Effect of Chronic Ethanol Administration on Hepatic eNOS Activity
and its Association with Caveolin-1 and Calmodulin in Female Rats

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Running head:
Alcohol modulation of eNOS activity in rat liver

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Abstract

Although chronic and excessive alcohol consumption is associated with liver disease, the mechanism of alcoholic liver injury is still not clear. Whether reduced hepatic production of nitric oxide (NO), which is evident in models of liver injury, is associated with alcohol-induced liver injury has not been investigated. We measured NOS activity in the liver of pair-fed rats receiving liquid diet with or without alcohol (3%, v/v) for 12 weeks. Compared with control rats, hepatic NOS activity was significantly reduced in alcohol-treated rats along with the evidence of liver injury. Interestingly, there was no difference in the hepatic expression of eNOS between ethanol-fed and pair-fed rats. We then tested the hypothesis that an imbalance between the binding of eNOS with inhibitory and stimulatory proteins may underlie the reduced activity of eNOS because eNOS catalytic activity is regulated partly through dynamic interactions with the inhibitory protein, caveolin-1, and the stimulatory protein calmodulin. We found that hepatic caveolin-1 was markedly increased in alcohol-treated rats compared with control rats while calmodulin remained unaltered. The binding of caveolin-1 and calmodulin with the eNOS was increased and decreased, respectively, in alcohol-treated rats. Our results suggest that chronic alcohol intake attenuates hepatic eNOS activity by increasing the expression of the inhibitory protein caveolin-1, and enhancing its binding with eNOS.
KEY WORDS: endothelial nitric oxide synthase; alcoholic liver injury; caveolin-1; calmodulin; protein interactions
Introduction

It is well established that chronic and excessive alcohol intake is associated with liver disease (13, 36). Alcoholic liver disease is categorized mainly in three major pathologic settings: alcoholic steatosis (alcoholic fatty liver), alcoholic hepatitis, and alcoholic fibrosis and cirrhosis (13, 36). The understanding of how alcohol damages the liver has expanded substantially over the last decade. Many factors have been implicated in alcohol-induced liver injury. However, the mechanisms underlying the alcoholic liver disease are still unclear (1, 7, 26, 36).

In the liver, nitric oxide (NO) synthesized by the endothelial NO synthase (eNOS) isoform, regulates hepatic blood flow and vascular resistance (30, 31). Numerous studies have shown that the production of NO is abnormal in injured liver (18, 27, 32, 37). Shah et al. have shown that in response to incremental increases in blood flow, cirrhotic animals produced significantly less NO than control animals because of reduced eNOS activity (32). Similarly, NO release from sinusoidal endothelial cells is reduced in liver cirrhosis, owing to impaired function of endothelial cell NOS (31, 37). Further, these data suggest that at the cellular level, abnormal production of NO in injured liver contributes to elevated intra-hepatic resistance and portal hypertension (27, 32, 37). However, whether reduced hepatic production of NO and NOS activity are associated with alcohol-induced liver injury remains unclear. There are, however, several indirect lines of evidence that support this possibility. Oshita et al. showed that alcohol increases portal pressure, which was associated with hepatic hypoxia and necrosis (25). Further, Kimura et al found that the production of NO in Kupffer cell was diminished by chronic alcohol administration (16). NO was expected to counteract the increase in hepatic vascular resistance elicited by
ethanol and, therefore, reduce liver injury, which highlights a protective role for NO in alcohol-induced liver disease (25). In support of this notion, treatment with a NOS inhibitor (L-NAME) increased the severity of liver injury by alcohol, whereas L-arginine supplementation completely prevented liver injury by alcohol (22). Most, if not all, of the reported studies were undertaken in male rats and none of the reported studies investigated the cellular mechanism implicated in alcohol-evoked reduction in hepatic NO production.

