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Introduction: The effects of ethanol intoxication on the heart have been extensively studied over the past century; however the consequences of ethanol withdrawal on the heart have not been documented. Myocardial adaptations to hypoxia can improve contractility, enhance antioxidant defense mechanisms, decrease lipid peroxidation induced by oxidative stress, and bolster cardiac resistance to ischemia-reperfusion. This study was conducted to determine if ethanol intoxication-withdrawal can harm the myocardium, and if intermittent hypoxia conditioning (IHC) can induce antioxidant enzymes and proteins that blunt these effects. **Hypothesis:** IHC increases myocardial antioxidant enzymes and other stress proteins, which could protect myocardium from ethanol intoxication-withdrawal. **Methods:** Four month old Sprague Dawley rats (n=61) were divided into 8 groups. Four groups were fed a 6.4% ethanol enriched diet, and the other four were fed an isocaloric dextrin diet. IHC and was initiated two weeks after the diets began. The antioxidant *N*-acetylcysteine (NAC) was injected throughout the IHC and sham conditioning programs to interrogate the role of reactive oxygen species in the effects of ethanol intoxication-withdrawal and IHC on myocardial proteins. Proteins were extracted from snap-frozen myocardium for assays of nitric oxide synthase (NOS), gluthathione peroxidase (GPx) and superoxide dismutase (SOD) activities, and immunoblot analyses of endothelial NOS (eNOS) and heat shock protein 70 (Hsp70).

Results: Ethanol withdrawal inactivated myocardial SOD, but did not affect activities of GPx and NOS, or contents of Hsp70 and eNOS. IHC lowered SOD activity by 55% in dextrin-fed rats, but partially protected SOD activity from ethanol intoxication-withdrawal. IHC did not affect myocardial GPx, NOS, and Hsp70 in dextrin and ethanol-withdrawn rats. NAC alone increased SOD activity in dextrin-fed and ethanol withdrawn rats and, when combined with IHC, NAC fully protected SOD activity from ethanol intoxication-withdrawal. **Conclusions:** Ethanol intoxication-withdrawal had statically significant effects on the antioxidant enzyme SOD. IHC and NAC selectively protected SOD activity from ethanol intoxication-withdrawal. This specificity suggests that the oxidative stress induced by ethanol intoxication-withdrawal is discrete in comparison to the massive oxidative stress inflicted on the brain under these conditions. The differences in the intensity of oxidative stress could be one of the factors influencing the divergence of results in comparison to previous investigations of ethanol intoxication-withdrawal.

RESPONSES OF MYOCARDIAL ANTIOXIDANT SYSTEMS TO

INTERMITTENT HYPOXIA AND ETHANOL WITHDRAWAL

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CHAPTER 1

Introduction and Background

Alcoholism and Its Prevalence

Approximately two-thirds of the United States population consumes alcohol, and 10% of these individuals abuse alcohol. Each year almost 1.5 million people are admitted to hospitals and alcohol treatment centers because of alcohol dependence and its medical sequelae [22]. A complex disorder, alcoholism results from an interplay of genetic, environmental, and social factors. Recent research indicates that the heritability of alcoholism centers on genes encoding alcohol dehydrogenase (ADH), cytochrome P450 (CYP2E1), and aldehyde dehydrogenase (ALDH), all of which participate in ethanol metabolism [31]. This research also focuses on brain chemistry and the neurotransmitters involved in addiction behavior. Specifically, the GABA receptor and neurotransmitters serotonin, dopamine, and glutamate are the foci of this neurochemistry research [28]. Alcoholism transcends race and socioeconomic status which underscores the role of hereditary factors in this disease; however, alcoholism is a complex disease that cannot be explained by genetics or neurochemistry alone. An individual's physical environment also plays an integral role in the development of alcohol addiction.

The World Health Organization defines two different types of alcoholics: alcohol abusers and alcohol-dependent individuals. Alcohol abusers have intense psychological

dependence on alcohol, leading to heavy consumption or binge drinking in response to environmental stimuli [23]. However, only alcohol-dependent individuals exhibit increased tolerance to alcohol and experience physical withdrawal symptoms upon abstaining from alcohol consumption [23]. Withdrawal symptoms typically manifest within 48 hours of ethanol abstinence and include headache, sweating, tremors, anxiety, nausea, disorientation, and sensitivity to light and sound. One of the most severe manifestations of withdrawal is delirium tremens (DT) which coincides with large increases in heart rate, respiration, and blood pressure. DT and its cardio-respiratory sequelae typically occur 2-4 days after the cessation of alcohol consumption [18].

In 2007 it was estimated that 7.6% of Americans met the criteria for alcohol-dependence, the majority in the 30-50 year age group [27]. Long term, heavy alcohol consumption and dependence can cause an array of internal disorders including hypertension, decreased cardiac ejection fraction, left ventricular hypertrophy, hepatic cirrhosis, and carcinomas. Excessive ethanol metabolism has profound effects on the liver, brain, and heart due in large part to oxidative stress that results from the oxidation of ethanol. The main pathways of alcohol metabolism are either oxidative or non-oxidative depending on the organ involved. The following sections examine the detrimental effects of ethanol intoxication and withdrawal on liver, heart and brain, emphasizing the harmful effects of reactive oxygen species (ROS) and other toxic products of ethanol metabolism and abrupt ethanol withdrawal.

Hepatic Ethanol Metabolism

Ethanol metabolism in liver yields acetaldehyde and free radicals, which reportedly interfere with mitochondrial redox state, protein structure, and antioxidant enzyme systems [8, 23, 30]. The majority of ingested ethanol is oxidized in the liver by ADH, an enzyme not present in myocardium. The other two enzymes responsible for oxidative metabolism of ethanol are CYP2E1 and catalase. In the liver, as well as other organs, CYP2E1 becomes an important ethanol-oxidizing enzyme when ADH is saturated, as in the case of heavy ethanol consumption. Ethanol oxidation by ADH generates acetaldehyde, a toxic compound (Figure 1.1). Normally, acetaldehyde is readily oxidized to acetate by ALDH in the mitochondria with concomitant reduction of $NADP^+$ to NADPH (Figure 1.2). Subsequent release of acetate into the circulation leaves a reduced cellular environment in the cytosol of hepatocytes [32]. The formation of NADPH provides reducing power for the production of superoxide anion ($\cdot O_2^-$), itself a free radical and the precursor of a host of ROS. Specifically, the conversion of acetaldehyde to acetate increases the NADPH:$NADP^+$ ratio, and the oxidation of NADPH to $NADP^+$ catalyzed by NAD(P)H oxidase generates $\cdot O_2^-$ by transfer of an electron to molecular oxygen [1]. As alcohol consumption intensifies, its increased oxidative metabolism augments the levels of $\cdot O_2^-$ and other ROS. Increased circulating ethanol concentrations have been found to upregulate hepatic expression of CYP2E1, another enzyme capable of generating ROS [31]. The formation of ROS as by-products of ethanol oxidation depletes intracellular antioxidant defenses, resulting in oxidative stress leading to apoptotic death of hepatocytes and eventually culminating in alcoholic liver disease [36].

By forming adducts with proteins, acetaldehyde can interfere with mitochondrial respiratory complexes and protein synthesis. Adducts are hybrid compounds formed by the interaction of both acetaldehyde and ROS with proteins and other complexes in the cell [29]. Not only does the formation of adducts impede normal protein function in the liver, it also elicits an immunogenic response in the tissues [29]. For the most part, adducts have been shown to primarily affect the liver; however, recent research shows that acetaldehyde interferes with protein synthesis in the myocardium, potentially implicating these circulating adducts in the cardiovascular sequelae of ethanol intoxication [29, 30].

Effects of Ethanol on the Heart

The impact of alcohol on the heart has been debated for over a century. In moderation, alcohol's protective capabilities against coronary artery disease (CAD) are well documented. Red wine, in comparison to beer or liquor, has been credited with the ability to protect against coronary atherosclerosis by suppressing LDL oxidation [37]. However, when consumed heavily, alcohol transitions from a protective agent into a destructive one and has profound toxic effects on the heart beginning at the mitochondrial level [30]. The heart lacks ADH, and there is no evidence that myocardium metabolizes ethanol [23]. However, the chronic oxidation of ethanol results in elevated circulating acetaldehyde concentrations, the suspected culprit in alcohol-induced cardiovascular injury [23].

Ethanol is metabolized mostly through oxidative pathways in the liver. However non-oxidative pathways of ethanol metabolism can also yield toxic products that affect

mitochondrial respiration. Fatty acid ethyl esters (FAEEs) are produced from the interaction of ethanol with fatty acids. FAEEs are present in plasma and tissues during intoxication, and persist long after ethanol is eliminated from the system [32]. In fact, FAEEs are often used as clinical markers of chronic ethanol intoxication [8]. As lipid soluble metabolites, FAEEs contribute to mitochondrial dysfunction by inserting into the mitochondrial membrane, and upon hydrolysis, releasing a free fatty acid into the inner mitochondrial membrane, producing a toxic detergent effect [30].

In addition to the toxic effects of acetaldehyde and FAEEs, activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) are decreased in myocardium of alcoholics, leading to an imbalance in the endogenous myocardial oxidant/antioxidant balance [37]. Not only is this affect a direct result of ethanol's depletion of myocardial glutathione (GSH) concentration, but the increased expression of CYP2E1 also contributes to the increased formation of ROS. The overproduction of ROS depletes endogenous antioxidant defenses and imposes lipid, DNA, and protein peroxidation [29, 31, 37], and the formation of acetaldehyde adducts leads to the disruption of protein synthesis and mitochondrial respiration [29].

The oxidative stress produced by ethanol metabolism also increases the expression of stress-induced chaperone proteins called heat shock proteins (Hsp), specifically Hsp70 [19]. Heat shock proteins are a highly conserved line of defense against cellular insults including oxidative stress, ischemia, changes in barometric pressure, and extreme temperatures. Heat shock proteins are responsible for repairing improperly folded or denatured proteins, stabilizing pre-folded structures during

translocation, and also are involved in directing damaged or improperly folded proteins to lysosomes for degradation [21]. Research also indicates that ethanol can directly trigger increases in cellular Hsp70 concentration in animal tissues and cultured cells [21].

In addition to Hsp 70, increased concentrations of nitric oxide (NO) have been found in alcoholics, possibly generated by increased inducible nitric oxide synthase (iNOS) activity produced as an inflammatory response to chronic ethanol intoxication [37]. While some research suggests that the production of NO is both cardio and neuroprotective [13], NO may also contradict this protection by suppressing expression of the cardioprotective endothelial NOS (eNOS) isoform by a classical negative feedback mechanism [13, 20]. Moreover, increased concentrations of NO can condense with superoxide ($\cdot O_2^-$) in an irreversible, biradical reaction, forming peroxynitrite ($ONOO^-$) which can initiate a cascade of lipid peroxidation and inactivate mitochondrial respiratory complexes [13].

