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ETHANOL PLUS THE JO2 FAS AGONISTIC ANTIBODY-INDUCED LIVER INJURY IS ATTENUATED IN MICE WITH PARTIAL ABLATION OF ARGININOSUCCINATE SYNTHASE

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Abstract

Background—Argininosuccinate synthase (ASS) is an enzyme shared by the urea cycle and the L-citrulline/nitric oxide (NO·) cycle. ASS is the rate-limiting enzyme in the urea cycle and along with nitric oxide synthase 2 (NOS2), it endows cells with the L-citrulline/NO· salvage pathway to continuously supply L-arginine from L-citrulline for sustained NO· generation. Thus, ASS conditions NO· synthesis by NOS2. Because of the relevance of NOS2 activation for liver injury, we examined the contribution of ASS to NO· generation and how it impacts liver injury.

Methods—Wild-type (WT) mice and *Ass*$^{+/-}$ mice (*Ass*$^{-/-}$ mice are lethal) were i.p. injected with ethanol at a dose of 2.5 g/kg body weight twice a day for 3 days. Two hours after the last dose of ethanol, mice were administered the agonistic Jo2 hamster anti-mouse Fas monoclonal Ab at a dose of 0.2 μg/g body weight. Mice were sacrificed 8 h after the Jo2 Ab injection. Markers of nitrosative and oxidative stress as well as liver damage were analyzed.

Results—Ethanol plus Jo2-induced liver injury as shown by serum ALT and AST activity, liver pathology, TUNEL and cleaved caspase-3 which were lower in *Ass*$^{+/-}$ mice compared with WT mice suggesting that ASS contributes to ethanol plus Jo2-mediated liver injury. CYP2E1 induction, decreased GSH and elevated TBARS were comparable in both groups of mice suggesting that CYP2E1-mediated oxidative stress is not linked to ASS-induced liver injury. In contrast, NOS2 induction, 3-nitrotyrosine adducts formation and elevated nitrites, nitrates and *S*-nitrosothiols were higher in livers from WT mice than from *Ass*$^{+/-}$ mice.

Conclusion—Decreased nitrosative stress causes lower ethanol plus Jo2-induced liver injury in *Ass*$^{+/-}$ mice.

Keywords

Nitrosative stress; nitric oxide synthase 2; urea cycle; L-citrulline/NO· cycle

INTRODUCTION

Nitric oxide (NO·) generation via nitric oxide synthase (NOS) is associated with epithelial cell injury, host immune defense and perpetuation of inflammatory responses (Nussler and

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Billiar, 1993). NOS catalyzes the formation of NO· via the L-citrulline/NO· cycle which comprises NOS, argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) (Flam et al., 2001). The role of ASS and ASL is to convert L-citrulline, a second product of NOS activity, back to L-arginine for sustained NO· production by NOS (Flam et al., 2001). There are three isoforms of NOS and while NOS1 and NOS3 are constitutive, NOS2 is inducible. Under physiological conditions the NOS2 gene is quiescent, yet toxins such as alcohol and lipopolysaccharide (LPS) can initiate and sustain its activation (Xie and Gross, 1997, Nathan and Xie, 1994). Heavy ethanol consumption causes liver damage in a NOS2-dependent manner (Cederbaum et al., 2009). Severe liver injury induced by the Fas/CD95 agonist Jo2 is associated with NOS2 (Chang et al., 2003). While an ethanol binge or Jo2 administration at low dose fails to induce liver damage, the combination of both induces severe liver injury, which can be partially blocked by the NOS2 inhibitor 1400W (Wang and Cederbaum, 2007) suggesting that NOS2 mediates ethanol plus Jo2-induced liver injury.

The urea cycle is a metabolic pathway in which ammonia produced during protein catabolism is converted to urea in the liver. The urea cycle involves five enzymatic reactions. ASS catalyzes the reversible ATP-dependent condensation of L-citrulline and aspartate to form argininosuccinate, the immediate precursor of L-arginine. Because ASS is the enzyme with the lowest *Vmax* in the urea cycle, it is the rate-limiting step in the cycle (Husson et al., 2003). In the liver, the L-citrulline/NO· cycle shares ASS and ASL with the urea cycle. Co-induction of NOS2 and ASS has been demonstrated *in vivo* in various tissues and in cells treated with LPS (Hattori et al., 1994, Hattori et al., 1995, Norris et al., 1995) (Nagasaki et al., 1996, Flodstrom et al., 1995). Hepatocytes can be stimulated to generate large amounts of NO· in addition to urea in response to sepsis or to LPS (Nussler and Billiar, 1993). We hypothesized that ASS could have an effect on ethanol plus Jo2-induced liver injury by regulating NOS2-mediated NO· production. In this study, we demonstrate that ASS contributes to ethanol plus Jo2-induced liver injury by increasing NOS2 and nitrosative stress.