The main objective of the present study was to test the hypothesis that alcohol reduces NO production by inhibiting the expression and/or activity of hepatic eNOS in female rats. To test this hypothesis, we designed the present study to determine (a) whether chronic alcohol exposure reduces the hepatic NOS activity (NO production) in our model, (b) if so, whether the reduced NOS activity is due to reduced expression of eNOS or due to abnormal association of eNOS with its regulatory proteins, caveolin-1 and/or calmodulin. We hypothesized that increased hepatic eNOS association with the inhibitory protein caveolin-1 or reduced association with the stimulatory protein calmodulin underlie ethanol-induced inhibition of eNOS catalytic activity. To this end, we performed a series of studies on the livers of rats that received liquid diet with or without alcohol (3%, v/v) for 12 weeks, as an experimental model to test this hypothesis.
Materials and Methods

Fourteen female Sprague-Dawley rats were used in the present study. The rats were obtained from Charles River (Raleigh, NC.) at their 11th-12th week of age (180-200g). The rats were randomly separated into two groups: (i) Ethanol treatment group: rats were fed the alcohol-containing liquid diet (3% v/v ethanol; Midwest Grain Products Co., Westoo, MO). (ii) Control group: rats were pair-fed regular liquid diet. Diets were prepared fresh every other day and stored in the refrigerator until dispensed. Diet intake (ml/100g) was measured every day and the rats were weighted once a week. Rats were maintained on the alcohol or control diet for 12 weeks. Experiments were performed in strict accordance with institutional animal care and use guidelines.

Blood Ethanol Concentration. The blood samples were obtained from conscious rats held in restrainers. A volume of 0.2 ml of blood was taken from a tail vein once a week. The ethanol content of the samples was measured using the enzymatic method described as in our previous studies (6).

NOS Activity Assays. The nitric oxide synthase assay kit (Calbiochem, La Jolla, CA) was used to determine the NOS activity. Briefly, the liver tissue was homogenized by polytron in homogenization buffer (25mM Tris-HCl, pH7.4, 1mM EDTA, 1mM EGTA). The tissue homogenate was centrifuged at 100,000×g for 60 min at 4°C. The pellet containing membrane-associated NOS was re-suspended in homogenization buffer. The samples were incubated with a reaction solution (50 mM Tris-HCL, pH 7.4, 6 µM tetrahydrobiopterin, 2 µM FAD, 2 µM FMN, 1.25 mM NADPH, 0.75 µM CaCl2) and 1µCi 3[H]-Arginine at 37°C. To determine the NOS activity, duplicate samples were incubated for 30 min in the presence of L-nitroarginine methyl ester (L-NAME 1mM) or
vehicle. The reaction was stopped by the addition of 400µl of cold stop buffer (50 mM HEPES, pH 5.5, 5 mM EDTA). 100 µl of the equilibrated resin was added into each reaction samples. After centrifugation, the radioactivity of elute was quantified. Radiolabeled counts per minute of L-citrulline generation were measured and used to determine L-NAME-inhibited NOS activity as in our previous study (35).

**Immunoblotting:** Liver tissue was homogenized in a homogenization buffer (50 mM Tris pH 7.5, 0.1 mM EGTA, 0.1mM EDTA, 2 µm leupeptin, 1mM phenylmethylsulfonyl fluoride, 1% (v/v) Nonidet P-40, 0.1% SDS, and 0.1% deoxycholate). After centrifugation (12,000×g for 5min), protein was quantified in the supernatant by a modified Lowry assay procedure (the Bio-Rad protein assay system). For the Western blot analysis of eNOS, caveolin-1 and calmodulin, the protein (100µg, 40µg and 40µg, respectively) was loaded onto SDS-PAGE gels with appropriate concentration (7.5%, 12% and 12%, respectively) for the best separation. Proteins were transferred to nitrocellulose membrane after electrophoresis. After blocking with 5% non-fat dry milk in Tris-buffered saline, the blots were incubated with the specified primary antibody (anti-eNOS dilution at 1:1000; anti-caveolin-1 dilution at 1:1000, Transduction Laboratories, San Diego, CA. Anti-calmodulin dilution at 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) in TBS buffer containing 5% non-fat dry milk overnight at 4 °C. After four washes, the blots were incubated with second antibody dilution in TBS buffer containing 5% non-fat dry milk. After three additional washes in TBS buffer with 0.1% (v/v) Tween-20, the blots were detected by ECL system, and exposed to x-ray film as in our previous study (35).