The Effects of Ethanol Withdrawal on the Brain

Research regarding the effects of ethanol withdrawal on the brain focuses primarily on the receptors and neurotransmitters involved in addiction, specifically the N-methyl D-aspartate (NMDA) and gamma-aminobutyric acid (GABA) receptors, and the neurotransmitters serotonin, dopamine, and glutamate. During intoxication, ethanol has an inhibitory effect on NMDA receptors, suppressing excitatory glutamate transmission in the brain [15]. This glutamatergic suppression elicits compensatory increases in NMDA receptor expression and incorporation into neuronal membranes [10]. Upon withdrawal

from ethanol, glutamate secretion increases sharply. When combined with the increased NMDA receptor density, the abrupt surge in glutamate secretion imposes a hyperexcitatory state that contributes to the neurological symptoms that manifest themselves upon ethanol withdrawal [10]. The binding of glutamate to NMDA receptors initiates a cascade of intracellular reactions mediated by Ca^{2+}, that ultimately leads to activation of the Ca^{2+}-dependent neuronal NOS (nNOS) isoform (Figure 1.3) [6]. The combination of NO and superoxide produces $ONOO^-$ which can inflict lipid peroxidation, DNA damage, protein tyrosine nitration, cysteine S-nitrosation, and the impairment of mitochondrial electron transport chain components [6, 8].

Although ethanol withdrawal has been extensively studied in the brain, its effects on the heart and liver remain unclear. Abstaining from ethanol consumption is imperative to the recovery from alcoholic cardiomyopathy (ACM) and liver disease [22, 23, 27]. Indeed, upon cessation of consumption, the prognosis of ACM patients improves significantly, as does that of patients diagnosed with alcoholic liver disease [22, 23, 27]. However, the time course of cardiac and hepatic recovery from the damage inflicted by intoxication is unknown, and it is also unclear whether ethanol withdrawal has detrimental effects on the heart and liver similar to its effects in the brain.

The Cardioprotective Effects of Intermittent Hypoxia Conditioning

Acute hypoxic episodes can be beneficial or detrimental to the cellular environment depending on the duration, frequency, and intensity of reduced FIO_2. Although associated with chronic pulmonary diseases such as asthma, sleep apnea, and

obstructive lung disease, intermittent hypoxia can be induced therapeutically by vigorous exercise, by brief exposures to hypobaric conditions, or by breathing hypoxic gas mixtures [13]. Not only is intermittent hypoxia under investigation as a powerful cardioprotective treatment [38, 11, 12], current studies by Jung *et al.* (2007, 2008) have shown intermittent hypoxia to be a neuroprotective intervention capable of blunting ethanol withdrawal symptoms. Intermittent hypoxia-reoxygenation produces moderate amounts of ROS that evoke expression of GPx, SOD, and Hsp70, via ROS signaling pathways [13]. Recent evidence indicates that intermittent hypoxia conditioning suppresses myocardial eNOS and iNOS content [20], and induces phosphorylation events that activate the remaining cardioprotective eNOS isoform [20]. The benefits of intermittent hypoxia conditioning (IHC) in ischemic injury have been extensively studied, prompted by epidemiological evidence that populations living at higher altitudes have decreased incidence and severity of cardiovascular disease morbidities [4]. However, the possibility that IHC may protect ROS-sensitive myocardial enzymes from oxidative stress imposed by ethanol withdrawal has never been tested.

Global Hypothesis and Specific Aims

Myocardial adaptations to hypoxia can improve contractility [13], enhance antioxidant defense mechanisms [13], decrease lipid peroxidation induced by oxidative stress [13], and greatly increase cardiac resistance to ischemia-reperfusion [11, 38]. Based on these findings, I hypothesize that intermittent hypoxia conditioning (IHC) increases

myocardial antioxidant enzymes and other stress proteins, and that these adaptations protect myocardium from the cytotoxicity caused by ethanol intoxication and withdrawal.

Aim 1: To test the hypothesis that a 20 day IHC program applied to rats consuming ethanol increases myocardial contents of antioxidant and anti-inflammatory proteins and decreases content of inducible nitric oxide synthase (iNOS), a pro-inflammatory enzyme.

a) Rats were sacrificed 24 hours after IHC treatments and discontinuing ethanol consumption. Hearts were excised and quickly freeze-clamped with Wollenberger tongs pre-cooled in liquid N_2. Frozen myocardium was pulverized to a fine powder under liquid N_2, and myocardial proteins were extracted in phosphate buffer.

b) A Coomassie Blue-based assay kit (Pierce; Rockford, IL) was used to measure total protein concentration of extracts according to the method of Bradford [(2)]. This information was used to normalize data from enzyme assays and immunoblots.

c) Total nitric oxide synthase (NOS) activity was measured in the extracts using a commercially available kit (EMD Bioscience; La Jolla, CA). The kit measures NOS activity based on the formation of NO_2^- detected at 540 nm in a microplate spectrophotomer.

d) Heat-shock protein 70 (Hsp70), endothelial NOS (eNOS) and inducible NOS (iNOS) were measured by immunoblotting using mouse anti-rat antibodies selective for the protein of interest.

Aim 2: To test the hypothesis that reactive oxygen species (ROS) act as signaling molecules modulating gene expression resulting in cardioprotective protein expression induced by IHC. This hypothesis was tested by daily *ip* injection of an antioxidant compound, *N*-acetylcysteine (NAC), to scavenge the ROS produced by hypoxia-reoxygenation.

a) Rats were sacrificed after a 20 day IHC + NAC regimen and a 24 hour ethanol withdrawal period. Hearts were freeze clamped and extracted in phosphate buffer as in specific aim 1.

b) A Coomassie Blue Bradford assay kit was used to measure total protein concentration of each sample extract. This data was used to normalize data from enzyme assays and immunoblots.

c) Activities of the antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD) were measured by spectrophotometry, as was total NOS activity.

d) Hsp70 (Hsp70), eNOS, and iNOS were measured by immunoblots using mouse anti-rat antibodies, as in specific aim 1.

Potential Clinical Impact

Because the cessation of alcohol consumption is imperative early in the course of alcoholic cardiomyopathy and other ethanol induced diseases [27], alcohol-dependent individuals must unavoidably endure alcohol withdrawal in order to halt the disease progression. Literature summarized herein supports the investigation of the effects of

ethanol intoxication-withdrawal on myocardial antioxidant enzyme systems, and the beneficial effects of intermittent hypoxia-induced cardioprotective protein expression in the myocardium. Current treatments of ethanol withdrawal's sequelae include the administration of anti-seizure and adrenergic medications, and sedatives [18]. Alternative treatments need to be investigated that not only aid in the neurological symptoms of ethanol withdrawal, but also protect the heart from the stress of intoxication-withdrawal. Intermittent hypoxia has been studied in the context of myocardial ischemia-reperfusion injury as well as in the area of ethanol withdrawal on the brain. Studies by my co-investigator show that ethanol withdrawal manifestations were blunted because of IHC [5, 6]. This study investigates, for the first time, the possibility that intermittent hypoxia could protect the heart from the ravages of ethanol withdrawal.

Figure 1.1: *Oxidative Metabolism of Ethanol in the Liver.* The main pathways of ethanol oxidation in hepatocytes are shown. ADH: alcohol dehydrogenase; CYP2E1: cytochrome P450 2E1.

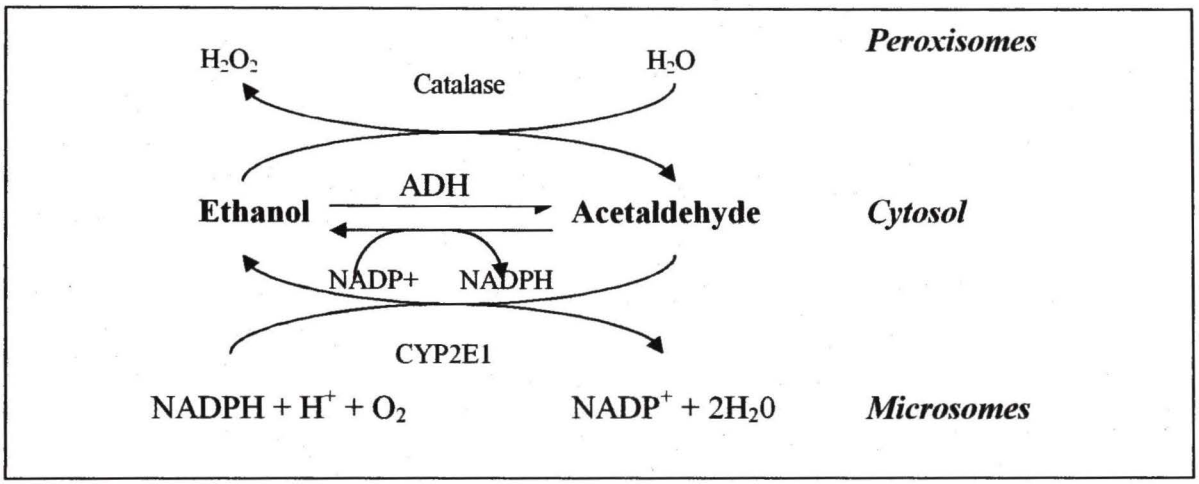


Figure 1.2: *Mitochondrial Metabolism of Acetaldehyde.* Acetate, generated in hepatocytes by mitochondrial oxidation of acetaldehyde, is released into the circulation. ALDH2: aldehyde dehydrogenase.