MATERIALS AND METHODS

In vivo model

Ass$^{+/-}$ mice in C57BL/6 and Sv129 genetic background were purchased from the Jackson Laboratory (Bar Harbor, MN). *Ass*$^{+/-}$ mice were originally generated as a model of citrullinemia (Patejunas et al., 1994). *Ass*$^{-/-}$ mice are embryonically lethal (Patejunas et al., 1994). Mice were i.p. injected with ethanol (Pharmaco, Brookfield, CT) at a dose of 2.5 g/kg b. wt. twice a day for 3 days. Two hours after the last dose of ethanol, mice were i.p. injected either saline solution or the agonistic Jo2 hamster anti-mouse Fas monoclonal Ab (BD Pharmingen, San Diego, CA) at 0.2 μg/g b. wt. These treatments at the selected doses do not cause significant liver injury when given alone (Wang et al., 2005; Wang and Cederbaum, 2007). Mice were sacrificed 8 h after the administration of the Jo2 Ab or saline solution. Blood was collected and serum was obtained to determine alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity using kits (Pointe Scientific, Canton, MI). Livers were excised into fragments; one fragment was fixed in 10% formalin solution for pathology analysis and the rest of the tissue was stored at −80°C for subsequent assays.

Liver pathology and immunohistochemistry

Liver samples were fixed in 10% neutral-buffered formalin solution and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin (H&E) for pathological evaluation by a liver pathologist blinded to the experimental conditions. Ten 100× fields were examined for necroinflammatory activity, which was scored as follows: 0,

none; 1, <2 foci per 100× field; 2, 2–4 foci per 100× field; 3, 5–10 foci per 100× field; 4, >10 foci per 100× field. Immunohistochemical staining for 3-nitrotyrosine adducts (3-NT), NOS2 and cleaved caspase-3, tumor necrosis factor-α (TNF-α) was performed using antibodies against 3-NT (Millipore, Billerica, MA), NOS2, cleaved caspase-3 (Cell Signaling, Beverly, MA) and TNF-α (R&D Systems, Minneapolis, MN) and the Histostain Plus detection system (Invitrogen, Carlsbad, CA). DNA fragmentation was determined by the TUNEL assay using the ApopTag *in situ* apoptosis detection kit (Chemicon, Temecula, CA). Samples were counterstained with hematoxylin. Positively stained nuclei were counted in 20 random fields per sample and averaged numbers were graphed.

Measurement of reduced glutathione

Liver homogenates were mixed with trichloroacetic acid (TCA) to a final concentration of 5% and incubated at 4°C for 30 min to precipitate proteins. The TCA extracts (10 μl) were added to 200 μl of methanol containing 1 mg/ml *o*-phthaldehyde and were incubated for 15 min at 37°C in the dark. Fluorescence was measured at 350/420 nm (excitation/emission). The concentration of glutathione (GSH) was determined from a GSH standard curve.

Quantification of thiobarbituric acid reactive substances

Liver homogenates supplemented with butylated hydroxytoluene to prevent further oxidation during the assay were incubated with 0.2 ml of 15% TCA-0.375% TBA-0.25 N HCl solution in a boiling water bath for 10 min. Following centrifugation at 500×*g* for 5 min the resulting supernatant was used to determine the generation of thiobarbituric acid reactive substances (TBARS) by measuring the absorbance at 535 nm. A standard curve was generated using 1,1,3,3-tetraethoxypropane treated as above.

Quantification of liver nitrites, nitrates and S-nitrosothiol

Liver nitrites plus nitrates were measured using a kit (Cayman Chemical, Ann Arbor, MI) to reflect hepatic levels of nitric oxides as described before (Lu et al., 2012). Total *S*-nitrosothiols (RSNO) measurement was carried out as described in (Kubes, et al., 1999). Briefly, fresh liver homogenate containing 10 μM diethylenetriaminepentaacetic acid was used for two reactions. In reaction 1, 200 μl of the homogenate were mixed with 200 μl of 1% (w/v) sulfanilamide in 0.5 M HCl. In reaction 2, 200 μl of the homogenate were mixed with 200 μl of 0.2% (w/v) $HgCl_2$ in the 1% sulfanilamide in 0.5 M HCl to liberate NO· from any RSNO (R is any molecule and *S*-NO is *S*-nitrosothiol). Both reactions were incubated in the dark at 37°C for 10 min. Next, 200 μl of 0.2% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride in 0.5 M HCl were added to both reactions, and the samples were incubated for an additional 10 min at 37°C in the dark. The samples were then read at 540 nm, and total RSNO were calculated as total RSNO (μM) = (OD reaction 2 − OD reaction 1)/0.05 and normalized by protein content.

Western blot analysis

Hepatic proteins were resolved by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes for blotting with anti-CYP2E1 (a gift from Dr. Jerome Lasker, Puracyp Inc, Carlsbad, CA), ASS and Calnexin (Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase-conjugated secondary antibody. Proteins were visualized with the Fujitsu Las4000 using the enhanced chemiluminescence Western blot detection reagent (Amersham Bioscience, Piscataway, NJ). Blots were quantified using the ImageJ software program from the National Institutes of Health. Protein expression was corrected by Calnexin used as internal control.

