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Nitric oxide and hypoxia exacerbate alcohol-induced mitochondrial dysfunction in hepatocytes

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ABSTRACT

Chronic alcohol consumption results in hepatotoxicity, steatosis, hypoxia, increased expression of inducible nitric oxide synthase (iNOS) and decreased activities of mitochondrial respiratory enzymes. The impact of these changes on cellular respiration and their interaction in a cellular setting is not well understood. In the present study we tested the hypothesis that nitric oxide (•NO)-dependent modulation of cellular respiration and the sensitivity to hypoxic stress is increased following chronic alcohol consumption. This is important since •NO has been shown to regulate mitochondrial function through its interaction with cytochrome *c* oxidase, although at higher concentrations, and in combination with reactive oxygen species, can result in mitochondrial dysfunction. We found that hepatocytes isolated from alcohol-fed rats had decreased mitochondrial bioenergetic reserve capacity and were more sensitive to •NO-dependent inhibition of respiration under room air and hypoxic conditions. We reasoned that this would result in greater hypoxic stress *in vivo*, and to test this, wild-type and iNOS$^{-/-}$ mice were administered alcohol-containing diets. Chronic alcohol consumption resulted in liver hypoxia in the wild-type mice and increased levels of hypoxia-inducible factor 1α in the peri-venular region of the liver lobule. These effects were attenuated in the alcohol-fed iNOS$^{-/-}$ mice suggesting that increased mitochondrial sensitivity to •NO and reactive nitrogen species in hepatocytes and iNOS plays a critical role in determining the response to hypoxic stress *in vivo*. These data support the concept that the combined effects of •NO and ethanol contribute to an increased susceptibility to hypoxia and the deleterious effects of alcohol consumption on liver.

1. Introduction

It has been shown that nitric oxide (•NO) regulates several mitochondrial functions, including respiration and biogenesis. These new insights have led to a deeper understanding of the cross talk between •NO signaling pathways and major regulatory and metabolic pathways in the cell [1,2]. For example, mitochondrial biogenesis can be regulated by the soluble guanylate cyclase pathway and •NO can modulate the response to hypoxia, depending on its concentration, through both mitochondrial-dependent and independent pathways [3–5]. However, under conditions associated with inflammation, increased reactive oxygen species (ROS) will decrease the concentration of •NO available to interact with cytochrome *c* oxidase and participate in reactions with other reactive species to generate secondary products that impair mitochondrial function through oxidation, nitration, and inactivation of mitochondrial proteins [6–8]. Chronic exposure to alcohol is particularly interesting in this respect since hepatotoxicity is associated with hypoxia, increased reactive nitrogen species (RNS) through induction of iNOS, protein nitration and lipid oxidation [9–12]. These oxidants are associated with oxidative damage to the mitochondrial respiratory chain and mtDNA particularly complexes I and II [7,10–15].

A role for the •NO-cytochrome *c* oxidase pathway in regulating oxygen (O_2) gradients has also been proposed based upon both theoretical modeling and the observation that •NO is a more effective inhibitor of

Abbreviations: iNOS, inducible nitric oxide; NO, nitric oxide; HIF-1α, hypoxia-inducible factor-1α; O_2, oxygen; ROS, reactive oxygen species; EtOH, alcohol/ethanol; OCR, oxygen consumption rate; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; CcOX, cytochrome *c* oxidase; CYP2E1, cytochrome P450 2E1; DetaNO, DetaNONOate; ALDH2, aldehyde dehydrogenase 2; RNS, reactive nitrogen species

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the most actively respiring (State 3) mitochondria [16,17]. This suggests that under normal conditions, the binding of •NO to cytochrome *c* oxidase limits O_2 consumption in the most actively respiring tissues, extending O_2 gradients in organs such as the heart or liver [7,16,17]. Since alcohol-dependent hepatotoxicity is associated with increased superoxide and induction of iNOS it is likely that peroxynitrite is formed [10–12,18]. Indeed, we have recently shown that amelioration of mitochondrial oxidant stress with a mitochondrial antioxidant, probably through scavenging peroxynitrite, can inhibit HIF-1α activation in response to chronic alcohol (EtOH) consumption [19]. Moreover, in response to ethanol (EtOH)-dependent hepatotoxicity, isolated liver mitochondria become more sensitive to respiratory inhibition by •NO [12,15]. This will further contribute to tissue hypoxia and oxidative stress through increasing production of superoxide within the respiratory chain [20]. In addition, it is now clear that as the tissue becomes hypoxic the respiratory chain is capable of generating superoxide at complex III which is also modified in ethanol-dependent hepatotoxicity [21,22].