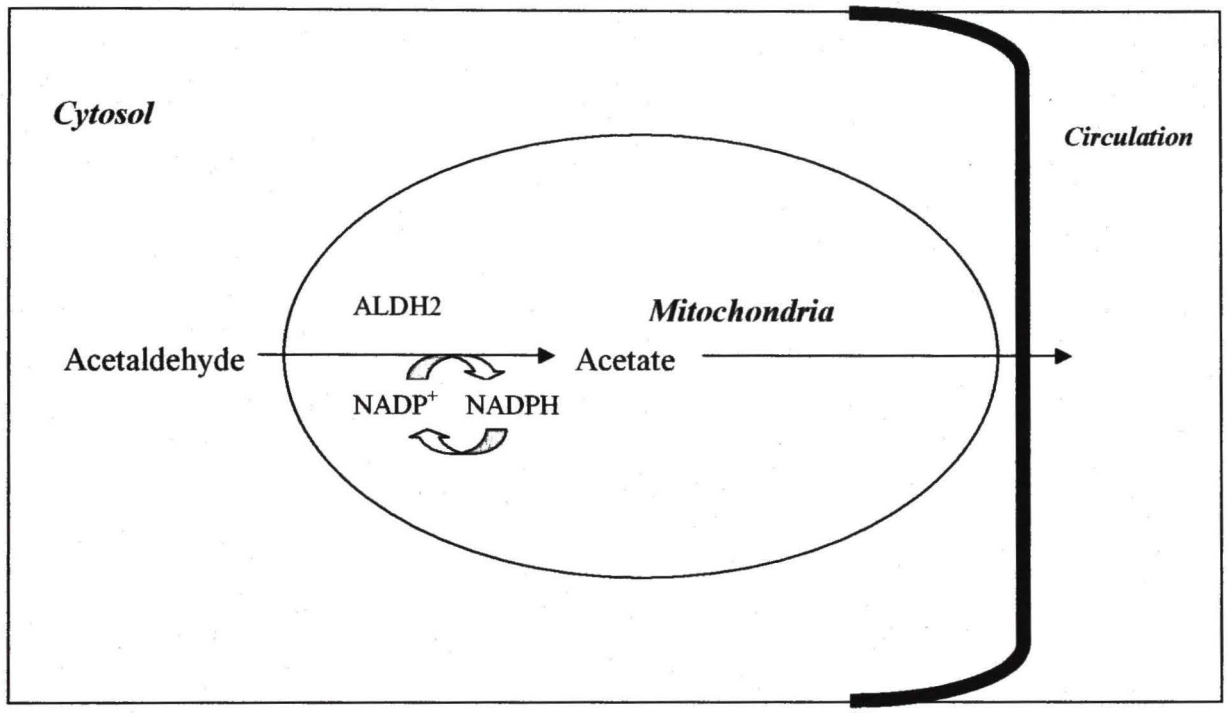
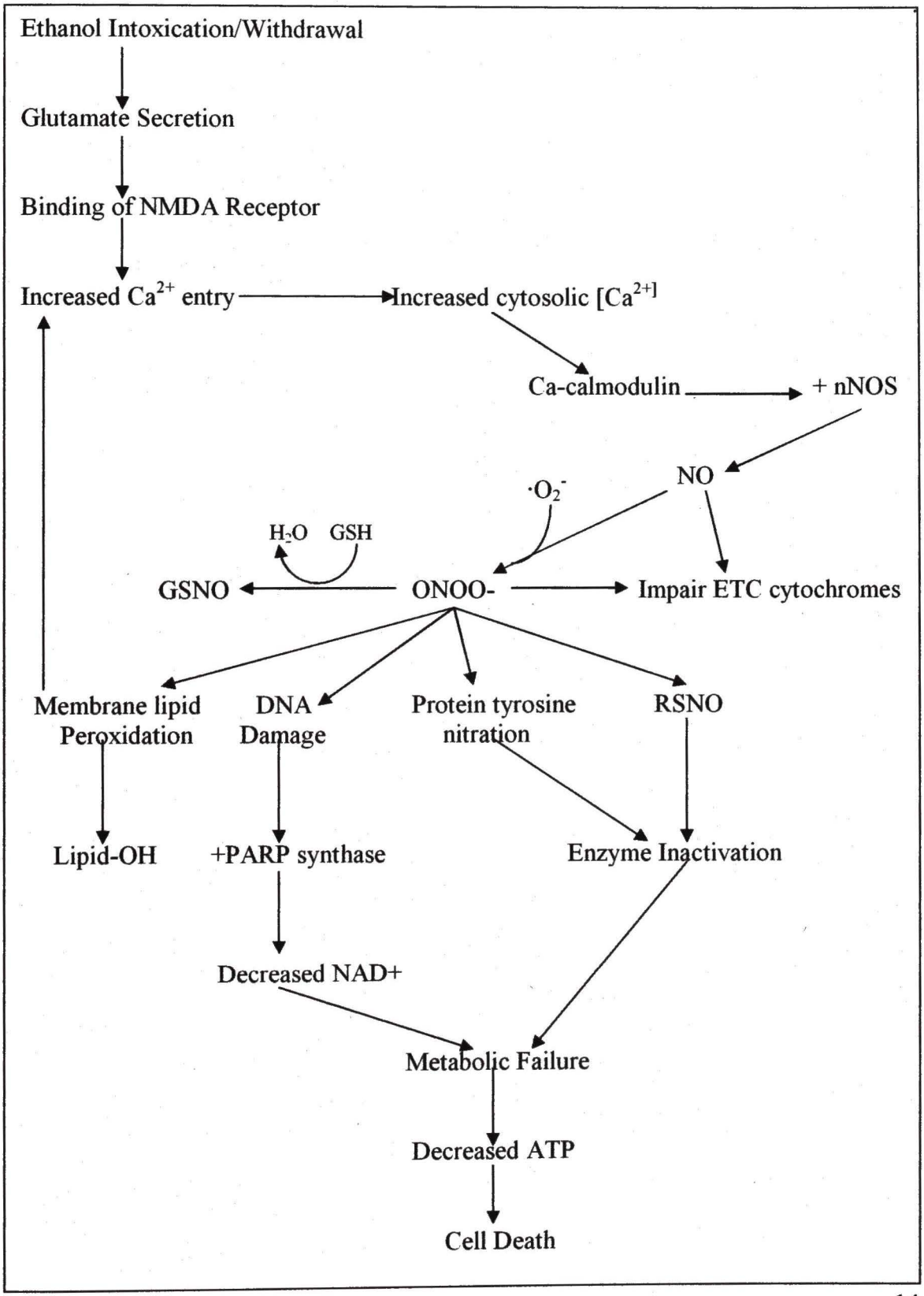


Figure 1.3: *Ethanol Withdrawal Glutamatergic-Induced Cascade.* This figure summarizes pro-oxidant cascades in the brain initiated by ethanol withdrawal. NMDA: N-methyl D-aspartate; nNOS: neuronal nitric oxide synthase; NO: nitric oxide; ONOO-: peroxynitrite; GSH: glutathione; GSNO: nitrosylated glutathione.



CHAPTER 2

Project Design and Methods

Housing and Diet of Animals

All procedures involving the rats were approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee. Four month old adult male Sprague Dawley rats weighing approximately 400 g were housed individually at controlled room temperature (22-25°C) and humidity (55%) with *ad libutum* access to water. These rats were randomly assigned to eight experimental groups (Table 2.1). Four groups were fed an ethanol diet, and the other four groups consumed an isocaloric control dextrin diet. Intermittent hypoxia conditioning (IHC) and *N*-acetylcysteine (NAC) injections were initiated two weeks after the diets began. Four groups (two ethanol, two dextrin) underwent a 20 day IHC program. These groups were subdivided further into those receiving daily *ip* injections of 20 mg NAC in 0.4 ml 0.9% NaCl, or 0.4 ml NaCl vehicle. These injections were administered 2 hours before IHC or sham conditioning sessions. Table 2.1 presents the number of rats in each group.

Ethanol Diet Composition

Ethanol was administered in a liquid diet. Each liter of this diet contained 42 g pulverized casein, 6 g l-methionine , 2.1 g vitamin mixture, 7.3 g mineral mixture, 25 g sucrose, 3 g xanthum gum, 0.4 g choline chlorine, 1 g Celufil cellulose, 10.5 g corn oil

and 65 g ethanol, resulting in a 6.5% (w/v) ethanol suspension [5]. Control rats received an isocaloric liquid dextrin diet in place of the ethanol suspension. Fresh bottles containing 100 ml of diet were placed in individual cages at the beginning of each day for *ad libitum* consumption. The volumes of ethanol or dextrin control diet consumed by each rat were recorded daily. 24 hours after terminating the ethanol or dextrin diets, physical signs of ethanol withdrawal were evaluated (Jung et al; 2007) and the rats were sacrificed immediately afterward. Consumption of the ethanol diet resulted in circulating blood ethanol concentrations of 1.00±0.06 mg/ml (Jung et al; 2007).

Intermittent Hypoxia Conditioning

The IHC protocol illustrated in Table 2.2 was conducted during the final 20 days of the 35 day ethanol diet. The conditioning program consists of 5-8 hypoxia exposures each day with intervening 4 minute periods of re-oxygenation. Cages containing 3-4 rats were placed in a 267 L acrylic chamber for IHC or sham conditioning. Nitrogen gas (N_2) was introduced into the chamber to lower FIO_2 to 10.0-9.5%, as specified by the protocol FIO_2 in the chamber was monitored with a precision O_2 sensor (Alpha Omega Instruments model 2000). Alongside the IHC protocol, a sham protocol was administered to non-hypobaric rats by placing rats into a separate acrylic chamber and releasing compressed air into the chamber for the same duration as the IHC protocol. The ethanol and dextrin mixtures were removed from the cages following the final IHC or sham exposure and the rats experienced 24 hours of withdrawal. A withdrawal sign test

was administered prior to sacrifice by applying the scoring system summarized in Table 2.3.

Tissue Preparation and Protein Extraction

Rats were anesthetized with ketamine (100mg/ml, 60mg/kg) and xylazine (20 mg/ml, 10mg/kg), and then decapitated. Hearts were exposed by bilateral thoracotomy, excised, and quickly freeze-clamped with Wollenberger tongs pre-cooled in liquid N_2.

Frozen hearts were pulverized into a fine powder under liquid nitrogen using a precooled mortar and pestle. Powdered tissue was added to 1 ml phosphate buffer (mM, pH 7.2), homogenized with a Teflon piston for one minute, and centrifuged at 100,000 *g* at 4°C for 20 minutes. The supernatant was collected and the pellet re-extracted twice in phosphate buffer. The three supernatant fractions were combined and stored at -80°C. A Coomassie Blue-based assay kit (Pierce; Rockford, IL) was used to measure total protein concentration of each extract according to the method of Bradford [(2)]. Extract protein concentrations were used to normalize data from enzyme assays and immunoblots.

Colorimetric Enzyme Assays

Superoxide dismutase (SOD) kits were used to quantify the enzyme's activity in myocardial extracts. This kit uses a tetrazolium salt for the detection of $\cdot O_2^-$. Extracts (10µl) were loaded onto a 96-well plate in triplicate and combined with 200µl buffer. Xanthine oxidase (20µl) was added, and after an incubation period of 20 minutes the plate was read at 450 nm in a microplate spectrophotometer (PowerWave XS; Winooski,

VT). The triplicate absorbances were averaged for determination of SOD activity in each extract.

Glutathione peroxidase (GPx) activity was measured by coupling glutathione disulfide (GSSG) formation to the rate of disappearance of NADPH in the presence of excess glutathione reductase (GR). Samples were diluted in a Tris buffer containing EDTA, GSH, NADPH, and GR and incubated at 37°C for 5 minutes. Next, the mixture was placed in a cuvette, and its absorbance was measured in a UV-vis spectrophometer. After basline absorbance was monitored for 50 seconds at 337 nm, 10 µl of a second buffer containing cumene hydroperoxide (5mg/ml) was added to the cuvette at 50 seconds to trigger the GPx reaction, and the reaction was allowed to progress for 300 seconds. GPx activities were calculated for each sample from the rate of absorbance decline which was proportional to the rate of NADPH oxidation.

Total NOS activity was detected by use of a commercially available kit (Calbiochem; La Jolla, CA) that measures the extract concentrations of nitrate and nitrite using a 96-well plate. Triplicates of each sample were assayed by combining 40 µl of extract, 20 µl of assay buffer solution, 10 µl NADPH solution, and 10 µl nitrate reductase in each well. After incubating the plate at room temperature for 1 hour, 10 µl of various cofactors combined in solution and lactate dehydrogenase (LDH) were added to each well and allowed to incubate for 20 minutes at room temperature. Next, 50 µl each of Griess reagents 1 and 2 were added consecutively to the wells and then the color was allowed to develop for 10 minutes at room temperature. Sample absorbences were

measured at 450 nm, for calculation of NOS activities (mU NOS/g protein). The triplicate activities were averaged to determine NOS activity in each extract.

