). EtOH treatment resulted in short, globular mitochondrial tubules. (B) Mito-eGFP-expressing cells either left untreated (top) or exposed for 30 h to 50 mM EtOH (bottom) were loaded with the mitochondrion-derived O_2^{-} indicator MitoSOX Red and imaged by confocal microscopy. EtOH-treated cells, but not control cells, displayed enhanced mitochondrial $O_2^{\bullet-}$ production. (C) Quantitation of mitochondrial ROS production in live cells. Following treatment, cells were loaded with the mitochondrial Ca^{2+} indicator rhod-2 for 45 min and stimulated with bradykinin (BK; 10 nM). Representative traces of mitochondrial Ca^{2+} uptake in response to bradykinin in (D) control and (E) EtOH-treated cells. EtOH-treated cells, but not control cells, displayed sustained mitochondrial Ca^{2+} elevation. f.a.u., fluorescence arbitrary units. (Reproduced with permission from © 2009 Madesh *et al*. Originally published in *Mol Cell Biol*. **2009** Jun;29(11):3099-112).

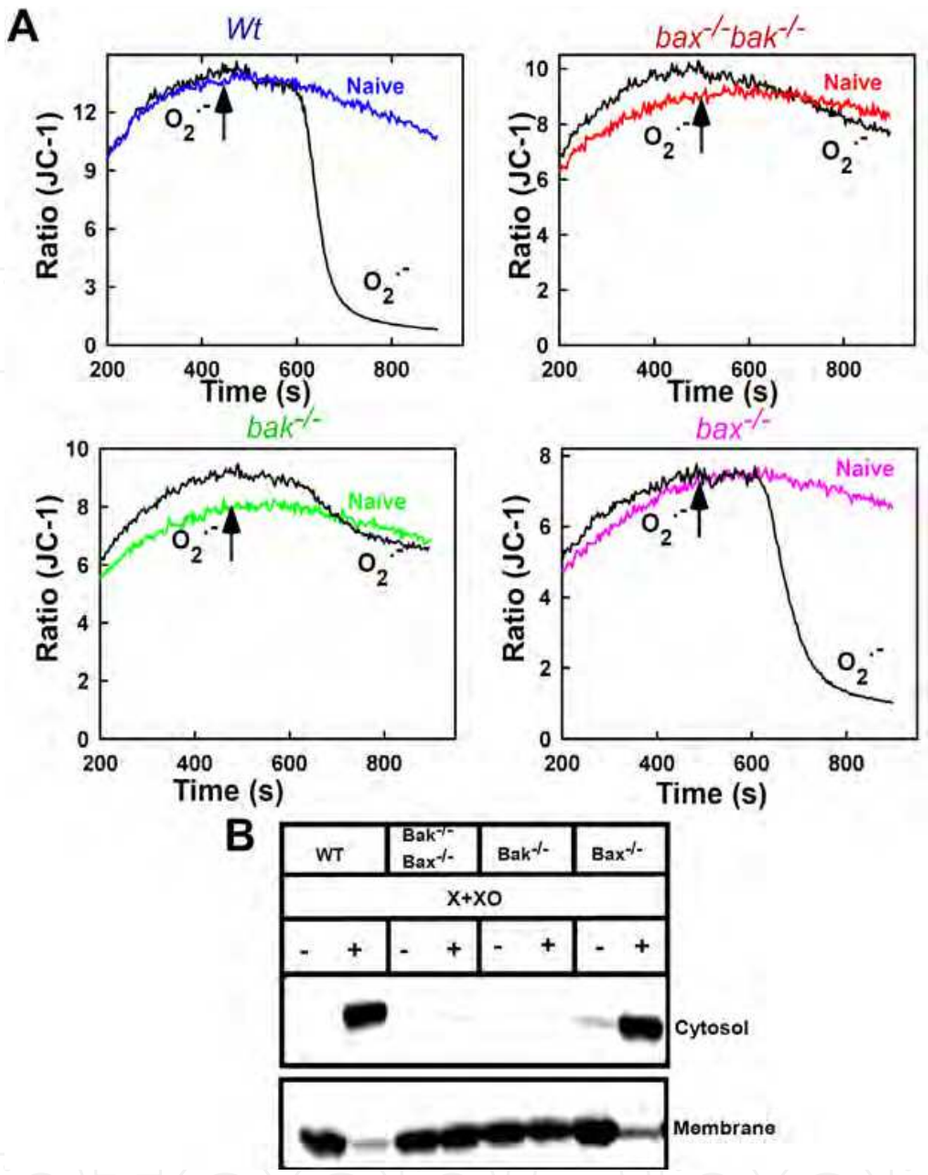

Fig. 3. (A) Wild type, *bax*-/- *bak*-/- double knockout, *bax*-/- and *bak*-/- MEFs were probed for cytochrome *c* in $O_2^{\bullet-}$-generating system. $\Delta\Psi_m$ was measured after $O_2^{\bullet-}$ treatment in permeabilized, TMRE-loaded *bax*-/- *bak*-/- MEFs expressing (B) GFP alone or together with (C) Bak or (D) Bax. Cells were exposed to the $O_2^{\bullet-}$-generating system or FCCP as indicated. (Reproduced with permission from © 2009 Madesh *et al*. Originally published in *Mol Cell Biol*. **2009** Jun;29(11):3099-112).

Taken together it is evident that $O_2^{\bullet-}$-evokes mitochondrial phase of apoptosis during chronic EtOH exposure. In addition, $O_2^{\bullet-}$ mediated tBid generation induces selective activation of mitochondrial Bak, triggering cytochrome *c* release and $\Delta\Psi_m$ loss that lead to apoptosis (*15*). Though mitochondria is known to play a crucial role in EtOH induced cell death, the upstream signaling molecules other than $O_2^{\bullet-}$ that target mitochondria is a open area of research in ALD.

3. Calcium and its role in ROS mediated apoptosis

