

# Chronic ethanol consumption increases the sensitivity of hepatocyte mitochondria to NO-dependent bioenergetic dysfunction.

Gloria A. Benavides<sup>1,2</sup>, Blake R. Zelickson<sup>1,2</sup>, Michelle S. Johnson<sup>1,2</sup>, Balu K. Chacko<sup>1,2</sup>, Aimee Landar<sup>1,2</sup>, Angela M. Betancourt<sup>1,3</sup>, Shannon M. Bailey<sup>1,3</sup>, Victor M. Darley-Usmar<sup>1,2</sup>

<sup>1</sup>Center for Free Radical Biology, <sup>2</sup>Department of Pathology, <sup>3</sup>Environmental Health Sciences. University of Alabama at Birmingham

## Introduction

Chronic alcohol consumption encompasses a complex liver pathology that features steatosis, steatohepatitis and cirrhosis, collectively termed as alcohol-induced liver disease (ALD).

Increased generation of reactive oxygen and nitrogen species (ROS/RNS) through the induction of CYP2E1, NADPH oxidase and inducible nitric oxide synthase (iNOS) have been shown to contribute to liver pathology associated with ALD in animal models.

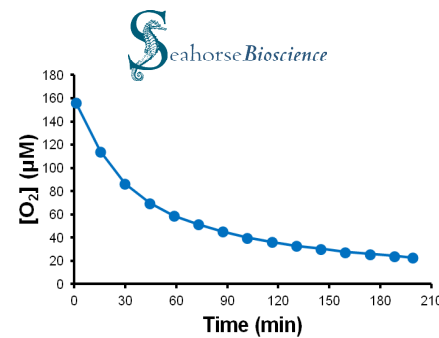
Chronic EtOH consumption causes mitochondrial dysfunction; however the underlying mechanisms resulting in increased generation of nitric oxide (NO) and ROS and their adverse effects on cellular respiration remain unclear.

We hypothesized that NO-dependent modulation of cellular respiration and the sensitivity to hypoxic stress is increased in hepatocytes from ethanol-fed rats.

## Methods

Male Sprague Dawley rats (200-250 g) or wild type (C57BL/6) and iNOS<sup>-/-</sup> (B6.129P2-NOS2 tm/au) mice were fed control or alcohol-containing Lieber-DeCarli liquid diets for 5 weeks and hepatocytes were isolated by liver perfusion and collagenase digestion.

To determine the effect of chronic alcohol consumption on hepatocyte bioenergetics, the Seahorse Bioscience extracellular flux analyzer (XF24) was used to measure O<sub>2</sub> consumption in hepatocytes. Cells from control and alcohol-fed rats were attached to specialized V7 plates coated with collagen. The XF24 analyzer was placed in a sealed glove box (Plas-Labs) equilibrated to 1% O<sub>2</sub> (11.5 μM O<sub>2</sub>). The OCR of hepatocytes was measured over time as the O<sub>2</sub> tension of the media decreased during equilibration from room air to 1% O<sub>2</sub> as shown in the graph [O<sub>2</sub>] vs. Time below.



Immunohistochemistry: After EtOH treatment, mice were administered pimonidazole (120 mg/ml) in saline (1 ml/Kg) via tail vein injection. Livers were harvested and fixed. Sections were incubated with anti-pimonidazole or anti-HIF-1α antibodies. Images were acquired using a Leica fluorescence microscope with IPLAB Spectrum.