Immunoblot Analysis of Myocardial Protein Content

Heat shock protein 70 (Hsp70), inducible NOS (iNOS), endothelial NOS (eNOS), and phospho-eNOS contents were measured by immunoblotting. Positive controls were: heat shocked HeLa cell lysate (Stressgen Bioreagents; Ann Arbor, MI) for Hsp70, mouse macrophage+IFNγ/LPS (BD Biosciences Laboratories; USA) for iNOS, HeLa cell lysate (Stressgen Bioreagents; Ann Arbor, MI) for eNOS, and human endothelial cell lysate (BD Biosciences Laboratories; USA) for phospho-eNOS. Proteins were separated by electrophoresis in a 10% SDS-polyacrylamide gel at 100 volts and maximum current. Proteins were then transferred onto a nitrocellulose membrane at 350 mA of current and maximum voltage. Membranes were shaken overnight in nonfat milk at 4°C, washed the following morning in 20 ml of tris-buffered saline (TBS) for 5 min, and then washed twice for 10 min in 20 ml tween tris-buffered saline (TTBS) to remove the milk. Next, the respective primary antibody solution was applied to the membrane at a dilution of 1:1000 with gentle shaking for a minimum of two hours. Following primary antibody exposure, the membranes were washed once in 20 ml TBS and twice in 20 ml TTBS, and then incubated with the appropriate secondary antibody with gentle shaking for one hour. Membranes were washed again in 20 ml TBS for 5 minutes, and then 5 times in 50 ml of TTBS. The membranes were exposed to a commercially available enhanced chemiluminescent (ECL) solution to detect antigen:antibody complexes, and

photographed. Band densities were measured in a densitometer and normalized to α-actin band densities measured on the same membrane. The band densities were averaged by experimental group and used for statistical analysis.

Statistical Analyses

Data are expressed as means ± standard error of the mean (SEM), with the exception of ethanol and dextrin diet consumption data which are expressed as means ± standard deviation. Ethanol withdrawal scores, diet consumption measurements, enzyme activities, and immunoblot band densities were compared between groups with a one-way ANOVA followed by Student-Newman-Keuls comparison test and/or Tukey's test. In the event that a one-way ANOVA failed to detect statistical significance, an ANOVA based on ranks was conducted followed by Student-Newman-Keuls comparison test and/or Tukey's test. Statistical significance is assumed at $P<0.05$. Comparisons between two groups were conducted by t-tests followed by post hoc Mann-Whitney Rank Sum tests.

Table 2.1: Distribution of Experimental Groups

Diet	IHC	NAC	*n*	Abbreviation
Dextrin	-	-	5	D
Dextrin	+	-	8	DI
Dextrin	-	+	8	DN
Dextrin	+	+	8	DIN
Ethanol	-	-	8	E
Ethanol	+	-	8	EI
Ethanol	-	+	8	EN
Ethanol	+	+	8	EIN

Table 2.1: *Distribution of experimental groups.* D: dextrin, E: ethanol withdrawal; I: intermittent hypoxia conditioning, N: *N*-acetylcysteine treated.

Table 2.2: Intermittent Hypoxia Conditioning Protocol

Session	*FIO_2*	*Hypoxia (min)*	*Normoxia (min)*	*Cycles*	*Hypoxia Time (min)*	*Session Time (min)*
1	10%	5	4	5	25	41
2	10%	5	4	6	30	50
3	10%	5	4	7	35	59
4	10%	5	4	8	40	68
5	10%	5	4	8	40	68
6	9.5%	6	4	7	42	66
7	9.5%	6	4	8	48	76
8	9.5%	6	4	8	48	76
9	9.5%	7	4	7	49	73
10	9.5%	8	4	7	56	80
11	9.5%	10	4	7	70	94
12	9.5%	10	4	7	70	94
13	9.5%	10	4	7	70	94
14	9.5%	10	4	7	70	94
15	9.5%	10	4	7	70	94
16	9.5%	10	4	7	70	94
17	9.5%	10	4	7	70	94
18	9.5%	10	4	7	70	94
19	9.5%	10	4	7	70	94
20	9.5%	10	4	7	70	94

Table 2.3: Ethanol Withdrawal Signs Scoring System

Withdrawal Signs	**Score**
Vocalization, Urination, and Defecation • One point each	0-3
Caudal Posture • 0 points for limp or normal tail. • 1 point for a stiff tail that curls around finger. • 2 points for a stiff tail, curls around finger, and stays elevated after release • 3 points for spontaneous, abnormal posture of the tail beyond stiffness, curling around finger, and remaining elevated after release.	0-3
Tremor • 0 points for no sign of tremor. • 1 point for mild tremor in one portion of the body (i.e., face). • 2 points for generalized, occasional tremors. • 3 points for constant generalized tremors.	0-3
Startle—reaction to a sudden loud noise (i.e. hand clap) • 0 points for no startle. • 1 point for a twitch. • 2 points for jumping or freezing. • 3 points for an exaggerated jump or freeze.	0-3
Handler induced convulsion	1
Spontaneous seizure	2
Death	10

CHAPTER 3

Results

Ethanol Withdrawal Scores and Consumption

Dextrin consumption rates were comparable in the IHC and non-hypoxic groups (Figure 3.1) between days 11 and 18 of the protocol in control dextrin rats (D: 94 ml±7 ml) when compared to dextrin/IHC rats (DI: 98 ml± 4 ml). Rats in the comparable ethanol groups (Figure 3.2) did not show any significant differences in consumption of the ethanol-laden diet (E: 77±12 ml; EI: 77±10 ml). However, ethanol fed rats consumed 18% less diet mass than their dextrin-fed counterparts ($P<0.05$; Figure 3.3). Thus, the IHC program per se did not alter intake of either the dextrin or ethanol diets.

Behavioral and cognitive signs of ethanol withdrawal reported by Jung *et al.* utilizing the scoring system presented in Table 2.3 indicate that IHC treatment result in significant neurological protection against the ravages of ethanol withdrawal[6]. Dextrin-consuming rats exhibited moderate dysfunction compared to the severe dysfunction that was exhibited by their ethanol withdrawn counterparts [6]. However, when the ethanol withdrawn rats were exposed to IHC, there was a remarkable suppression of cognitive dysfunction and neurological manifestations of ethanol withdrawal[6].

Superoxide Dismutase and Glutathione Peroxidase

In dextrin-fed rats IHC lowered SOD activity by 59%, from 63±10 to 26±4 U/g protein ($P<0.05$). Documented control SOD activity in male rats is 73±6 U/g protein according to Seiva *et al.* [24] Ethanol withdrawal produces a three-fold decrease in SOD activity when compared to dextrin rats (E: 24±1 U/g protein) (Figure3.4). Interestingly, the IHC (EI: 49±9 U/g protein) and NAC (EN: 45±8 U/g protein) treatments given separately partially protected SOD activity in ethanol withdrawn rats (Figure 3.5). Within dextrin groups, the NAC administration also slightly decreased SOD activity (DN: 43±11 U/g protein), and when NAC and IHC treatments were combined SOD activity continued to be suppressed (DIN: 34±4 U/g protein) (Figure 3.5). When both treatments were combined in the ethanol withdrawal groups, SOD activity was completely protected from ethanol intoxication-withdrawal (EIN: 64±7 U/g protein) and was essentially identical to the dextrin control value obtained in this study (Figure 3.5). It appears that IHC and NAC each have protective effects on SOD activity in the face of ethanol intoxication-withdrawal, and combining the two treatments is more effective than either treatment alone.

No statistically significant glutathione peroxidase differences were detected within dextrin and ethanol withdrawn rats (Figure 3.6). Zang *et al.* reports control GPx levels at .120 U/mg protein, which is confirmed by this study [33]. Upon further analysis, student's t-test was performed and significant differences between the dextrin/NAC, dextrin control, and ethanol/NAC groups ($P=0.01$ EN vs DN, $P=0.04$ D vs DN) was

found indicating that ethanol intoxication-withdrawal could partially inactivate GPx when combined with NAC (Figure 3.7).

Total NOS Activity and Immunoblots of NOS Isoenzymes

There were no statistically significant differences in NOS activities among the dextrin and ethanol intoxication-withdrawal groups, despite the implementation of an IHC regimen (Figure 3.8). Upon treatment with NAC, ethanol withdrawn and dextrin groups showed opposite responses. Dextrin/NAC groups increased their concentration of NOS and ethanol withdrawn/NAC groups decreased their NOS activity (DN: 270±52 mU /g protein; EN: 140±16 mU /g protein) (Figure 3.9). Myocardial contents of the iNOS and constitutive eNOS isoenzymes were examined by immunoblot. Inducible NOS was undetectable in any group. In contrast, substantial eNOS content was evident in all 8 groups, although no treatment effects were detected (Figure 3.10A and 3.10B). Phosphorylated eNOS isoforms, i.e. P-ser^{1117} and P-thr^{495}, also were studied by immunoblot. No discernable bands were detected for P-ser^{1117} or P-thr^{495} (data not shown). Thus, neither ethanol intoxication-withdrawal, IHC, nor NAC materially affected NOS activity, myocardial contents of NOS isoforms, or eNOS phosphorylation.

Heat Shock Protein 70

Immunoblotting revealed distinct bands for Hsp70 in all 8 groups (Figure 3.11A). However, there were no statistically significant differences in Hsp70 contents among the treatments (Figure 3.11B).

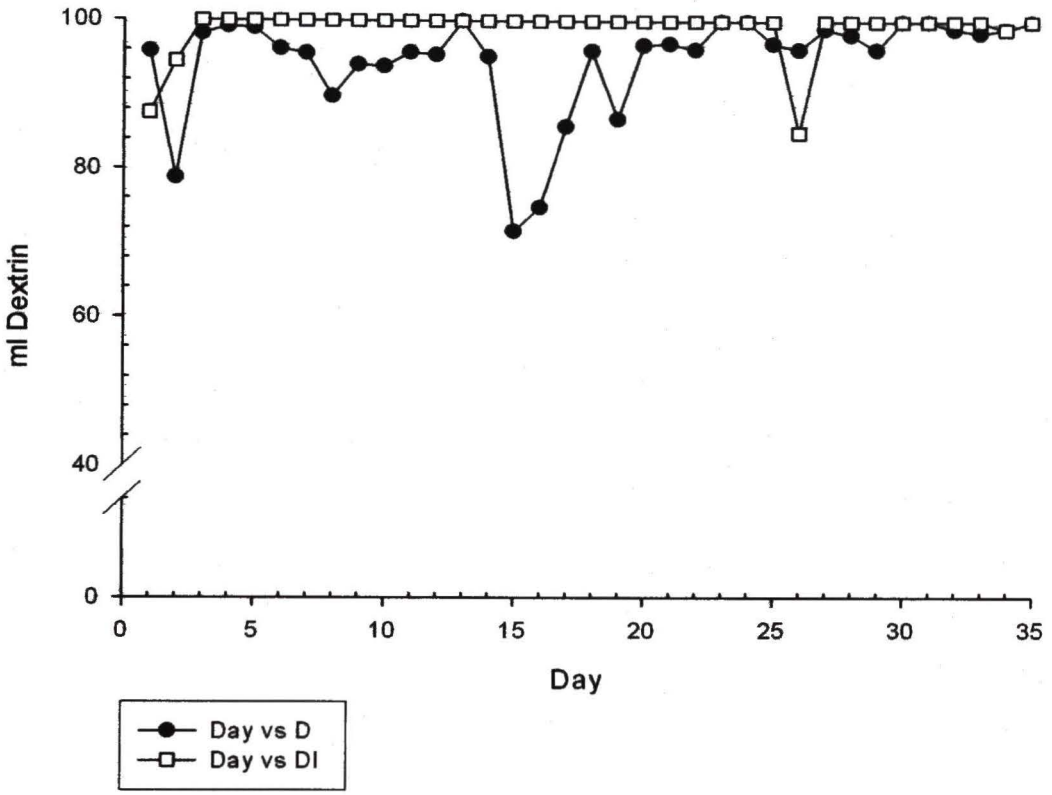


Figure 3.1: *Diet consumption of dextrin rats and dextrin/IHC rats.* Diet consumption was measured daily prior to IHC or sham treatments. D: dextrin (n=5); DI: dextrin/IHC (n=6). Values are means of each group's daily consumption.