Statistical analysis

Values are expressed as means ± SEM (n=6). One-way ANOVA (subsequent *post hoc* comparisons) analysis was performed using the Excel Data Analysis tool package. $p<0.05$ were considered statistically significant.

RESULTS

Ethanol plus Jo2-induced liver injury is lower in *Ass*$^{+/-}$ mice than in WT mice

ASS expression in WT mice was 2-fold higher than in *Ass*$^{+/-}$ mice, either at basal levels or following treatment with ethanol plus Jo2 Ab (Figure 1A). After treatment with ethanol plus Jo2, serum ALT and AST activity was significantly elevated in both WT and *Ass*$^{+/-}$ mice but were 2-to-3-fold higher in WT mice compared to *Ass*$^{+/-}$ mice (Figure 1B–1C). H&E staining shows that necrotic foci were observed mostly around central veins (Figure 1D) and more necrosis (~2.5-fold) was detected in liver sections from WT mice than from *Ass*$^{+/-}$ mice (Figure 1D–1E). Ethanol plus Jo2 induced significant apoptosis. TUNEL staining shows that like the necrotic foci, the apoptotic cells were observed mostly in zones 2 and 3 (Figure 2A) and the apoptosis index was ~2-fold higher in WT mice than in *Ass*$^{+/-}$ mice (Figure 2B). Likewise, immunohistochemistry analysis showed positive staining for cleaved caspase-3 in liver sections from WT mice, which when quantified was 2-fold higher than in *Ass*$^{+/-}$ mice (Figure 2C–2D). These results suggest that ethanol plus Jo2-induced liver injury was lower in *Ass*$^{+/-}$ mice compared to WT mice.

The lower liver injury induced by ethanol plus Jo2 in *Ass*$^{+/-}$ mice is not due to differences in CYP2E1-mediated oxidative stress

Ethanol can induce CYP2E1 and CYP2E1-dependent oxidative stress can then mediate ethanol plus Jo2-induced liver injury (Wang and Cederbaum, 2007). Therefore, we examined whether the decreased liver injury induced by ethanol plus Jo2 observed in *Ass*$^{+/-}$ mice was due to changes in CYP2E1-mediated oxidative stress. First, we measured CYP2E1 expression. As shown in Figure 3A, CYP2E1 induction was comparable in WT mice and in *Ass*$^{+/-}$ mice after ethanol plus Jo2 injection. To further evaluate the extent of oxidative stress induced by ethanol plus Jo2, GSH levels and lipid peroxidation-end products were measured. GSH depletion and lipid peroxidation-end products were identical in WT mice and in *Ass*$^{+/-}$ mice after ethanol plus Jo2 treatment (Figure 3B–3C). These results indicate that the decreased liver injury provoked by ethanol plus Jo2 in *Ass*$^{+/-}$ mice is not due to differences in CYP2E1-mediated oxidative stress.

The lower liver injury induced by ethanol plus Jo2 in *Ass*$^{+/-}$ mice is not due to changes in tumor necrosis factor-α (TNF-α)

Ethanol increases gut permeability and the translocation of Gram-negative bacteria and LPS from the gut into the portal circulation. LPS activates Kupffer cells to produce noxious cytokines such as TNF-α (Lu et al., 2005). Yet, while serum TNF-α levels are not always detectable (Lu et al., 2008; 2010), intrahepatic TNF-α expression could be detectable by immunohistochemistry (IHC) (Ge et al., 2013). Serum TNF-α levels were undetectable (not shown) but as shown in Figure 4, after ethanol plus Jo2 administration, TNF-α positive staining increased with minor differences between WT mice and *Ass*$^{+/-}$ mice (Figure 4A–4B). Therefore, these results suggest that TNF-α plays a minimal role in the difference in ethanol plus Jo2-induced liver injury between WT mice and *Ass*$^{+/-}$ mice.

Nitrosative stress is responsible for the lower liver injury induced by ethanol plus Jo2 in *Ass*$^{+/-}$ mice compared to WT mice