**Immunoprecipitation.** Liver tissue was homogenized in a buffer containing 150 mM NaCl, 1% IGEPAL CA-630, 50 mM Tris, pH 8.0, 0.1 mM PMSF, 1×protease inhibitor
cocktail (Roche, Mannheim, Germany). After centrifugation (12,000×g, 5min), protein was quantified by using a modified Lowry assay procedure (the Bio-Rad protein assay system) The sample containing 5mg of protein were pre-cleared by incubating with Protein A-Sepharose for 1h at 4°C. Anti-eNOS polyclonal antibody (250µg/ml) or rabbit IgG (3.5µg, control) was added and mixed for 1h at 4°C. 50% Protein A-Sepharose slurry was added to the mixture and incubated, with rotation, at 4°C overnight. The preparation of the immunoprecipitation was analyzed by Western blot as described above.

**Densitometric analysis** was performed on a video based computerized system. Protein levels were quantified by measuring the optical density of the specific bands using the public domain NIH Image program version 1.60 (http://rsb.info.nih.gov/nih-image/). The NIH Image program has a built in "Calibrate" command. This command allows transforming pixel values captured by directly the camera, from a scale which is linear with respect to transmission, into an integrated optical density scale that correlates to concentration (33). Briefly, to calibrate the system, (i) the Gel Plotting Macros was chosen, (ii) an appropriate coverage area was used to outline the first lane, (iii) the same area was copied and pasted to outline each of the other lanes in succession, (iv) plot lanes command was used to generate the lane profile plots, and (v) the areas of the peaks were measured by clicking inside each one in succession with the wand tool. Finally, the measurements were recorded and are presented as Integrated Optical Density, which are related to the concentration in each measured lane. A stabilized fluorescent light box model B 95 (Imaging Research Inc. St. Catharines, Ontario, Canada) was used with a high performance monochrome CCD camera model 495-2010 (COHU Inc, San Diego, California) to capture the image on the developed film. To achieve a reliable comparison
between treatment and control results, all extracts from the treatment and control rats (n=6 each) were assayed in the same Western blot. Further, each extract was assayed at least two times. Therefore, the protein and immunoprecipitation data presented in the figures constitute the average (+ SEM) of the individual values obtained from the alcohol-treated and control rats under the same experimental conditions.

**Histological/Immunohistochemical Analysis.** The liver tissue was obtained after the rat was euthanized by a fatal dose of pentobarbital and fixed in 4% PBS buffered paraformaldehyde. Then the tissues were transferred to 20% sucrose solution at 4°C until the tissues sank. The sucrose-infiltrated tissues were frozen and sectioned (10 µm) by microtome (HM 505E, Microm international, GmbH). The hematoxylin and eosin (H&E) staining for pathological examination was performed as reported (22). For immunohistochemical analysis, the sections (3-4 sections from each animal) were immunostained with anti-caveolin-1 polyclonal antibody (1:300, Transduction Laboratories, San Diego, CA) using a modification of the avidin-biotin-complex method (ABC) following the manufacture’s instruction (Vector Laboratories, Inc. Burlingame, CA). Sections from treatment and control groups (6 sections from each rat) were subjected to the same experimental conditions (incubation time, washings, etc.). Control sections treated with the mouse IgG exhibited no positive staining (data not shown). Similarly, no positive staining was detected in sections treated with the primary or the secondary antibody alone (data not shown).