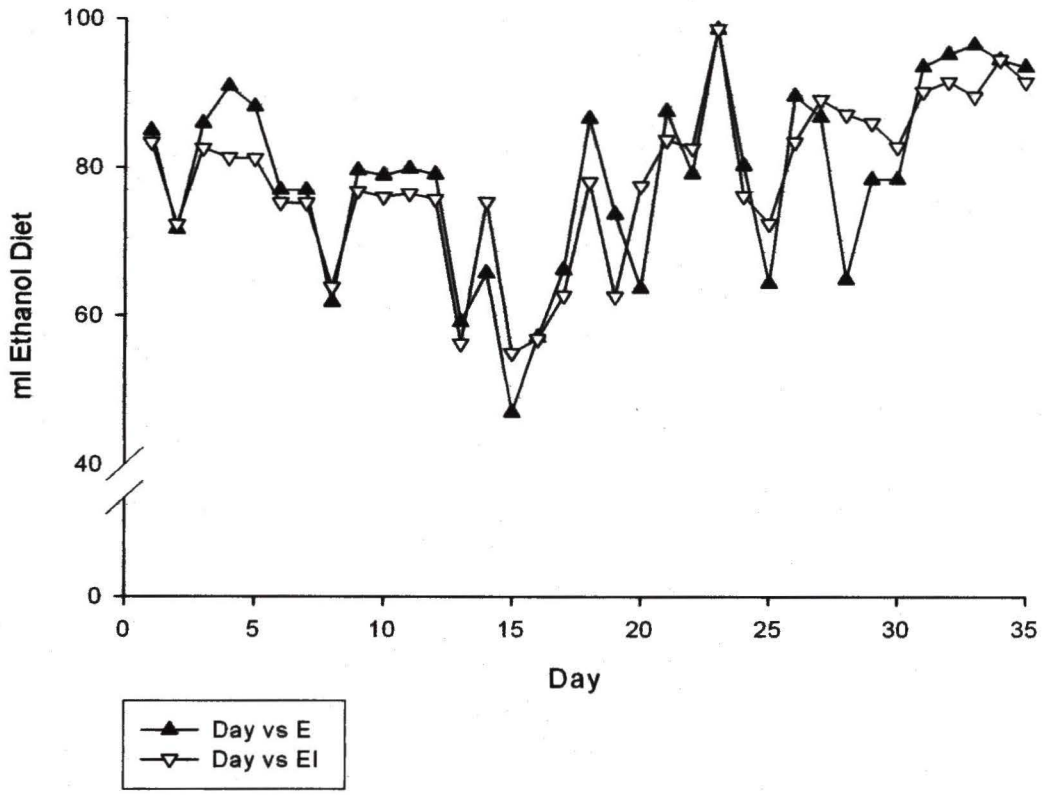


Figure 3.2: *Daily ethanol diet consumption by non-hypobaric and IHC rats.* E: ethanol (n=7); EI: ethanol/IHC (n=8). No statistically significant differences were detected between these two groups.

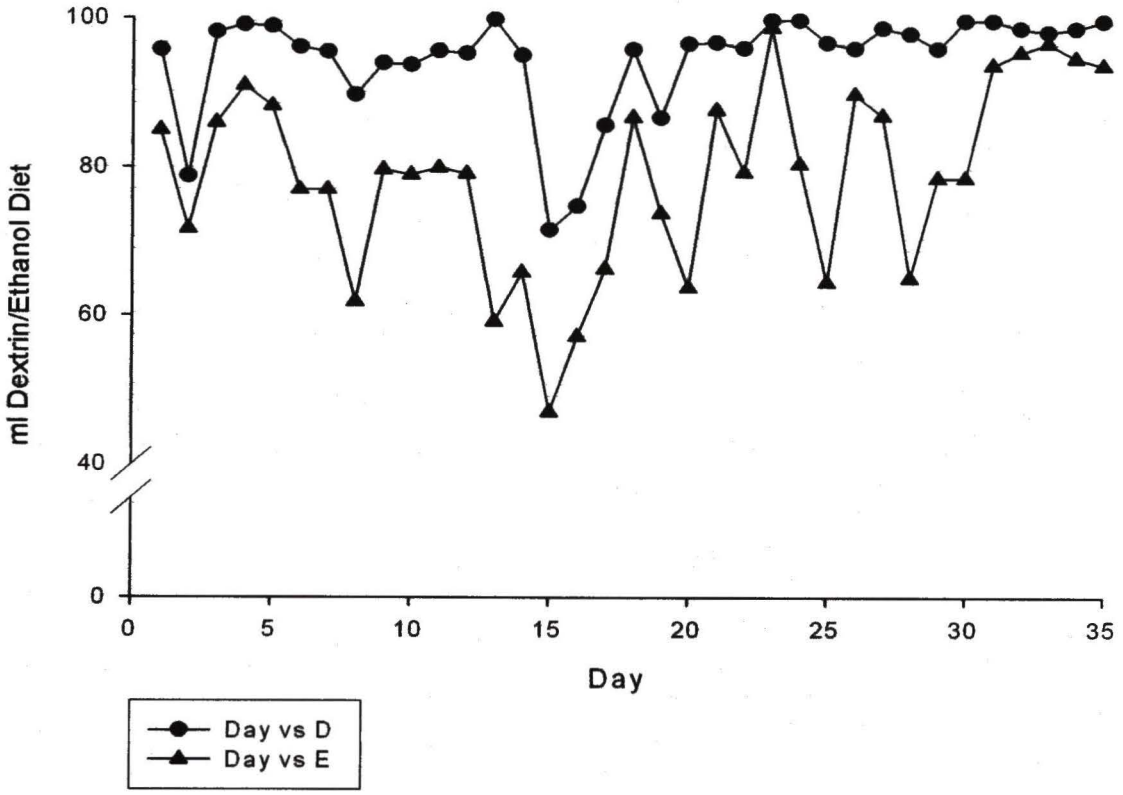


Figure 3.3: *Daily consumption of dextrin rats versus ethanol diets by non-hypobaric rats.* D: dextrin (n=5); E: ethanol (n=7). Plotted values are means of each experimental group's daily consumption.

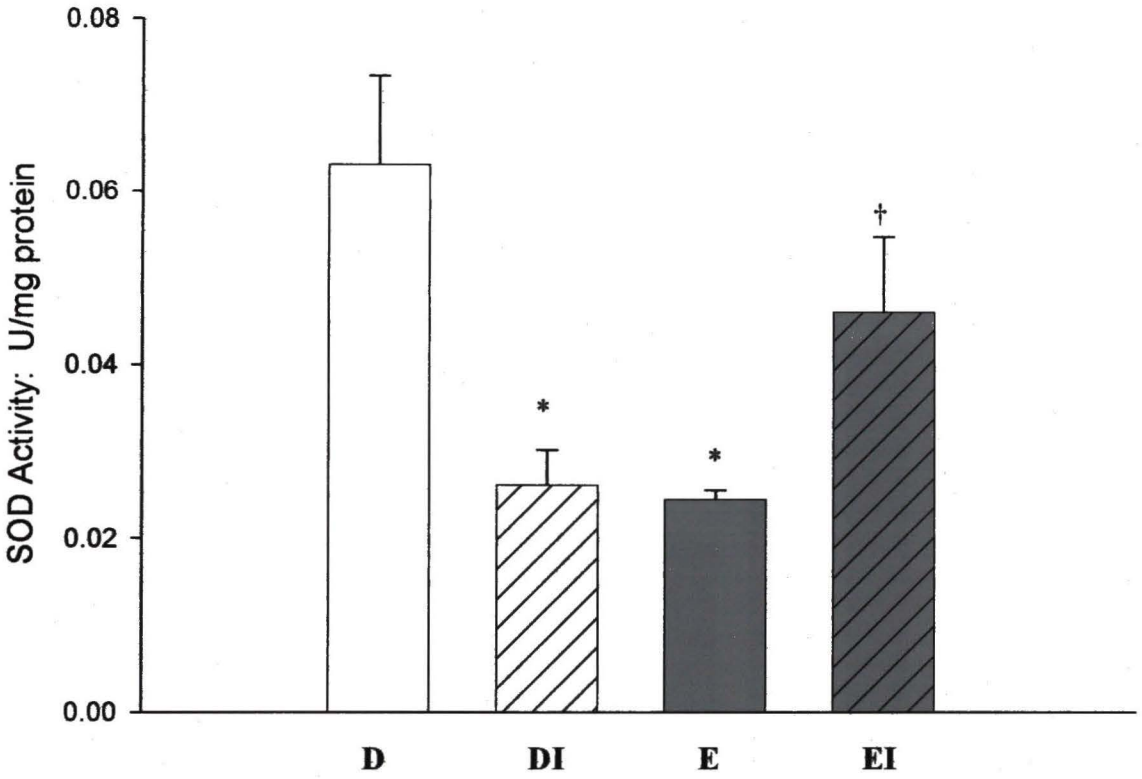


Figure 3.4: *Myocardial superoxide dismutase (SOD) activities in dextrin and ethanol rats.* SOD was measured in triplicate by colorimetric assays. D: dextrin, E: ethanol intoxication-withdrawal, I: intermittent hypoxia conditioned. *P<0.05 D vs E, D vs DI, †P<0.05 EI vs E.