$[Ca^{2+}]_m$ signals are known to control variety of responses in liver including apoptosis. Chronic EtOH exposure in rats leads to sustained Ca^{2+} elevation that triggers MPTP opening. MPTP opening leads to Ca^{2+} overload in the mitochondria and results in mitochondrial swelling a phenomenon observed in EtOH fed rats but not in control rats (*45*). Cells at basal metabolic rate tightly regulate free Ca^{2+} in the range of 100 to 200 nM in both cytosol and mitochondria through NCX (Na^+/Ca^{2+} exchanger), PMCA (Plasma membrane Ca^{2+}-ATPase) and SERCA (Sarcoendoplasmic reticulum Ca^{2+}-ATPase) pumps. Mitochondria play an important role in rapid uptake of Ca^{2+} through a uniporter and is then released slowly back into the cytosol (*46-48*). EtOH is known to induce elevated $[Ca^{2+}]_i$ by altering the $[Ca^{2+}]_m$ buffering capacity. Endothelial cells lining the capillaries and veins are first to encounter ethanol. Ethanol exposure activates the endothelial cells which are known to signal the immune cells. Our studies have previously shown ROS generation by activated macrophages evoked an $[Ca^{2+}]_i$ transient in endothelial cells (*28*). However sustained increase in $[Ca^{2+}]_i$ coupled with altered mitochondrial Ca^{2+} handling capacity leads to irreversible cell injury (*16, 28, 49*). Though, the exact source of increased cellular Ca^{2+} in ALD is poorly understood, several pathways have been proposed for the increased calcium flux. Receptor mediated pathways (G Protein-Coupled Receptor and tyrosine kinase receptor) that generate second messengers like $InsP_3$ which binds to $InsP_3R$ on endoplasmic reticulum trigger Ca^{2+} release (*50*). Further the $[Ca^{2+}]_m$ uptake was directly proportional to the magnitude of $[Ca^{2+}]_c$. Under pathophysiological conditions, the GPCR (G Protein-Coupled Receptor) Ca^{2+} linked mROS is essential for leukocyte/endothelial cell adhesion (*50*). EtOH exposure in HepG2 cells induces $[Ca^{2+}]_m$ overload that triggers mROS (fig 2D & E). In the cellular milieu, Ca^{2+} is compartmentalized as gradients in different organelles in the range of μM to nM (Ca^{2+}=ER>mitochondria>lysosomes>cytosol=nucleus). During ALD the alterations in Ca^{2+} homeostasis leads to $[Ca^{2+}]_m$ overload. Under pathological or physiological conditions $[Ca^{2+}]_m$ levels dictate the cells to program either towards cell death or survival signals in the liver. Accumulation of Ca^{2+} in mitochondria beyond the transition threshold opens the MPTP, resulting in $\Delta\psi_m$ loss, mitochondrial swelling, mROS overproduction and finally leading to cell death (*51*).