**Immunofluorescence.** The liver tissue cryostat sections (10 µm) were prepared using the method described above for histological analysis. Briefly, sections (6 from each rat) were blocked with normal goat serum, incubated with primary antibody over night at 4°C,
washed three times with PBS, incubated with the secondary antibody for 1 h at room temperature, and washed three times with PBS. The Golgi apparatus was identified by incubating the slices with the anti-GM130 monoclonal antibody (BD Biosciences, Franklin Lakes, NJ) and AMCA (Coumarin)-conjugated Donkey anti-goat IgG (1:50 dilution; Jackson ImmunoResearch Laboratory, West Grove, PA). eNOS was detected with a polyclonal antibody (1:50; Transduction Laboratories, San Diego, CA) and a goat anti-rabbit–Texas Red secondary antibody. For triple staining, caveolin-1 was identified with an anti-mouse monoclonal antibody (1:50; Transduction Laboratories, San Diego, CA) and a goat anti-mouse–FITC secondary antibody while eNOS and the Golgi apparatus were detected as described above. The intracellular localization of these proteins was visualized on a Nikon E600 fluorescence microscope with appropriate filters and images captured with an Orca II CCD camera (Hamamatsu, Bridgewater, NJ) and analyzed with Metaview image analysis and acquisition software (Universal Imaging Corporation, Downingtown, PA). The color images presented are in pseudo color. Control sections that were only incubated with the primary or secondary antibody showed no fluorescence (data not shown).

**Statistical Analysis.** All data are presented as “mean ± SE”. The two-tailed Student t-test and ANOVA (time-course data) were used in the analysis of data with the level of significance set at p<0.05.
Results

Figure 1 shows the liquid diet daily intake, body weights and blood ethanol concentration over the course of the experiment. The daily liquid diet intake was similar in the two groups of rats that subsequently received ethanol or was pair-fed and received isocaloric diet (week 0; Fig. 1). Compared with baseline data, the addition of alcohol (3% v/v) to the liquid diet caused a significant reduction in daily diet intake (week 1; Fig. 1). Alcohol-treated and control rats had similar baseline body weights and there was a steady increase in body weights in both groups (Fig. 1). In the alcohol group, the average daily intake of alcohol was 6.1±0.2 g/kg and the blood alcohol concentration was relatively stable over the course of the study (Fig. 1). The average blood alcohol concentration was relatively low at 13.2±1.6 mg/dl. Notably, blood sampling occurred before providing the alcohol containing liquid diet. This level of blood alcohol is comparable with the low levels during daytime in rats fed the same diet. Alcohol was not detectable in the blood of control group rats.

Histological changes of the liver

To determine whether chronic alcohol intake caused liver injury, potential pathological changes in the liver were examined by H&E stain. A comparison of liver sections obtained from rats fed alcohol or control diet revealed signs of fatty liver in the alcohol-fed rats, which demonstrated the ability of chronic (12-week) alcohol feeding to produce pathological liver injury (Fig. 2 upper panels).

Effect of alcohol on NOS activity and eNOS protein level in liver tissue

The membrane-associated NOS activity (conversion of radiolabeled L-arginine to L-citrulline) was significantly reduced in liver tissues of alcohol-treated rats compared
with control rats (Fig. 3A). To investigate whether the decreased hepatic NOS activity in chronic alcohol-treated rats was the result of reduction of eNOS protein level, eNOS expression in liver tissue was examined by immunoblotting. As shown in the representative blot (Fig. 3B), and the densitometry analysis (n = 6 for each group; Fig. 3B), no significant difference in eNOS protein level was detected in alcohol-treated compared with control rats. Neither iNOS nor nNOS protein was detected (Western blotting) in liver tissues of control or alcohol-fed rats (data not shown).