Since ASS is shared by the urea cycle and the L-citrulline/NO· cycle, ASS could drive NOS2 induction and thus condition nitrosative stress. Ethanol plus Jo2-induced liver injury involved nitrosative stress because 3-NT and NOS2 have been previously shown to be increased, and most importantly, ethanol plus Jo2-induced liver injury was blocked by 1400W, a specific NOS2 inhibitor (Wang and Cederbaum, 2007). Here, we examined whether the prevention of liver injury induced by ethanol plus Jo2 in *Ass*$^{+/-}$ mice was due to a decrease in nitrosative stress. Immunohistochemical staining showed that NOS2 induction by ethanol plus Jo2 treatment occurred mainly in zones 2 and 3 both in WT mice and in *Ass*$^{+/-}$ mice (Figure 5A–5B), which is consistent with the distribution of the necrotic foci and of the apoptotic cells. Yet, less positive staining was observed in ethanol plus Jo2-treated *Ass*$^{+/-}$ mice (Figure 5A–5B), suggesting that ASS is necessary for NOS2 induction by ethanol plus Jo2. Consistently, a significant increase in NO· production measured as nitrites plus nitrates were observed in livers from both WT mice and *Ass*$^{+/-}$ mice following the ethanol plus Jo2 treatment. Higher NO· production was detected in WT mice than in *Ass*$^{+/-}$ mice (Figure 5C). Newly generated NO· could react with tyrosine residues to form 3-NT adducts (Gow et al., 2004, Ischiropoulos, 1998). IHC staining showed that similar to NOS2 induction, 3-NT adducts appeared around zones 2 and 3 (Figure 5D–5E) and less positive staining was observed in ethanol plus Jo2-treated *Ass*$^{+/-}$ mice (Figure 5D–5E). As expected, intrahepatic RSNO levels increased after ethanol plus Jo2 treatment more in WT mice than in *Ass*$^{+/-}$ mice (Figure 5F). These results suggest that there is less nitrosative stress in *Ass*$^{+/-}$ mice compared to WT mice, which may explain why less liver injury occurred by ethanol plus Jo2 in *Ass*$^{+/-}$ mice compared to WT mice.

DISCUSSION

Binding of Fas to its ligand (FasL) or Fas antibody results in cell apoptosis via cellular pathways including receptor oligomerization and recruitment of the Fas-associated proteins with death domain, which eventually leads to activation of caspase-8 and downstream caspases such as caspase-3 (Chen et al., 2001, Tagawa et al., 1998, Feldstein et al., 2003). The Fas agonistic antibody Jo2 can activate the Fas-associated apoptotic signaling in the liver in a dose-dependent manner (Ogasawara et al., 1993, Wang et al., 2005). The Fas/Fas ligand complex plays a central role in ethanol-induced hepatic apoptosis (Zhou et al., 2001, Minana et al., 2002, Sosa et al., 2005). While either a low dose of Jo2 Fas agonistic Ab (0.2 μg/g) or acute ethanol (2.5g/kg) cannot induce obvious liver injury, the combination of both increases hepatic apoptosis and liver injury induced by Jo2 Ab alone (Wang and Cederbaum, 2007). In this study we found that ethanol plus Jo2-induced liver injury and apoptosis as demonstrated by pathological changes, serum transaminases, TUNEL and cleaved caspase-3 positive staining, which were lower in *Ass*$^{+/-}$ mice compared to WT mice, suggesting that ASS contributes to ethanol plus Jo2-induced liver injury by enhancing NOS2-mediated nitrosative stress.

TNF-α participates in cell survival and cell death pathways (Chang et al., 2006). The balance between NFκB and JNK determines the biological outcome of TNF-α, while NFκB promotes survival by inducing the expression of cFLIP, an inhibitor of caspase-8, JNK enhances cell death by promoting cFLIP-L ubiquitination and proteasomal degradation (Chang et al., 2006). Previously we found that ASS contributes to LPS-stimulated TNF-α secretion and in turn up-regulates apoptosis induced by pyrazole plus LPS treatment (Lu et al., 2012). However, in the present study we observed that after ethanol plus Jo2 treatment, TNF-α production in the liver is increased in both WT and *Ass*$^{+/-}$ mice and it was slightly increased in WT mice compared to *Ass*$^{+/-}$ mice. These results suggest that TNF-α plays a

minor role, if any, in the ASS-mediated liver injury and apoptosis in this model. In the pyrazole plus LPS model, LPS was injected at 4 mg/kg, but in the ethanol plus Jo2 model no exogenous LPS was administrated, which may be one of the reasons for the difference in the contribution of TNF-α to liver injury.

CYP2E1 can be induced by ethanol, exhibits enhanced NADPH oxidase activity, and elevates the rate of generation of superoxide radical ($O_2^{-}\cdot$) and of H_2O_2. Catalase decomposes H_2O_2; yet, GSH and GSH peroxidase are generally considered quite relevant for H_2O_2 removal (Lu et al., 2005). Ethanol plus Jo2 treatment causes CYP2E1-mediated oxidative stress, which leads to liver injury (Wang and Cederbaum, 2007). Nevertheless, it seems that the difference in ethanol plus Jo2-induced liver injury between *Ass*$^{+/-}$ mice and WT mice does not involve CYP2E1-mediated oxidative stress since the expression of CYP2E1, GSH levels and TBARS (a measurement of lipid peroxidation-end products) in the liver did not exhibit a significant difference between WT mice and *Ass*$^{+/-}$ mice. Liver damage may be dependent on the balance of the local production of NO· and ROS such as $O_2^{-}\cdot$ (Lu et al., 2005). Markers of nitrosative stress including the expression of NOS2, the formation of 3-NT adducts, the concentration of nitrates plus nitrites and the levels of RSNO in the liver were higher in WT mice than in *Ass*$^{+/-}$ mice, suggesting that ASS contributes to ethanol plus Jo2-induced nitrosative stress.