4. Mitochondrial permeability transition

Ca^{2+}-linked cell death program in ALD may be either apoptotic or necrotic phenomenon determined by OMM permeabilization and MPTP opening respectively. Ca^{2+} overload leads to oxidative stress that permanently leads to MPTP opening exposing the mitochondrial inner membrane permeable to all solutes of molecular weight up to 1.5Kd (39). Furthermore, the persistent MPTP opening leads to irreversible mitochondrial depolarization. Mitochondrial depolarization, in conjunction with mROS overproduction and subsequent inner mitochondrial membrane (IMM) damage sets the stage for apoptosis (*52*). A major pathway that leads to mitochondrial damage in a broad spectrum of inflammatory or ischemia-related conditions results from the amplification of mitochondrial and cytosolic $O_2^{\bullet-}$ production (*53*). ROS mediated cell death, in particular $O_2^{\bullet-}$-mediated apoptosis, begins with rupture of the outer mitochondrial membrane (OMM) and cytochrome *c* release that subsequently trigger MPTP opening resulting in mitochondrial swelling. MPTP opening is also known to be involved in initiation of the apoptotic machinery without damage to the OMM. ROS and $[Ca^{2+}]_m$ overload acts synergistically to trigger MPTP opening, and evokes cytochrome *c* release and subsequent activation of caspases (10).

$O_2^{\bullet-}$ or H_2O_2 exposure amplifies the Ca^{2+}-induced MPTP opening in a permeabilized cell system which in turn could be attenuated with either $O_2^{\bullet-}$ scavengers SOD or SOD mimetic, MnTBAP, or H_2O_2 scavenger catalase (fig 4A & B). However, $O_2^{\bullet-}$ -induced cytochrome *c* release was insensitive to inhibitors of MPTP (*16*). Thus, MPTP opening is not essential for $O_2^{\bullet-}$-induced cytochrome c release. In addition, exogenous delivery of cytochrome *c* eliminated the $O_2^{\bullet-}$ -induced $\Delta\Psi_m$ loss. These data suggest that integrity of the IMM and matrix space was preserved during $O_2^{\bullet-}$-induced cytochrome *c* release *(15, 16)*.