Previous studies have shown that chronic EtOH consumption causes marked bioenergetic defects in both peri-venous and peri-portal hepatocytes. Upon exposure to hypoxia, which occurs in EtOH-induced hepatotoxicity, these defects become more pronounced and are associated with decreased aerobic and anaerobic ATP production [23–26]. Interestingly, it is now clear that mitochondria do not generally function close to their maximal respiratory function (State 3 in isolated mitochondria) in cells but a more intermediate respiratory state we have termed "state apparent" [27–29]. We have proposed that decreases in the specific activity of mitochondrial respiratory chain proteins decrease the bioenergetic reserve or spare capacity but this has not been shown *in vivo*. This is important given that it makes the cell potentially more susceptible to stress since we and others have shown reserve capacity is used at times of oxidative stress or increased work load [28–30]. Previous studies have shown with isolated mitochondria that the specific activities and respiratory control ratio of mitochondria isolated from alcohol treated animals are decreased [15,31]. Based on this, we hypothesized that hepatocytes from chronic ethanol-fed animals will have a decreased reserve capacity. Furthermore, since the reserve capacity is decreased then mitochondria will be metabolically more active and closer to State 3 to maintain bioenergetic homeostasis under which conditions mitochondria are more sensitive to NO [29]. We hypothesized that the sensitivity to NO-dependent inhibition would therefore increase and this would be exacerbated by hypoxia (which is a feature of alcohol-dependent hepatotoxicity). In support of this it has been shown that inducible nitric oxide synthase (iNOS) is known to be increased in response to EtOH consumption, and mitochondria isolated from EtOH-treated animals are more susceptible to •NO-dependent inhibition of respiration [12,15]. Given that induction of iNOS is also associated with protein nitration, we reasoned that ˙NO would exacerbate the effects of hypoxia and pathological effects of alcohol in the hepatocytes from EtOH-exposed animals [6,9,10,32].

In support of this concept, we and others have demonstrated that EtOH-dependent hepatotoxicity is suppressed in iNOS$^{-/-}$ animals [10,12]. These data suggest an important link between increased •NO formation from iNOS during chronic EtOH intoxication, enhanced sensitivity of mitochondrial respiration to •NO, and hypoxia. The adaptive response to hypoxia is orchestrated through hypoxia-inducible factor-1 (HIF-1), [33]. The effects of NO and iNOS on hypoxia and cellular bioenergetics were tested in a model of chronic EtOH-induced hepatotoxicity using hepatocytes isolated from Sprague–Dawley rats and studies examining effects in liver tissue from C57BL/6 and iNOS$^{-/-}$ mice.

2. Materials and methods

2.1. Reagents and antibodies

All chemicals were purchased from Sigma-Aldrich (St.-Louis, MO) unless stated otherwise and were of the highest grade available.

2.2. Alcohol feeding

Male Sprague–Dawley rats or wild type (C57BL/6) and iNOS$^{-/-}$ (B6.129P2-NOS2 tm/lau) mice were pair-fed an isocaloric liquid diet, with and without ethanol (4% for mice and 5% for rats), for 5–6 weeks as described previously [12,22]. EtOH consumption was uniform throughout the study between wild type and iNOS$^{-/-}$ mice (data not shown). Animals were handled in accordance to "The Guide for the Care and Use of Laboratory Animals" approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

2.3. Hepatocyte preparations

Primary rat hepatocytes were isolated as previously described [23]. The viability of hepatocytes at isolation was 93 ± 1% and was not different between control and EtOH-fed rats. In initial experiments we found that the efficiency of adherence for the ethanol treated cells was variable and compensated for this difference by seeding with a higher number of cells than the control. However, this resulted in experimental variation in the basal O_2 consumption between hepatocyte preparations. Where feasible, this was corrected for by measurement of protein levels in the wells. To allow comparison of the data between conditions, changes are therefore expressed as a% of the basal OCR in each case and the range of OCR consumption in the individual experiments reported in the text.

2.4. Immunoblot analysis

Briefly, hepatocyte homogenates (20 μg) were separated using 12.5% SDS-PAGE followed by immunoblotting onto nitrocellulose. Antibody dilutions were 1/3000 for CYP2E1 (Millipore, Billerica, MA), 1/5000 for porin/VDAC (Invitrogen Carlsbad, CA), and 1/2000 for cytochrome *c* oxidase subunit IV (CcOX-IV) (Invitrogen Carlsbad, CA) in Tris-buffered saline containing 0.05% Tween-20 followed by HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (GE Healthcare, Piscataway, NJ). The efficiency of protein loading and protein transfer was monitored using Ponceau S staining which was then used for normalization. The intensities of protein bands were quantified prior to image saturation using AlphaEaseFC software (Alpha Innotech, Santa Clara, CA).

2.5. Mitochondrial bioenergetics and enzyme activities

Citrate synthase and CcOX activities were measured as previously described [13,22]. Citrate synthase activities are expressed in units of enzyme activity, where 1 unit = 1 μmol thionitrobenzoate generated/min. CcOX activities are expressed in *k*/s, where *k* is the first order rate constant for the oxidation of cytochrome *c*. An XF24 analyzer (Seahorse Bioscience, Billerica, MA) was used to measure hepatocyte O_2 consumption [29]. Hepatocytes from control and EtOH-fed rats were adhered to collagen-coated V7 plates (Seahorse Bioscience) for 24 h. The seeding density was optimized for both control and EtOH-fed rat hepatocytes (Supplemental Fig. 1), with 20,000 and 40,000 cells/well chosen for control and EtOH groups, respectively. XF24 assays were performed in unbuffered DMEM (pH 7.4) supplemented with 5.5 mM D-glucose, 1 mM sodium pyruvate, and 4 mM L-glutamine. Cellular mitochondrial function was measured as described previously using sequential injections of oligomycin (1 μg/mL), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, 0.3 μM), and antimycin A (10 μM) plus rotenone (1 μM) [29]. The concentrations used were determined by titrating to yield their optimal effects (data not shown).