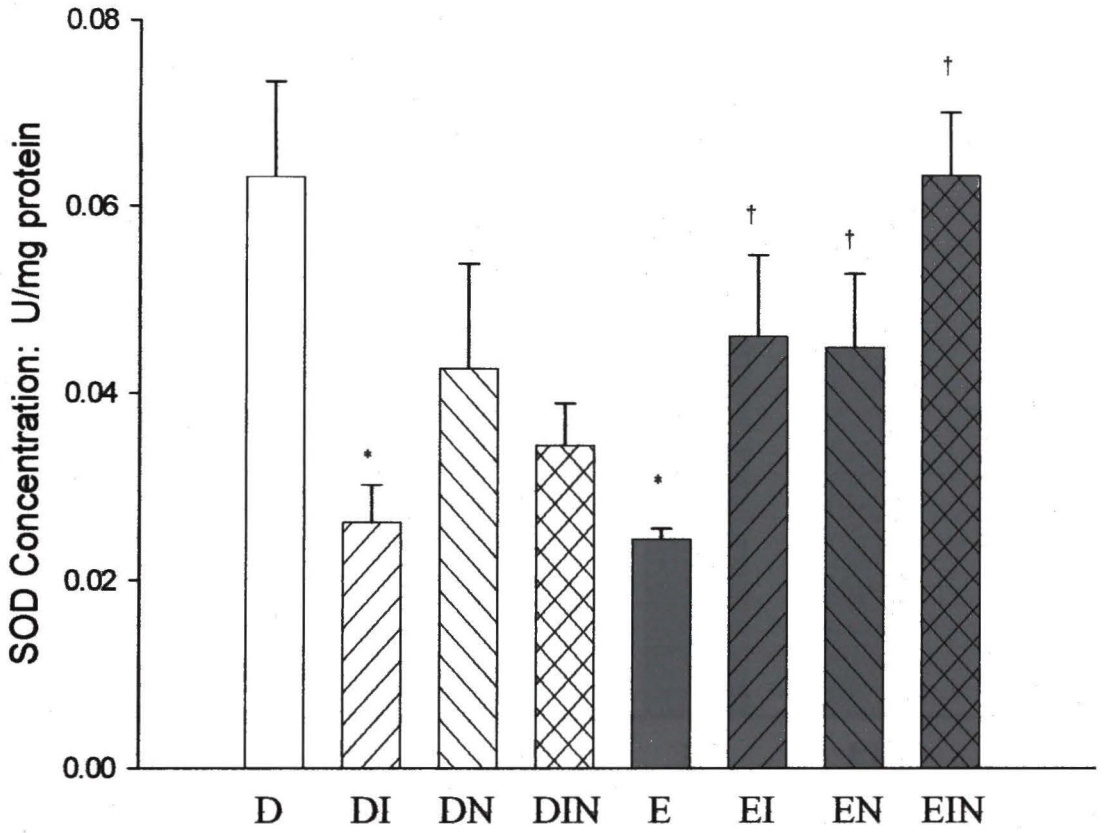


Figure 3.5: *Myocardial superoxide dismutase (SOD) activities.* SOD was measured in triplicate by colorimetric assays. D: dextrin, E: ethanol intoxication-withdrawal, I: intermittent hypoxia conditioned, N: *N*-acetylcysteine treated. *P<0.05, D vs E, D vs DI. †P<0.05, EIN vs E, EI vs E, EN vs E.

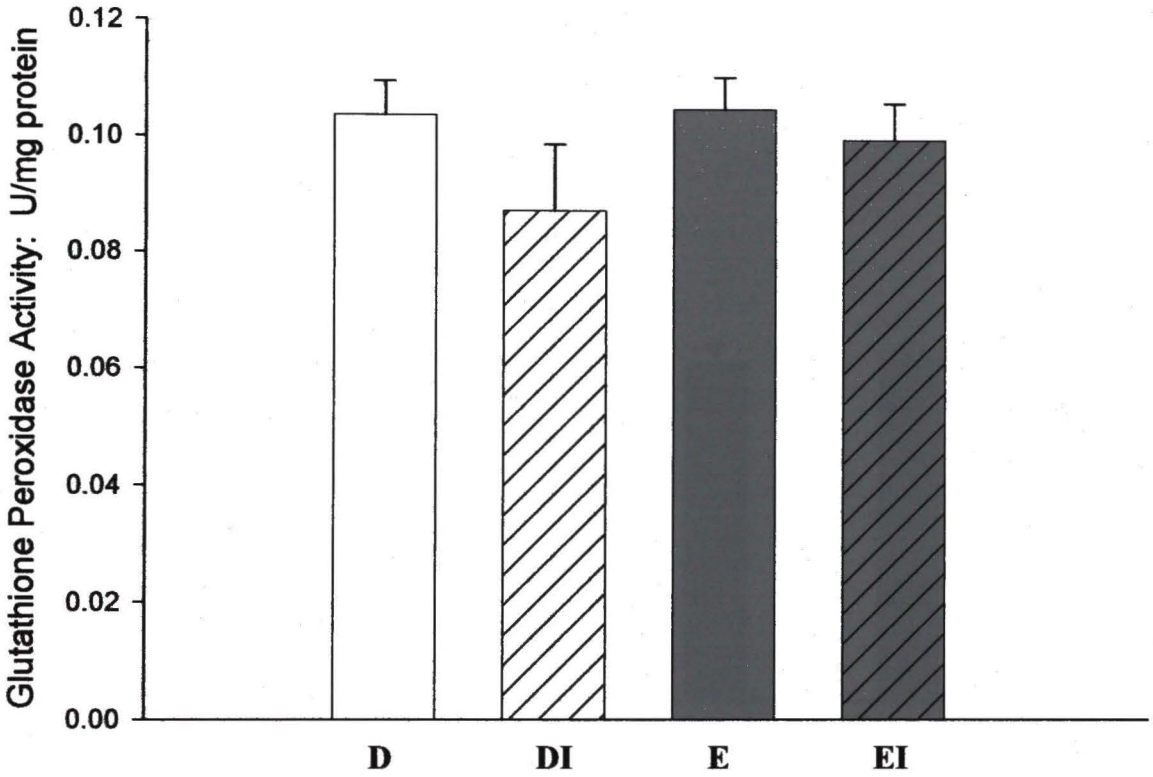


Figure 3.6: *Myocardial glutathione peroxidase activity in dextrin and ethanol rats.* GPx was measured via UV-spectrophotometry. D: dextrin, E: ethanol intoxication-withdrawal, I: intermittent hypoxia conditioned. There were no statically significant differences detected.

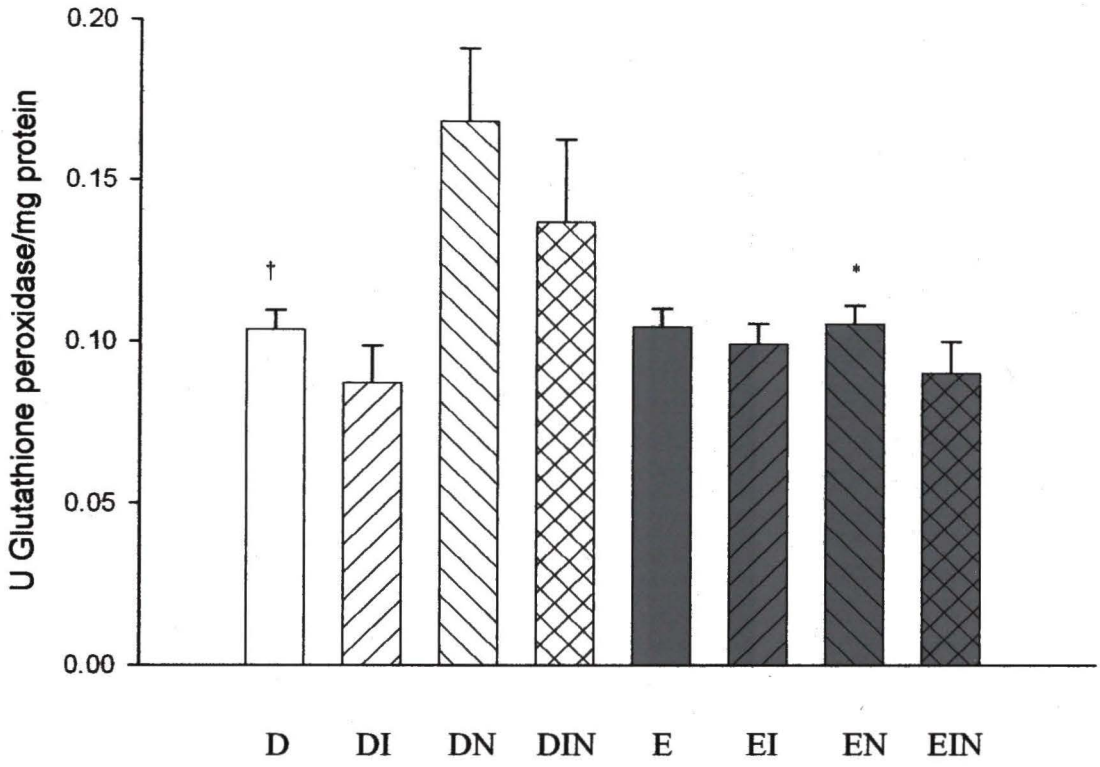


Figure 3.7: *Myocardial glutathione peroxidase activity.* GPx was measured via UV-spectrophotometry. D: dextrin, E: ethanol intoxication-withdrawn, I: intermittent hypoxia conditioned, N: *N*-acetylcysteine treated. When compared by t-test, *P=0.01 EN vs DN, †P=0.04 D vs DN.

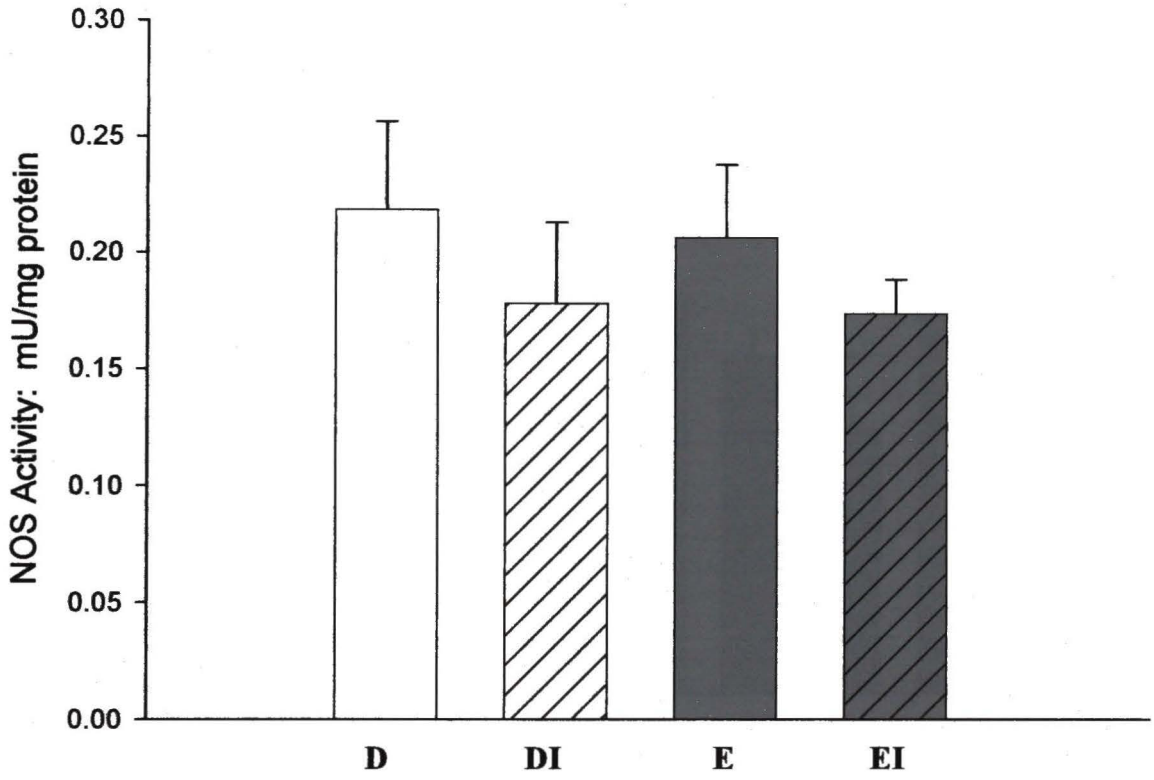


Figure 3.8: *Total myocardial NOS activity in dextrin and ethanol rats.* D: dextrin, E: ethanol intoxication-withdrawal, I: intermittent hypoxia conditioned. There were no statically significant differences detected.