As the rate-limiting enzyme from the urea cycle, ASS contributes to the synthesis of arginine, which generates urea by the action of arginase in the liver. ASS is also a critical enzyme in the L-citrulline/NO· cycle to provide arginine for NO· generation by NOS2. Simultaneous induction of NOS2 and ASS has been demonstrated *in vivo* in various tissues and cells treated with LPS (Flodstrom et al., 1995, Hattori et al., 1994, Hattori et al., 1995, Nagasaki et al., 1996, Norris et al., 1995). ASS overexpression leads to enhanced ability for NO· production (Xie and Gross, 1997). Here we found that partial ablation of ASS could decrease NO· synthesis by NOS2. Once NOS2 is highly induced, NO· production is determined by the intracellular concentration of L-arginine, the only physiological nitrogen donor for NO· production (Nussler and Billiar, 1993) and by other co-factors for NOS2 activity. An active urea cycle provides L-arginine for NO· synthesis in the liver. *Ass* deficiency decreased the expression of NOS2 and the availability of L-arginine. Consequently, after ethanol plus Jo2 treatment, hepatic nitrites plus nitrates, a stable and soluble readout for NO· production, were lower in *Ass*$^{+/-}$ mice than in WT mice. Peroxynitrite generated by the rapid reaction between NO· and $O_2^{-}\cdot$ can induce nitration of free and protein-associated tyrosine residues and cause the formation of 3-NT protein adducts, the footprint of nitrosative stress (Ischiropoulos, 1998), which can then explain why 3-NT adduct formation increased after ethanol plus Jo2 treatment in WT mice compared with *Ass*$^{+/-}$ mice. Therefore, it could be suggested that ASS activity contributes to nitrosative stress.

NOS2-mediated NO· production can be either beneficial or detrimental. Whether NO· is protective or detrimental is determined by the source and by the amount of NO· production and the cellular redox status of the liver (Chen et al., 2003). NOS2-derived NO· generation participates in the pathogenesis of alcoholic liver disease (Dey and Cederbaum, 2006, McKim et al., 2003, Venkatraman et al., 2004). NOS2 catalyzes the NADPH-dependent conversion of L-arginine to NO· plus L-citrulline (Ignarro, 1996). While the NOS2 gene lies quiescent in cells at rest, NOS2 transcription can be initiated by various immunostimulants, cytokines and growth factors (Nathan and Xie, 1994, Xie and Gross, 1997). Overexpression of NOS2 mediates a variety of pathological conditions (Radi et al., 2002, Gross and Wolin, 1995). After ethanol consumption, NOS2 is required to produce NO·-derived pro-oxidants (Venkatraman et al., 2004, McKim et al., 2003). In the model of ethanol plus Jo2 administration, it appears that ASS contributes to liver injury by enhancing nitrosative

stress. Yet, this needs to be further evaluated by the use of pharmacological NO· donors or NOS2 inhibitor or *Nos2*$^{-/-}$ mice.

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REFERENCES

Cederbaum AI, Lu Y, Wu D. Role of oxidative stress in alcohol-induced liver injury. Arch Toxicol. 2009; 83:519–548. [PubMed: 19448996]

Chang B, Nishikawa M, Sato E, Inoue M. Mice lacking inducible nitric oxide synthase show strong resistance to anti-Fas antibody-induced fulminant hepatitis. Arch Biochem Biophys. 2003; 411:63–72. [PubMed: 12590924]

Chang L, Kamata H, Solinas G, Luo JL, Maeda S, Venuprasad K, Liu YC, Karin M. The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. Cell. 2006; 124:601–613. [PubMed: 16469705]

Chen D, McKallip RJ, Zeytun A, Do Y, Lombard C, Robertson JL, Mak TW, Nagarkatti PS, Nagarkatti M. CD44-deficient mice exhibit enhanced hepatitis after concanavalin A injection: evidence for involvement of CD44 in activation-induced cell death. J Immunol. 2001; 166:5889–5897. [PubMed: 11342603]

Chen T, Zamora R, Zuckerbraun B, Billiar TR. Role of nitric oxide in liver injury. Curr Mol Med. 2003; 3:519–526. [PubMed: 14527083]

Dey A, Cederbaum AI. Alcohol and oxidative liver injury. Hepatology. 2006; 43:S63–S74. [PubMed: 16447273]

Feldstein AE, Canbay A, Guicciardi ME, Higuchi H, Bronk SF, Gores GJ. Diet associated hepatic steatosis sensitizes to Fas mediated liver injury in mice. J Hepatol. 2003; 39:978–983. [PubMed: 14642615]

Flam BR, Hartmann PJ, Harrell-Booth M, Solomonson LP, Eichler DC. Caveolar localization of arginine regeneration enzymes, argininosuccinate synthase, and lyase, with endothelial nitric oxide synthase. Nitric Oxide. 2001; 5:187–197. [PubMed: 11292368]

Flodstrom M, Niemann A, Bedoya FJ, Morris SM Jr, Eizirik DL. Expression of the citrulline-nitric oxide cycle in rodent and human pancreatic beta-cells: induction of argininosuccinate synthetase by cytokines. Endocrinology. 1995; 136:3200–3206. [PubMed: 7628352]