## Results

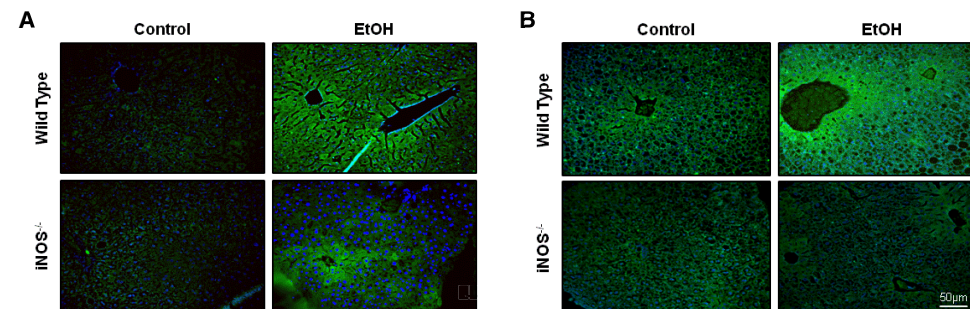


Figure 1. Inducible nitric oxide synthase (iNOS)-derived nitric oxide (NO) is required for chronic alcohol-induced liver hypoxia. (A) Pimonidazole staining, and (B) Fluorescence microscopy to detect HIF-1α stabilization were used in liver sections from control and alcohol (EtOH)-fed wild type and iNOS<sup>-/-</sup> mice.

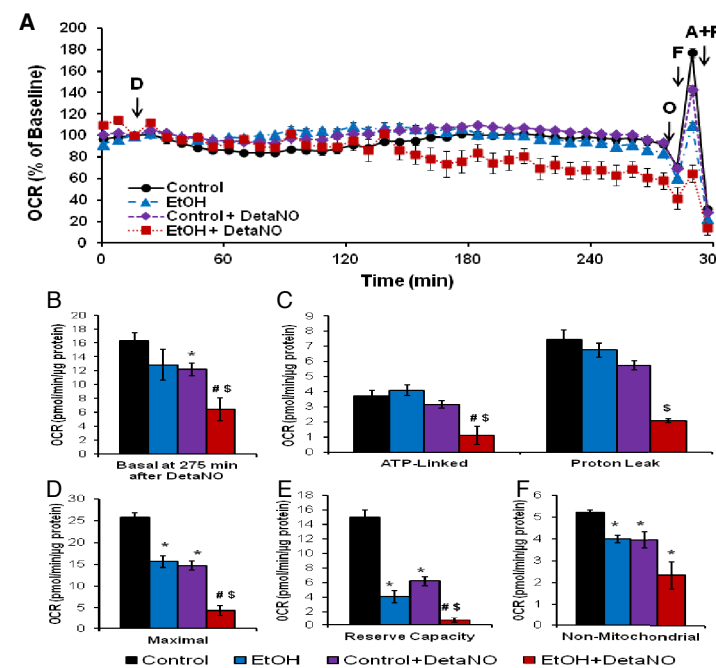


Figure 2. Chronic alcohol consumption sensitizes hepatocytes to NO-induced mitochondrial dysfunction. (A) Bioenergetic profile of isolated hepatocytes from control and EtOH-fed rats treated with 500 μM DetaNONOate (D) for 4h followed by subsequent injections of oligomycin (O), FCCP (F), and antimycin-A plus rotenone (A+R). (B) Basal OCR after DetaNONOate injection (C) ATP-linked respiration is equal to the oligomycin-induced decrease in OCR, with the remaining OCR due to proton leak. (D) Maximal OCR was measured following FCCP injection. (E) The reserve capacity was calculated from the difference between the maximal and basal OCR. (F) The non-mitochondrial OCR was determined by injecting antimycin-A plus rotenone simultaneously to fully inhibit the electron transport chain. Results are mean ± SEM. n=5 per group. \*p<0.005 compared to Control. #p<0.05 compared to EtOH. \$p<0.05 compared to Control + DetaNO.