**Effect of alcohol on the association of caveolin-1 and calmodulin with eNOS in liver**

Whether the association of eNOS with caveolin-1 and calmodulin contributed to the reduction in hepatic NOS activity in the alcohol-treated rats was investigated by immunoprecipitation. Enhanced binding of caveolin-1 with eNOS occurred in the liver tissue obtained from alcohol-treated compared with control rats (Fig. 3E). On the other hand, diminished calmodulin binding was evident in primary eNOS immunoprecipitates prepared from the liver tissue of alcohol-treated compared with control rats (Fig. 3E).

**Effect of alcohol on expression of caveolin-1 and calmodulin in liver**

As shown in the representative blot and the densitometry analysis (Fig. 3C), hepatic caveolin-1 expression was significantly increased in alcohol-treated compared with control rats. Comparable results were obtained from immunohistochemical study using the same antibody. The sections from control rats displayed weak granular positive staining (Fig. 2A, lower panels). The sections from alcohol-treated rats showed stronger staining for hepatic caveolin-1 compared with that of control rats (Fig. 2B, lower panels). In contrast to the increase in caveolin-1 expression (Fig. 3C), the calmodulin expression in the liver was not affected by chronic ethanol administration (Fig. 3D).
Co-localization of eNOS and caveolin-1 in hepatocytes

In this experiment, we investigated whether eNOS is trapped in the Golgi apparatus in hepatocytes and whether chronic ethanol administration influences: (i) eNOS association with the Golgi membrane and (ii) the co-localization of eNOS and caveolin-1 with the Golgi apparatus. Triple-label immunofluorescence analyses demonstrated the co-localization of eNOS and caveolin-1 with the Golgi apparatus in hepatocytes (Fig. 4). eNOS association with caveolin-1 was increased in hepatocytes of alcohol-treated compared with control rats (Fig. 4). Furthermore, as shown in Figure 4, eNOS co-localization with the Golgi membrane was increased in hepatocytes of alcohol-treated compared with control rats.
Discussion

The cellular mechanisms implicated in NO production in injured liver constitute an important area of investigation because these processes may provide potential avenues for therapeutic intervention. The present study dealt with the chronic effects of alcohol on eNOS-NO signaling in the rat liver. The study was extended to investigate the effect of alcohol on two counterbalancing allosteric proteins, caveolin-1 and calmodulin, which modulate the activity of eNOS activity. We found that histological changes consistent with liver injury in alcohol-treated rats were associated with: (1) a reduction in hepatic NOS activity (NOS-derived NO); (2) increased hepatic expression of caveolin-1; (3) increased and decreased association of eNOS with the inhibitory protein, caveolin-1, and the stimulatory protein, calmodulin, respectively. The increased production of caveolin-1 and its enhanced association with hepatic eNOS may contribute to the reduction of hepatic NOS activity in alcohol-treated rats. These results provide the first analysis of molecular mechanisms underlying the alcohol-related regulation of eNOS activity in a rat model of alcoholic liver injury.

Whether the reduction in eNOS-derived NO contributes to, or caused by, the liver injury in alcohol-treated rats cannot be ascertained from the present findings. It is likely, however, that the reduced eNOS-NO signaling in our model plays a contributory role in alcoholic liver injury for two reasons. First, liver injury observed in ethanol-fed rats was associated with significant reduction in eNOS catalytic activity. Second, reported evidence suggests that adaptive changes in NO synthesis in the liver confer beneficial or protective role to counter the deleterious effect of ethanol on the liver. In support of this notion are the reported findings that treatment with a NOS inhibitor (L-NAME) increases the
severity of liver injury, whereas NO supplement with L-arginine completely prevented the liver injury caused by alcohol (22). Further, Kimura et al. showed that the evoked production of NO by Kupffer cells is diminished by chronic alcohol administration (16). Together, these results suggested that NO is likely to be involved in alcohol-mediated modulation of the function of hepatic cells during alcohol intake. The mechanism by which hepatic NO confers protection against ethanol-induced liver injury is not fully understood. Suggested mechanisms include: (i) NO plays a significant role in stabilizing the hepatic microcirculation and protecting the liver from oxidative injury (22, 23); (ii) A counterbalance exists in vivo between hepatic levels of NO and superoxide level (2); and (iii) NO counteracts tumor necrosis factor (TNF) dependent activation of caspase 3 (15), which plays a pivotal role in liver injury (5). Notably, TNF is known to be involved in alcoholic liver injury (5). Indeed, in alcohol-fed rats, the transition from steatosis to steatohepatitis is associated with an increase in the hepatic expression of TNF (4, 19). Thus, the reduced catalytic activity of hepatic eNOS activity, we observed in the present study, may contribute to the pathophysiology of alcohol-induced liver injury.