Ge X, Lu Y, Leung TM, Sørensen ES, Nieto N. Milk osteopontin, a nutritional approach to prevent alcohol-induced liver injury. Am J Physiol Gastrointest Liver Physiol. 2013; 304:G929–G939. [PubMed: 23518682]

Gow AJ, Farkouh CR, Munson DA, Posencheg MA, Ischiropoulos H. Biological significance of nitric oxide-mediated protein modifications. Am J Physiol Lung Cell Mol Physiol. 2004; 287:L262–L268. [PubMed: 15246980]

Gross SS, Wolin MS. Nitric oxide: pathophysiological mechanisms. Annu Rev Physiol. 1995; 57:737–769. [PubMed: 7539995]

Hattori Y, Campbell EB, Gross SS. Argininosuccinate synthetase mRNA and activity are induced by immunostimulants in vascular smooth muscle. Role in the regeneration or arginine for nitric oxide synthesis. J Biol Chem. 1994; 269:9405–9408. [PubMed: 7511585]

Hattori Y, Shimoda S, Gross SS. Effect of lipopolysaccharide treatment in vivo on tissue expression of argininosuccinate synthetase and argininosuccinate lyase mRNAs: relationship to nitric oxide synthase. Biochem Biophys Res Commun. 1995; 215:148–153. [PubMed: 7575582]

Husson A, Brasse-Lagnel C, Fairand A, Renouf S, Lavoinne A. Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. Eur J Biochem. 2003; 270:1887–1899. [PubMed: 12709047]

Ignarro LJ. Physiology and pathophysiology of nitric oxide. Kidney Int Suppl. 1996; 55:S2–S5. [PubMed: 8743501]

Ischiropoulos H. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch Biochem Biophys. 1998; 356:1–11. [PubMed: 9681984]

Kubes P, Payne D, Grisham MB, Jourd-Heuil D, Fox-Robichaud A. Inhaled NO impacts vascular but not extravascular compartments in postischemic peripheral organs. Am J Physiol. 1999; 277:H676–H682. [PubMed: 10444494]

Lu Y, Leung TM, Ward SC, Nieto N. Partial deletion of argininosuccinate synthase protects from pyrazole plus lipopolysaccharide-induced liver injury by decreasing nitrosative stress. Am J Physiol Gastrointest Liver Physiol. 2012; 302:G287–G295. [PubMed: 22052013]

Lu Y, Wu D, Wang X, Ward SC, Cederbaum AI. Chronic alcohol-induced liver injury and oxidant stress are decreased in cytochrome P4502E1 knockout mice and restored in humanized cytochrome P4502E1 knock-in mice. Free Radic Biol Med. 2010; 49:1406–1416. [PubMed: 20692331]

Lu Y, Zhuge J, Wang X, Bai J, Cederbaum AI. Cytochrome P450 2E1 contributes to ethanol-induced fatty liver in mice. Hepatology. 2008; 47:1483–1494. [PubMed: 18393316]

McKim SE, Gabele E, Isayama F, Lambert JC, Tucker LM, Wheeler MD, Connor HD, Mason RP, Doll MA, Hein DW, Arteel GE. Inducible nitric oxide synthase is required in alcohol-induced liver injury: studies with knockout mice. Gastroenterology. 2003; 125:1834–1844. [PubMed: 14724835]

Minana JB, Gomez-Cambronero L, Lloret A, Pallardo FV, Del Olmo J, Escudero A, Rodrigo JM, Pelliin A, Vina JR, Vina J, Sastre J. Mitochondrial oxidative stress and CD95 ligand: a dual mechanism for hepatocyte apoptosis in chronic alcoholism. Hepatology. 2002; 35:1205–1214. [PubMed: 11981771]

Nagasaki A, Gotoh T, Takeya M, Yu Y, Takiguchi M, Matsuzaki H, Takatsuki K, Mori M. Coinduction of nitric oxide synthase, argininosuccinate synthetase, and argininosuccinate lyase in lipopolysaccharide-treated rats. RNA blot, immunoblot, and immunohistochemical analyses. J Biol Chem. 1996; 271:2658–2662. [PubMed: 8576237]

Nathan C, Xie QW. Regulation of biosynthesis of nitric oxide. J Biol Chem. 1994; 269:13725–13728. [PubMed: 7514592]

Norris KA, Schrimpf JE, Flynn JL, Morris SM Jr. Enhancement of macrophage microbicidal activity: supplemental arginine and citrulline augment nitric oxide production in murine peritoneal macrophages and promote intracellular killing of Trypanosoma cruzi. Infect Immun. 1995; 63:2793–2796. [PubMed: 7790103]

Nussler AK, Billiar TR. Inflammation, immunoregulation, and inducible nitric oxide synthase. J Leukoc Biol. 1993; 54:171–178. [PubMed: 7689630]

Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S. Lethal effect of the anti-Fas antibody in mice. Nature. 1993; 364:806–809. [PubMed: 7689176]