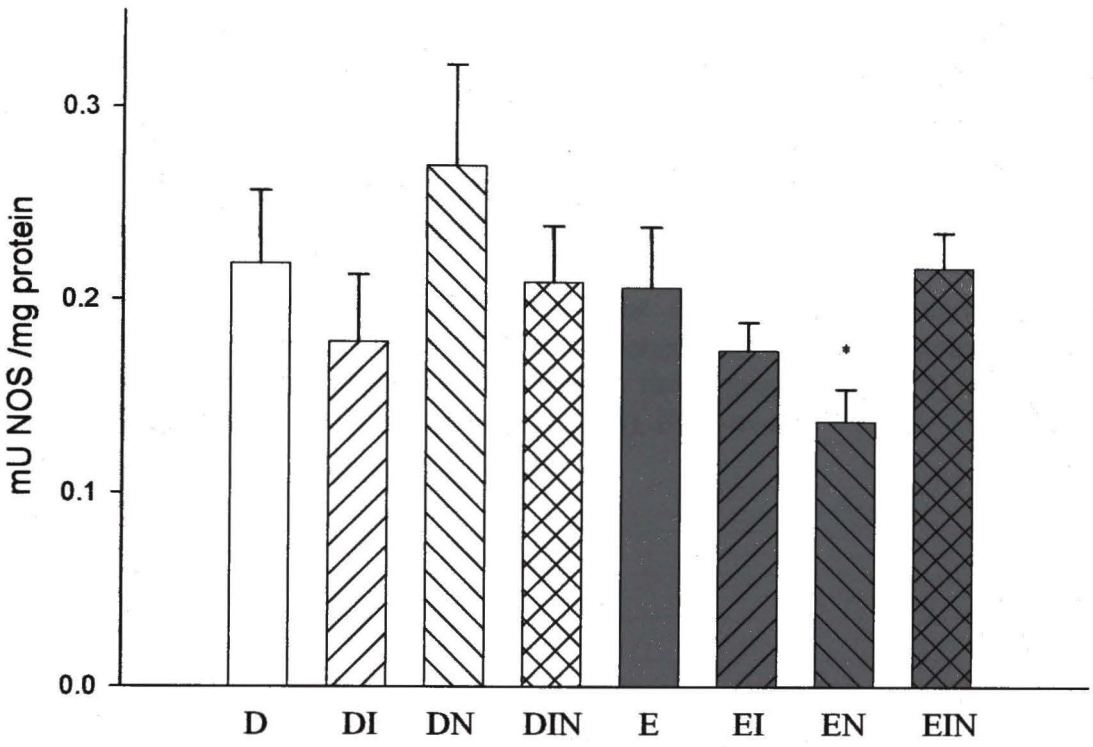


Figure 3.9: *Total myocardial NOS activity.* D: dextrin, E: ethanol intoxication-withdrawal, I: intermittent hypoxia conditioned, N: *N*-acetylcysteine. There were no statistically significant differences between the eight experimental groups. When compared by a t-test, *P=0.01 DN vs EN.

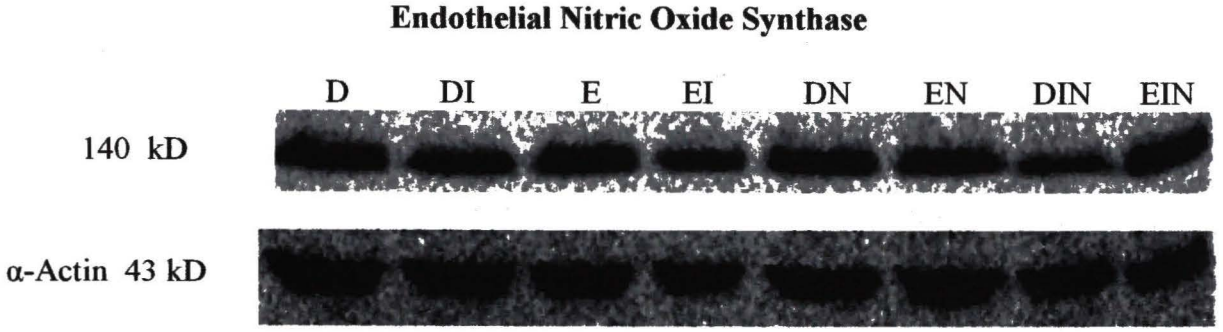


Figure 3.10A: *Endothelial NOS immunoblot.* D: dextrin; E: ethanol intoxication-withdrawal; I: intermittent hypoxia conditioned; N: *N*-acetylcysteine treated. No significant differences were detected among the 8 groups. D (n=4), DI (n=4), DN (n=4), DIN (n=4), E (n=4), EI (n=4), EN (n=4), EIN (n=4).

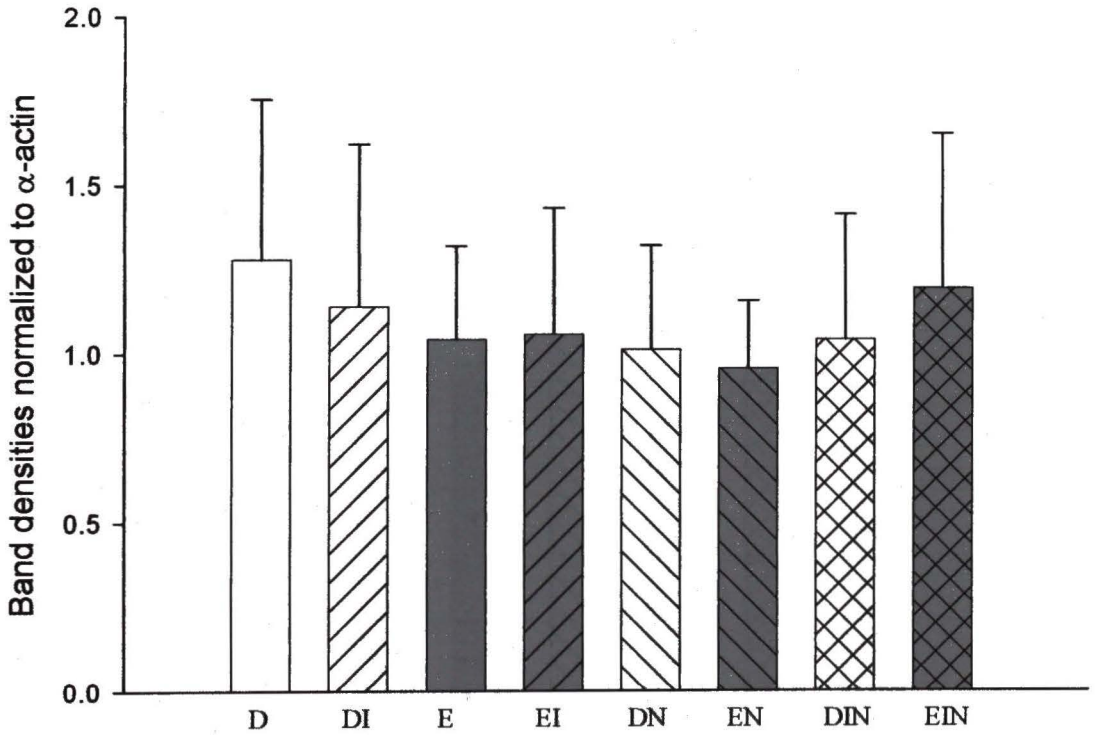


Figure 3.10B: *Endothelial NOS (eNOS) content.* All bands were analyzed by densitometry and normalized to α-actin. D: dextrin; E: ethanol intoxication-withdrawal; I: intermittent hypoxia conditioned; N: *N*-acetylcysteine treated. No significant differences were detected among the 8 groups. D (n=4), DI (n=4), DN (n=4), DIN (n=4), E (n=4), EI (n=4), EN (n=4), EIN (n=4). The same experiments were used in Figure 3.9.

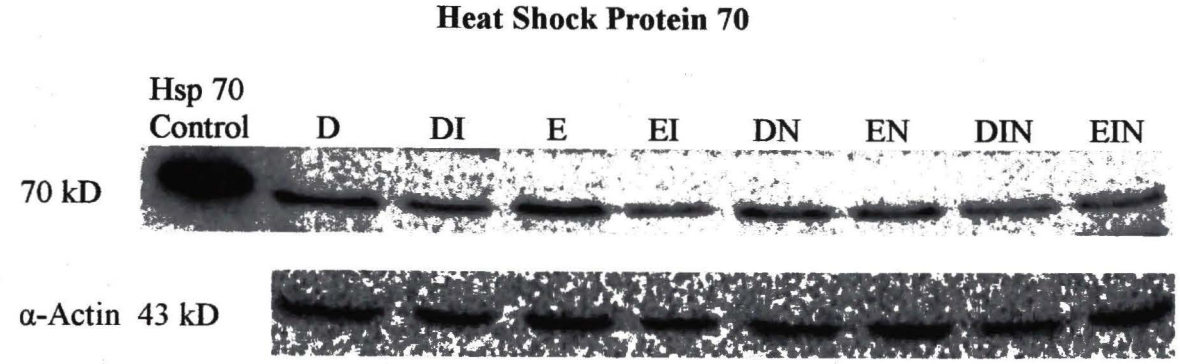


Figure 3.11A: *Heat shock protein 70 immunoblot.* D: dextrin; E: ethanol intoxication-withdrawal; I: intermittent hypoxia conditioned; N: *N*-acetylcysteine treated. No significant differences were detected among the 8 groups.