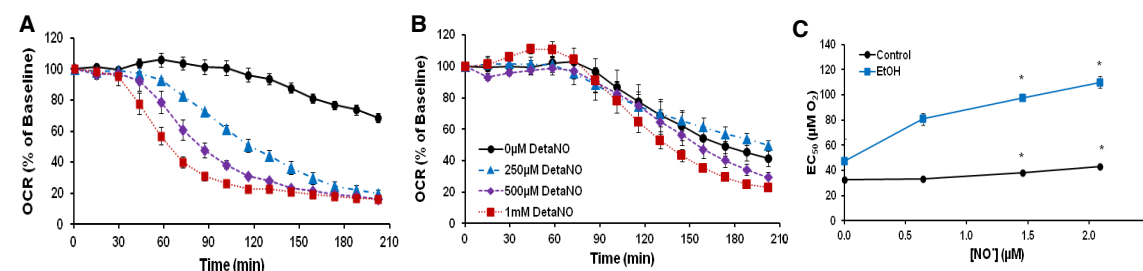


Figure 3. The susceptibility to NO-induced inhibition of respiration was increased in alcohol hepatocytes during hypoxia. (A) The effects of 0-1000 μM DetaNONOate added prior to the start of the assay on OCR of hepatocytes from control rats and, (B) EtOH-fed rats over time. (C) The EC<sub>50</sub> were calculated by fitting the data to a sigmoidal curve. Results are mean ± SEM. n=5 per group.

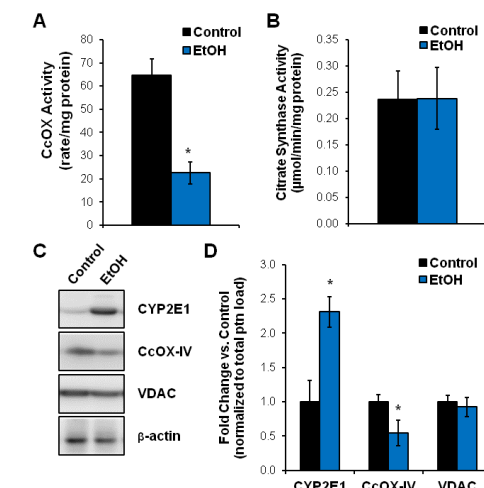


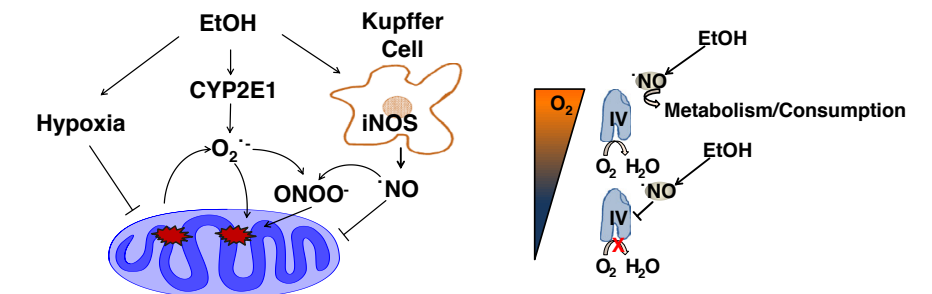
Figure 4. Effects of alcohol consumption on mitochondrial protein levels and activity. (A) Cytochrome c oxidase (CcOX) and (B) Citrate synthase activity in isolated hepatocytes from control and EtOH-fed rats. (C) Western Blot and (D) Densitometric quantification of protein levels of cytochrome P450 2E1 (CYP2E1), cytochrome c oxidase subunit IV (CcOX-IV), voltage-dependent anion channel (VDAC), and β-actin from primary hepatocytes isolated from control and EtOH-fed rats. Data are the mean ± SEM. n=6 for each group. \*p<0.05 compared to control.

## Summary

Chronic alcohol consumption significantly increased the binding of the hypoxia markers pimonidazole and HIF-1α in the low O<sub>2</sub> zones of the liver; however the binding of these markers was negligible in EtOH-fed iNOS<sup>-/-</sup> mice.

The oxygen consumption was unaltered at the basal level but exhibited a significantly decreased in the maximal and reserve capacity of the mitochondrial respiration in the EtOH-fed rats. Mitochondrial dysfunction occurred by addition of a second stressor (NO).

Under hypoxic stress, the addition of NO to EtOH hepatocytes increased inhibition of mitochondrial respiration.



Taken together, these data provide evidence for the role of NO as an important regulator of mitochondrial respiration under conditions of hypoxic stress in intact hepatocytes.

Reference: Zelickson, BR et al. *Biochim. Biophys. Acta.* 2011 Sep 24.

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