Patejunas G, Bradley A, Beaudet AL, O'Brien WE. Generation of a mouse model for citrullinemia by targeted disruption of the argininosuccinate synthetase gene. Somat Cell Mol Genet. 1994; 20:55–60. [PubMed: 8197477]

Radi R, Cassina A, Hodara R. Nitric oxide and peroxynitrite interactions with mitochondria. Biol Chem. 2002; 383:401–409. [PubMed: 12033431]

Sosa L, Vidlak D, Strachota JM, Pavlik J, Jerrells TR. Rescue of in vivo FAS-induced apoptosis of hepatocytes by corticosteroids either associated with alcohol consumption by mice or provided exogenously. Int Immunopharmacol. 2005; 5:301–314. [PubMed: 15652761]

Tagawa Y, Kakuta S, Iwakura Y. Involvement of Fas/Fas ligand system-mediated apoptosis in the development of concanavalin A-induced hepatitis. Eur J Immunol. 1998; 28:4105–4113. [PubMed: 9862346]

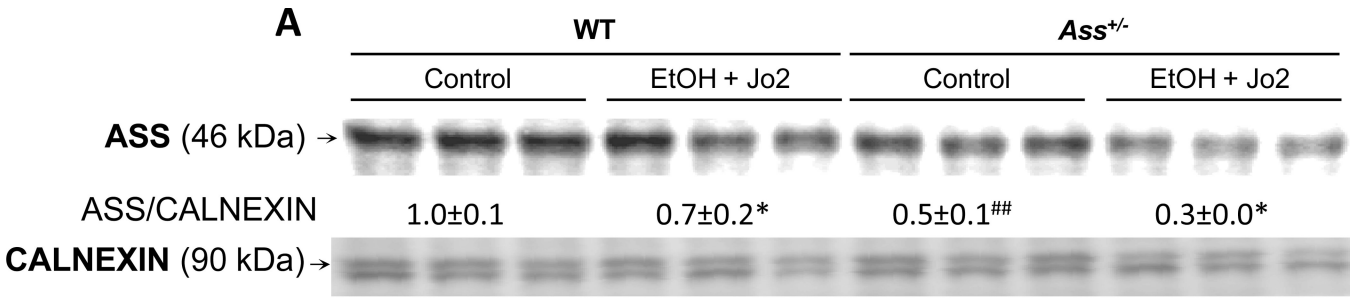
Venkatraman A, Shiva S, Wigley A, Ulasova E, Chhieng D, Bailey SM, Darley-Usmar VM. The role of iNOS in alcohol-dependent hepatotoxicity and mitochondrial dysfunction in mice. Hepatology. 2004; 40:565–573. [PubMed: 15349894]

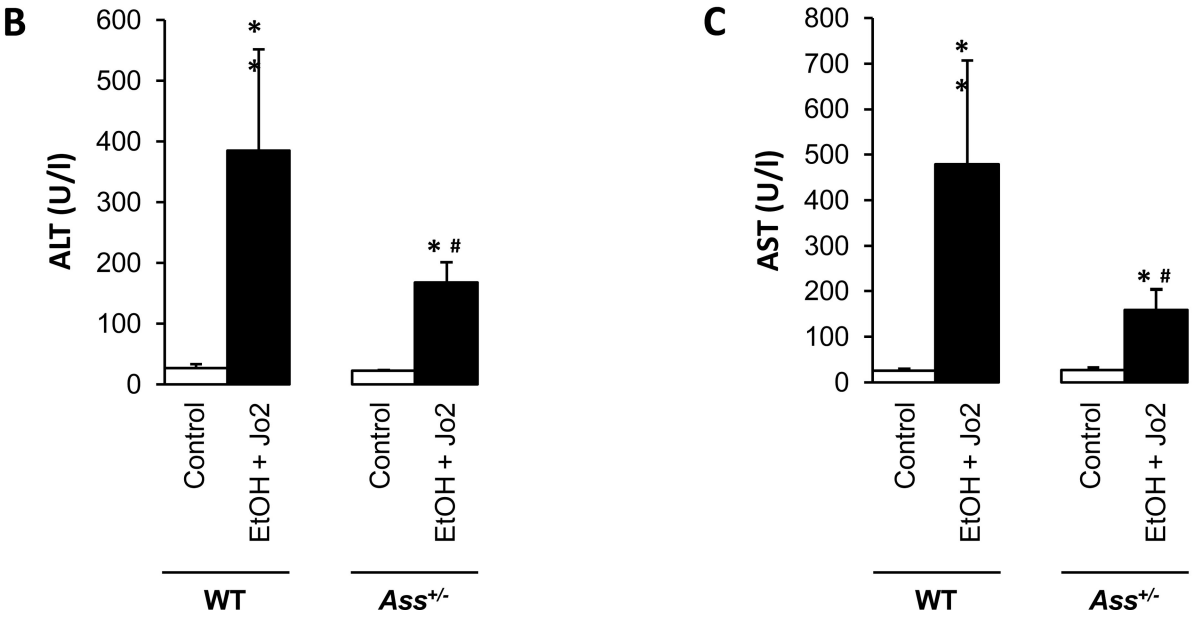
Wang X, Cederbaum AI. Acute ethanol pretreatment increases FAS-mediated liver injury in mice: role of oxidative stress and CYP2E1-dependent and -independent pathways. Free Radic Biol Med. 2007; 42:971–984. [PubMed: 17349925]