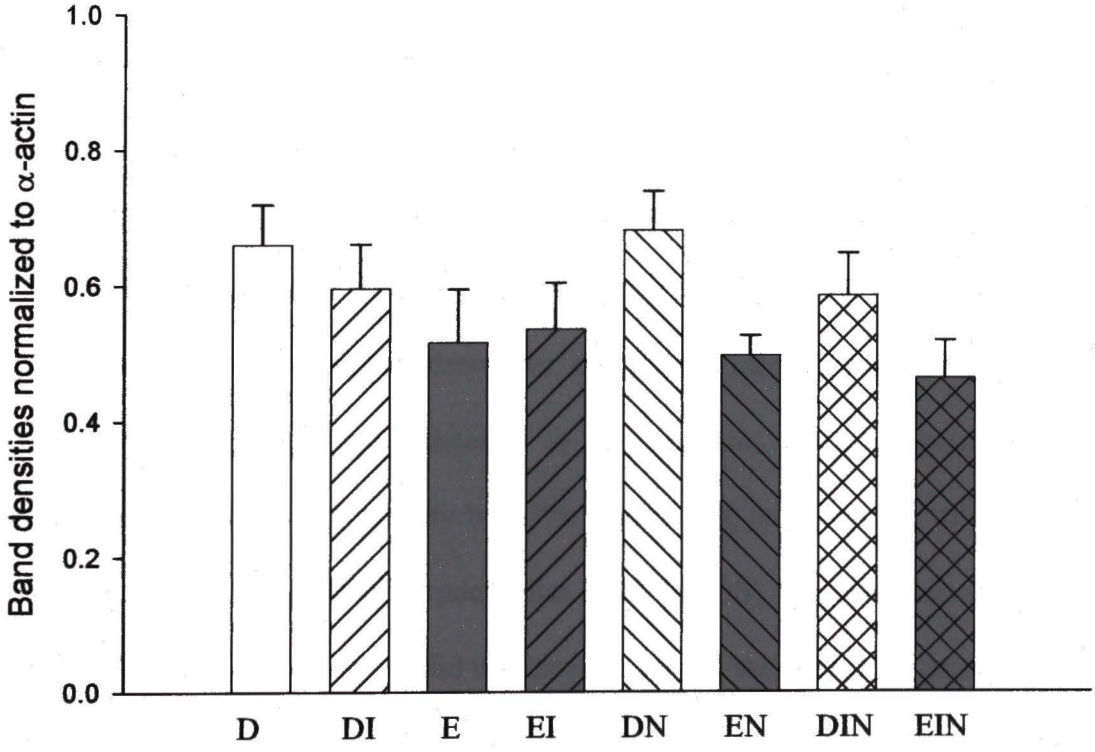


Figure 3.11B: *Heat shock protein 70 (Hsp70) content.* Results of densitometry were normalized to α-actin. D: dextrin; E: ethanol intoxication-withdrawal; I: intermittent hypoxia conditioned; N: *N*-acetylcysteine treated. No significant differences were detected among the 8 groups.

CHAPTER 4

Discussion and Conclusions

This study was conducted to determine if ethanol intoxication and withdrawal have the same harmful effects on the heart as they do on the brain, whether intermittent hypoxia conditioning (IHC) can induce antioxidant enzymes and proteins that blunt the damaging effects of ethanol intoxication and withdrawal, and if the administration of *N*-acetylcysteine (NAC) a broad spectrum antioxidant, interferes with IHC induction of beneficial proteins in a rat model of ethanol intoxication and withdrawal. Ethanol intoxication-withdrawal sharply lowered myocardial activity of the key antioxidant enzyme superoxide dismutatse (SOD). When administered separately, IHC and NAC partially protected SOD activity, and when the treatments were combined, SOD activity was completely protected from ethanol intoxication-withdrawal. Glutathione peroxidase (GPx) activities in ethanol withdrawn vs. dextrin-fed rats were unaffected by IHC and NAC. However, when administered to ethanol-free, dextrin-consuming rats, the antioxidant increased GPx activity by 43%. There were no detectable differences in heat shock protein 70 (Hsp70) and endothelial NOS (eNOS) contents among the treatment groups. Thus, the IHC protocol did not increase any of the beneficial proteins of interest, although it did partially protect SOD activity from ethanol intoxication-withdrawal.

Impact of Ethanol Withdrawal and IHC on Myocardial Antioxidant Enzymes

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are endogenous antioxidant enzymes that function to protect myocardium from ROS generated as by products of oxidative metabolism. Hepatic ethanol metabolism releases into the circulation ROS [31] which reduces the activities of GPx and SOD in myocardial tissue [23]. In this study, ethanol intoxication-withdrawal decreased myocardial SOD activity. This study demonstrated, for the first time, IHC's ability to protect the key antioxidant enzyme SOD in ethanol withdrawn rats. NAC is a potent, promiscuous antioxidant that scavenges a variety of ROS. In ethanol withdrawn rats, SOD was partially protected by IHC and NAC treatments given separately, and when IHC and NAC treatments were combined, protection of SOD activity was essentially complete. A 20-day program of IHC evokes cardioprotection against ischemic injury in dogs by ROS signaling generated during the hypoxia-reoxygenation cycles [12]. IHC dramatically lowered myocardial infarction and ventricular tachyarrhythmia produced by coronary artery occlusion/reperfusion in dogs [20]. However, dietary supplementation with NAC abrogated IHC induced cardioprotection in the dog model. In rats undergoing ethanol intoxication-withdrawal ± IHC, NAC produced additive protection of SOD when combined with IHC, although it had no effect on GPx activity or Hsp70 and eNOS contents. The finding that NAC not only did not abrogate but actually enhanced IHC protection of SOD activity is surprising in light of the recent finding in our laboratory that NAC blunted anti-ischemic cardioprotection in dogs [12]. This divergence indicates either that SOD activity is unrelated to myocardial susceptibility to ischemia-reperfusion,

or that ethanol intoxication-withdrawal is a fundamentally different stress than ischemia-reperfusion.

Ethanol depletes mitochondrial glutathione (GSH) by interfering with the carrier mechanism responsible for transporting GSH from the cytosol across the inner mitochondrial membrane [31]. This GSH-depleting effect of ethanol deprives mitochondrial glutathione peroxidase of its source of reducing power to neutralize mitochondrial ROS. There was no significant difference in GPx activities between the ethanol withdrawn and dextrin control rats in this study. It should be kept in mind, however, that GPx activities were measured under ideal conditions in a buffer, in the presence of non-limiting concentrations of GSH and other reactants. These activities might not reflect impairment of the reaction *in situ* due to limited GSH supply. Moreover, statistically significant increases in GPx were found in dextrin groups treated with NAC. Recent research suggests that GPx activity recovers quickly (within 17 hours) after ethanol is withdrawn in male rats [34]. In this study, rats were withdrawn from ethanol for 24 hours, which could conceivably allow GPx to recover. The increased GPx activity in dextrin/NAC rats *in vivo* may be attributed to the sulfhydryl content of NAC and its conversion to L-cysteine, a precursor for the synthesis of GSH [9]. However, because GPx was assayed in the presence of non-limiting GSH concentrations, more investigation into the *in vivo* concentrations of GSH is necessary to draw a definitive conclusion.

Impact of Ethanol Withdrawal and IHC on Myocardial Heat Shock Protein 70

Another potentially beneficial protein is heat shock protein 70 (Hsp70), which facilitates protein folding and helps target damaged proteins for lysosomal degradation [25]. Hsp70 was detectable by immunoblot in all 8 experimental groups; however, there were no statistically significant differences in the band densities among the groups. The lack of increased expression in the IHC groups suggests that the total duration or intensity of the hypoxia stimulus is not enough to modify Hsp70 expression, at least in rat. Previous studies by Meerson *et al.* demonstrated that IHC increases myocardial Hsp70 content in rats, however the IHC protocol of Meerson *et al.* is longer than the IHC protocol used in the present study [16, 17]. However, Mohan *et al.* [17] reported that the expression of Hsp70 in guinea pig myocardium was reduced specifically in the atria, and remained unchanged in the ventricles following completion of a 21-day IHC program [17]. Zhong *et al.* [35] reported that increasing duration of hypoxia exposure from 14 to 42 days (6 hours/day, 11.1% FIO_2) progressively increases Hsp70 expression in rat myocardium, and was cardioprotective against ischemia-reperfusion imposed by left anterior descending coronary artery occlusion-reperfusion [35]. This group also reported that the elevated Hsp70 remained detectable 2 weeks after the rats completed the hypoxia protocol [35]. The equivocality in these reports combined with our finding that Hsp70 contents were unaltered by ethanol withdrawal, IHC or NAC, indicates that the duration of hypoxia conditioning, and the exact nature of the stressor (broad oxidative stress, e.g. ethanol intoxication-withdrawal, vs. localized stress to ischemic tissue) may affect the time course and intensity of Hsp70 expression. Global stressors such as ethanol induced

oxidative stress may require increased IHC duration and/or intensity to confer appreciable benefit to the myocardium in the form of increased Hsp70 expression.

Impact of Ethanol Withdrawal and IHC on Myocardial NOS Isoforms

Nitric oxide synthase (NOS) activity was unaffected by IHC in this study. Indeed, the rats had no discernable iNOS content, which could be attributed to the moderate intensity of the IHC protocol or the removal of ethanol from the diet. In contrast, potentially beneficial eNOS expression was evident in all 8 groups. Previous studies in this laboratory have reported increased eNOS and decreased iNOS in the canine myocardium upon completion of the same 20 day IHC protocol applied to rats in this study [20]. Ethanol intoxication elicits an inflammatory response which induces iNOS and generates NO as a byproduct of inflammation [23, 37]. Either the IHC suppressed iNOS activity in the current study, or the inflammatory response induced by ethanol intoxication rapidly subsided upon the removal of ethanol. More investigation is needed to determine the true cause of the absence of iNOS from rat myocardium.

Conclusions

Ethanol intoxication is responsible for non-ischemic dilated cardiomyopathy, which accounts for 21-36% of all congestive heart failure cases, and is currently the third leading cause of preventable death in the United States [27]. Despite many efforts, the exact mechanism of ethanol's toxic effect on the myocardium remains to be elucidated. IHC and NAC protection against ethanol intoxication and withdrawal proved to be highly

selective. Among the enzymes measured, superoxide dismutase (SOD) activity was the only one affected by ethanol withdrawal. It was partially protected by NAC and IHC, and it was completely restored upon the combination of NAC and IHC. The remaining measurements differ from reports in myocardium of ethanol intoxicated rats. According to the literature, GPx is decreased in intoxication but was unaffected by withdrawal in this study. Likewise, Hsp70 is reportedly increased in intoxication, but was unaffected by withdrawal. Intoxication also reportedly increases myocardial iNOS which produces excess NO; however iNOS was undetectable in ethanol intoxication-withdrawal myocardium during this study.

The diversity of cardioprotection between animal models suggests that the type of oxidative stress that inflicts injury is important when determining the effectiveness of IHC and NAC treatments. The systemic oxidative stress produced by ethanol intoxication-withdrawal versus the targeted oxidative stress that ischemia-reperfusion induces could be one of the major factors influencing the apparent divergence of the results of the current investigation from those of previous IHC studies.

Future Directions

Due to the profound myocardial effect that ethanol metabolism has, an investigation into the cardiac performance during ethanol withdrawal using isolated perfused hearts would be an area of interest. In particular, imposition of global ischemia-reperfusion on hearts isolated from the 8 groups of rats would enable assessment of the protective vs. detrimental effects of the different treatments on myocardial susceptibility

to ischemia-reperfusion. Moreover, the ability of ethanol withdrawn hearts to withstand ischemic injury, and whether or not IHC and NAC administration confers myocardial protection against ischemic injury, are potential areas of expansion for this lab.

The effects intoxication without withdrawal and withdrawal periods greater than 24 hours are also interesting expansions for this project. Analysis of enzyme systems and other beneficial myocardial proteins during ethanol intoxication, prior to withdrawal, would further elucidate the effects that 24 hours of withdrawal has on the myocardium. Moreover, extending the withdrawal period to 48 hours provides the opportunity to determine the recovery rate of antioxidant defenses and myocardial proteins from the damage inflicted upon ethanol intoxication.

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