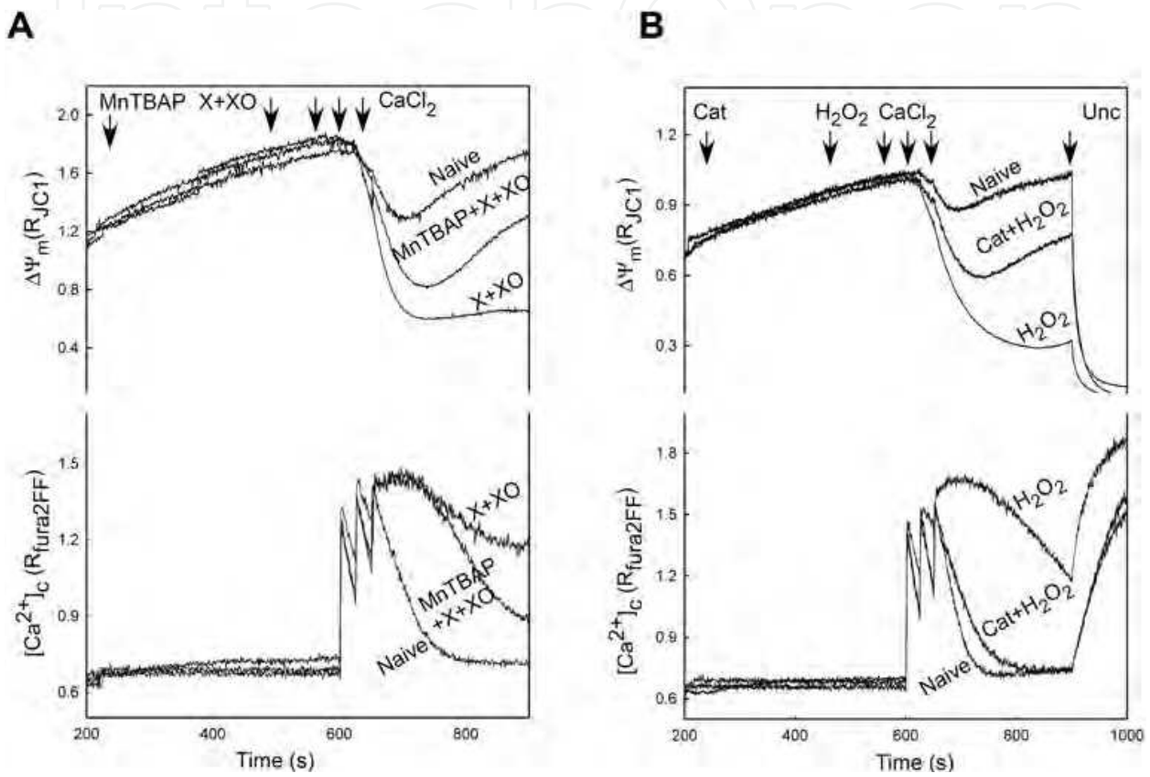

Fig. 4. Effect of ROS on Ca^{2+}-induced PTP opening and Cytochrome *c* release in permeabilized HepG2 cells. (A) $O_2^{\bullet-}$-generating system (xanthine [0.1mM] plus xanthine oxidase [20 mU/ml]) and (B) H_2O_2 (90 mM) augmented Ca^{2+}-induced depolarization (three pulses, 30 M $CaCl_2$ each) and decreased mitochondrial Ca^{2+} uptake. These effects were inhibited by an $O_2^{\bullet-}$-scavenger, MnTBAP (20 μM; 68 ±4.5% decrease in depolarization and 78 ±13% decrease in $[Ca^{2+}]_c$ rise at 900 s; *n*= 3), and catalase (Cat; 2500U/ml), respectively. At the end of the measurements, cells were exposed to FCCP (Unc; 1μM), a protonophore that caused rapid and complete dissipation of $\Delta\Psi_m$. (Reproduced with permission from © 2001 Madesh and Hajnóczky. Originally published in *J. Cell Biol.* 155:1003-1015).

5. Role of Bcl-2 family proteins in ROS-induced $\Delta\psi_m$ loss

Although ROS-induced Ca^{2+} dependent MPTP opening is associated with cytochrome *c* release, in particular, superoxide selectively triggers OMM permeabilization and cytochrome *c* release independent of Ca^{2+} dependant MPTP opening. $O_2^{\bullet-}$ produced by the mitochondrial respiratory chain has been reported to cause cardiolipid destruction in the IMM and dissipation of the $\Delta\Psi_m$ (*54, 55*). However, $O_2^{\bullet-}$ produced under various

pathophysiological conditions including ALD, causes OMM permeabilization in a Bax/Bak dependant manner. Antiapoptotic Bcl-2 family protein Bcl-x_L prevents $O_2^{\bullet-}$-induced $\Delta\Psi_m$ loss and cytochrome *c* release, implying a role for proapoptotic Bcl-2 proteins Bax and Bak. Despite their high homology, Bax and Bak have distinct subcellular localization and functional regulation. Bax is largely a cytosolic protein that undergoes conformational change that is prerequisite for mitochondrial phase of apoptosis. In contrast, Bak is a mitochondrial integral membrane protein which undergoes oligomerization upon activation by proapoptotic BH3-only proteins (tBid). $O_2^{\bullet-}$ -induced mitochondrial functional changes require either Bax or Bak. BH3 which constitute a subset of pro-apoptotic members of the Bcl-2 protein family are necessary to induce apoptosis (*10, 56*). $O_2^{\bullet-}$-mediated $\Delta\Psi_m$ loss and cytochrome *c* release is absent in Bax/Bak (*bax-/- bak-/-*) doubly deficient cells. Interestingly, Bak is necessary and sufficient for $O_2^{\bullet-}$-induced $\Delta\Psi_m$ loss and cytochrome *c* release. Mitochondria isolated from heart of *bak-/-* mice are resistant to $O_2^{\bullet-}$-induced mitochondrial depolarization. Further, *bid-/-* deficient MEFs are also insensitive to $O_2^{\bullet-}$ -induced mitochondrial phase of apoptosis. Conversely, mitochondria from Bax-deficient mice display $O_2^{\bullet-}$-induced mitochondrial depolarization. Upon TNF, Fas ligand or $O_2^{\bullet-}$ challenge, the cytosolic BH3-only protein Bid undergoes proteolytic processing (caspase 8 and caspase 2) to generate active form of Bid-tBid. tBid elicited $O_2^{\bullet-}$-induced mitochondrial depolarization and cytochrome *c* release requires Bak. Taken together, these findings