Another important finding from the present study is the differential effect of alcohol on the association of eNOS with its regulatory proteins, caveolin-1 and calmodulin. It is well established that the binding of eNOS with the ubiquitous calcium-regulatory protein calmodulin promotes NO production (28). By contrast, an association between caveolin-1, the structural protein of plasmalemmal caveolae, and eNOS leads to a reduction in the catalytic activity of the enzyme (8, 14). Based on these studies, a molecular paradigm has been proposed whereby NO production is regulated through a reciprocal and competitive interaction of calmodulin and caveolin with eNOS, with NOS
activation enhanced by binding with calmodulin and inhibited by binding with caveolin-1 (14, 20). In this study, we show an enhanced and diminished binding of eNOS with caveolin-1 and calmodulin, respectively, along with the reduced NOS activity in the liver form alcohol-fed rats. A similar alteration in the interactions of eNOS with its regulatory proteins has been found in the other models of liver injury such as cirrhotic liver (32).

It was important to determine whether chronic alcohol feeding influences the hepatic expression of caveolin-1 or calmodulin protein to gain insight into the mechanism by which chronic ethanol feeding alters the association between hepatic eNOS and both proteins. We report, for the first time, an increase in caveolin-1 protein level, but not calmodulin, in the livers of alcohol-fed rats. Prominent increase in hepatic caveolin-1 protein level has also been found in experimental models of liver disease, suggesting a pathophysiological role for caveolin-1 upregulation in the liver (10, 32). The mechanisms of ethanol-evoked enhancement of hepatic caveolin-1 expression remain unclear. It is possible that a link that exists between increased cholesterol and subsequent increase in caveolin-1 elicited by ethanol in cultured cells (3, 12, 29) may explain, at least partly, the significant increase in hepatic caveolin-1 in our model. Interestingly, the hypolipidemic drug fenofibrate dose-dependently reduced alcoholic fatty liver in rats fed ethanol in liquid diet (34); ethanol feeding paradigms used in the reported study (34) and the present study were very similar. Future studies will determine whether ethanol enhancement of caveolin-1 expression in liver involves lipid metabolism.

In endothelial cells, the majority of eNOS is found in the membrane fraction, predominantly trapped in the Golgi apparatus with the rest in the plasma membrane (24). The organized spatial localization of eNOS on the caveolae in plasma membrane and
Golgi complex in endothelial cells is considered an important regulator of its activity (9, 11, 21). We report a similar pattern in hepatocytes where eNOS localization with caveolin-1 and the Golgi apparatus was evident in both alcohol-treated and control rats. However, eNOS localizations with caveolin-1 and with the Golgi membrane were increased in hepatocytes from alcohol-treated compared with control rats (Fig. 4). The mechanisms of ethanol-evoked enhancement of hepatic eNOS localization with caveolin-1 remain unclear. It is possible that the enhanced caveolin-1 expression in the liver of ethanol fed rats contributed to the enhanced association between eNOS and caveolin-1 demonstrated by the immunoprecipitation and immunofluorescence/merging findings. Further, reported studies suggest that morphological changes in endothelial cells may affect the interaction between eNOS and cell membrane, which may lead to the increased eNOS localization with caveolin-1 and ultimately leads to reduced enzyme inactivity (21). Finally, it is possible that impaired membrane function in hepatocytes of ethanol-treated rats (17) contribute to the altered spatial localization of eNOS in hepatocytes obtained from ethanol-fed rats in the present study.

**Perspective.** Results of the present study demonstrated, for the first time, that alcoholic fatty liver is associated with diminished hepatic NOS-derived NO in a model of ethanol-induced liver injury. The diminished catalytic activity of hepatic eNOS is a result of increased binding of hepatic eNOS with the inhibitory protein caveolin-1 and reduced binding with the facilitator protein calmodulin in alcohol-treated rats. Increased caveolin-1 production in the livers of alcohol-treated rats along with altered subcellular localization of eNOS may contribute to the enhanced binding between eNOS and caveolin-1, which ultimately leads to reduced eNOS activity. By contrast, calmodulin level remained
unaltered in spite of a significant reduction in its binding with eNOS in the livers of ethanol-fed rats. Our findings may help elucidate the molecular mechanism underlying the inhibitory effects of alcohol on hepatic eNOS activity, which seems to be implicated in alcoholic liver injury.
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References:


Figure Legends

Figure 1. Liquid diet intake (upper panel), and body weights (middle panel) of the ethanol-fed and control rats (n=6 each) over the 12-week treatment period. Weekly blood ethanol concentrations in the ethanol-fed rats are shown in the lower panel.

Figure 2. Upper panels: Representative sections from the livers of a rat fed control diet (A) or ethanol in liquid diet (B) for 3 months. Fatty deposits (H&E stain), consistent with liver injury, are evident in the liver section obtained from the ethanol-fed rat. Lower panels: Immunohistochemical detection of caveolin-1 in the liver of an alcohol treated or a control rat. Sections of liver tissue were obtained from alcohol-treated and control rats and fixed in 4% paraformaldehyde/PBS solution for immunohistochemical staining for caveolin-1. A: The section from control rats displayed diffused background with granular positive focal staining. B: the section from alcohol-treated rat liver showed stronger staining for caveolin-1 compared with that of control rats (The solid line in panel A represents 50µm). Control sections that were treated with the mouse IgG or only primary or the secondary antibody exhibited no positive staining (data not shown).

Figure 3. Effect of chronic ethanol (3%, v/v) intake for 3 months on: (A) NOS activity measured by \(^{3}\)H-labeled L-citrulline generation in homogenized liver tissue. Membrane-associated NOS activity was significantly (p < 0.05) reduced in rats fed ethanol compared with rats fed control diet (n = 6 for each group); (B) hepatic eNOS, (C) caveolin-1, and (D) calmodulin protein expression. Protein samples were prepared from liver tissues from rats fed ethanol or control diet. Densitometric data (n=6, each group) are presented as
means ± SE. (E): Hepatic eNOS association with caveolin-1 or calmodulin in rats fed alcohol or control diet measured by immunoprecipitation (IP). Proteins were separated and analyzed by immunoblotting as detailed under methods. The association of eNOS with caveolin-1 and calmodulin was increased and decreased, respectively, in alcohol-treated compared with control rats. Results are mean ±SE (n=6 for each group).

Figure 4. Co-localization of eNOS and caveolin-1 in Golgi apparatus in hepatocytes of alcohol-treated and control rats using triple staining and merging technique. Caveolin-1 was detected with an anti-mouse monoclonal antibody (1:50) and a goat anti-mouse–FITC secondary antibody (green). eNOS was detected with a polyclonal antibody (1:50 dilution) and a goat anti-rabbit–Texas Red secondary antibody. The Golgi apparatus was detected with goat-polyclonal to ARAP1 and AMCA (Coumarin)-conjugated Donkey anti-goat IgG (1:50 dilution) (blue).
**Fig. 1**

- **Liquid Diet Intake (ml/100g/day)**
  - Control
  - ETOH

- **Body Weight (g)**
  - Control
  - ETOH

- **Blood ethanol concentration (mg/dL)**
  - ETOH
Fig. 2
Fig. 4