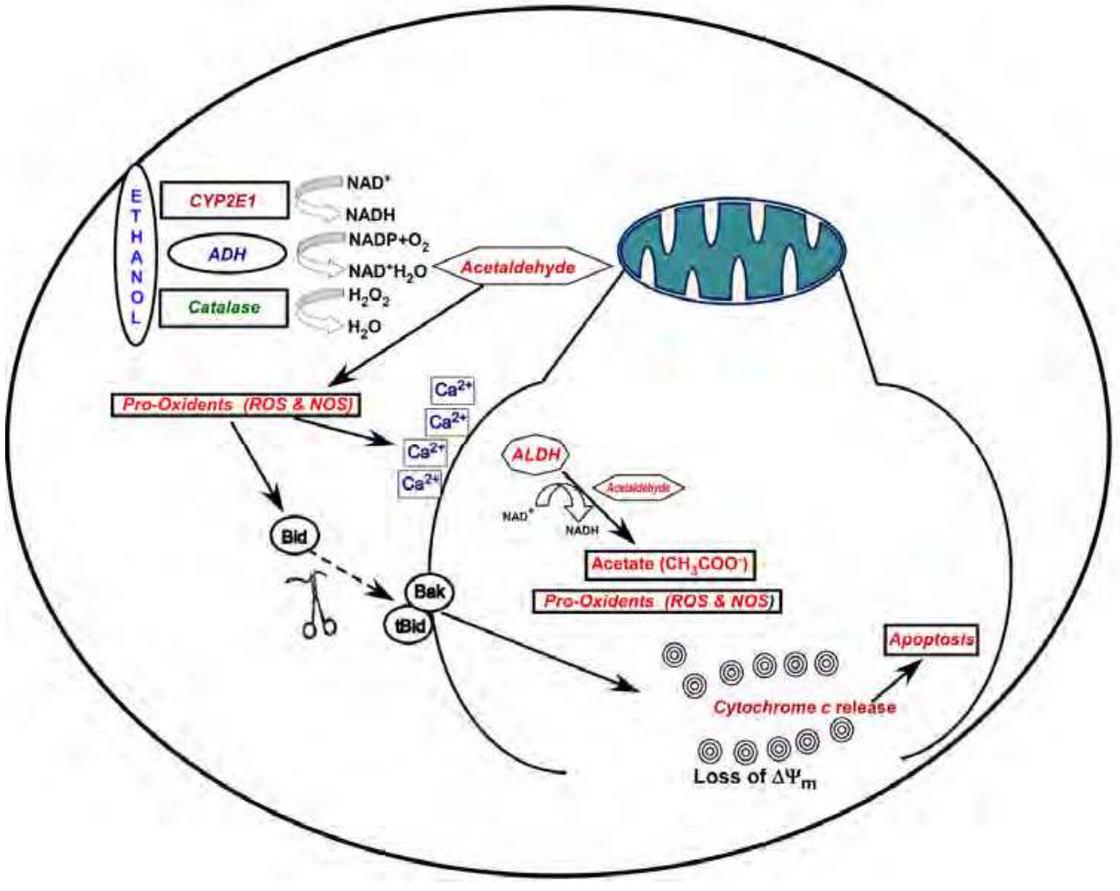

Fig. 5. Mitochondria are prime target for EtOH-induced cell death-Scheme.

implicate the requirement of Bak and Bid for $O_2^{\bullet-}$-induced $\Delta\Psi_m$ loss and cytochrome *c* release (*15, 16, 24, 10, 57*).

6. Conclusion

The aberrant rate of cell death is a hallmark of ALD. It is evident that ethanol induced ROS mediated oxidative stress is responsible for induction of apoptosis. The sequential events such as changes in redox status, increase in cytosolic ROS, sustained $[Ca^{2+}]_m$ elevation and translocation of pro-apoptotic proteins from cytosol to mitochondria are intimately linked with ethanol metabolism (fig 5). Major cell death pathways such as apoptosis, necrosis and the recently described necroptosis are associated with oxidative stress. Though, ROS production is proposed as a major factor in ethanol induced cell death little is known about the downstream mechanisms of the multimode cell death. In conclusion, mitochondria are prime target where multiple stress signaling pathways converge to induce cell death in the context of ALD.

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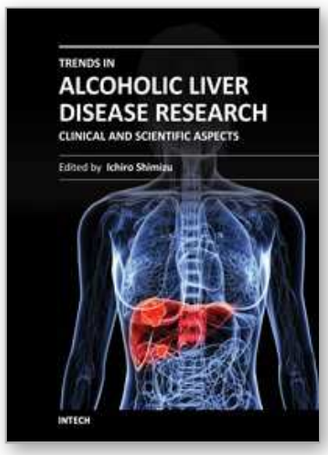

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Alcoholic liver disease occurs after prolonged heavy drinking. Not everyone who drinks alcohol in excess develops serious forms of alcoholic liver disease. It is likely that genetic factors determine this individual susceptibility, and a family history of chronic liver disease may indicate a higher risk. Other factors include being overweight and iron overload. This book presents state-of-the-art information summarizing the current understanding of a range of alcoholic liver diseases. It is hoped that the target readers - hepatologists, clinicians, researchers and academicians - will be afforded new ideas and exposed to subjects well beyond their own scientific disciplines. Additionally, students and those who wish to increase their knowledge will find this book a valuable source of information.

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