Wang X, Lu Y, Cederbaum AI. Induction of cytochrome P450 2E1 increases hepatotoxicity caused by Fas agonistic Jo2 antibody in mice. Hepatology. 2005; 42:400–410. [PubMed: 16025513]

Xie L, Gross SS. Argininosuccinate synthetase overexpression in vascular smooth muscle cells potentiates immunostimulant-induced NO production. J Biol Chem. 1997; 272:16624–16630. [PubMed: 9195976]

Zhou Z, Sun X, Kang YJ. Ethanol-induced apoptosis in mouse liver: Fas- and cytochrome c-mediated caspase-3 activation pathway. Am J Pathol. 2001; 159:329–338. [PubMed: 11438480]





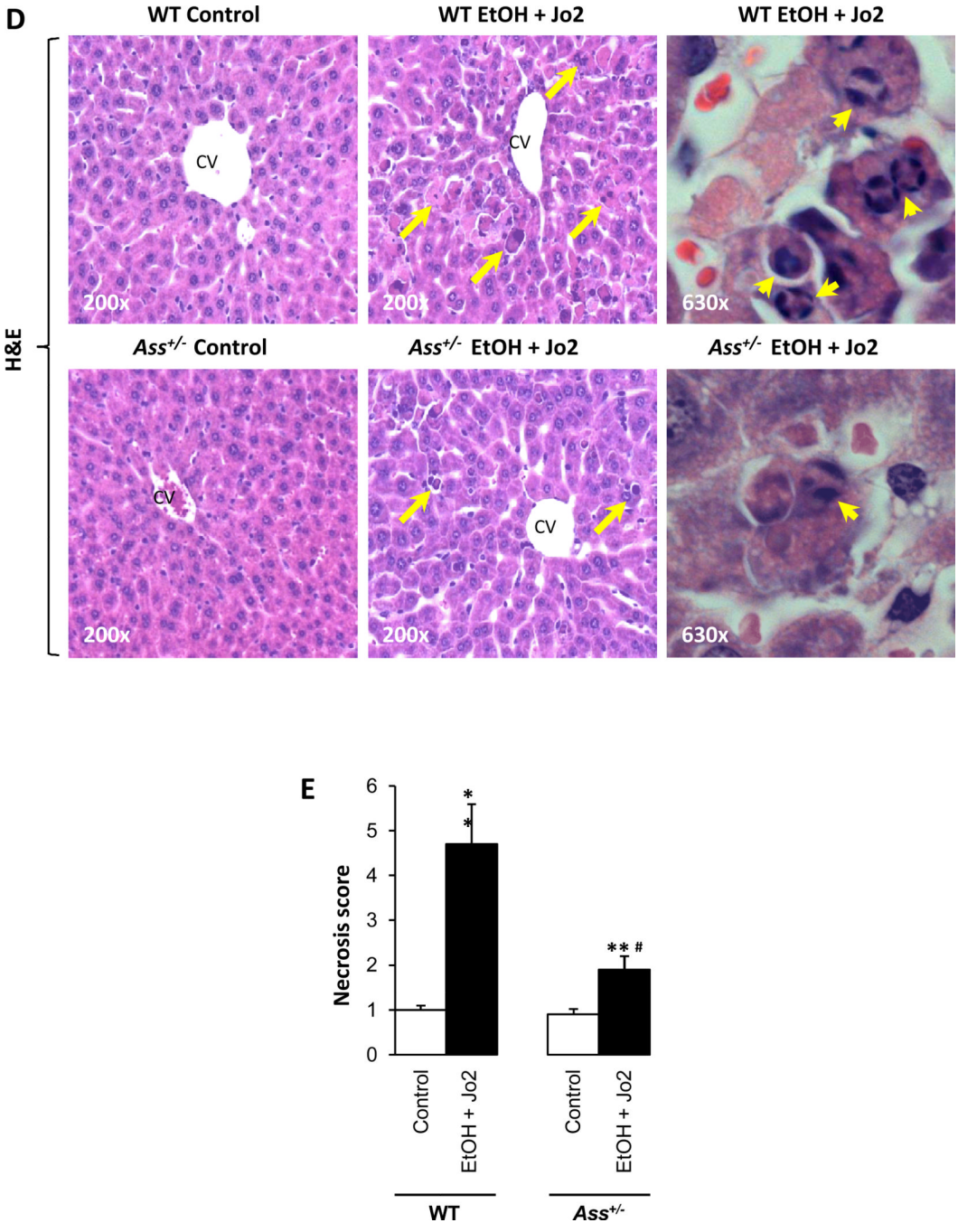
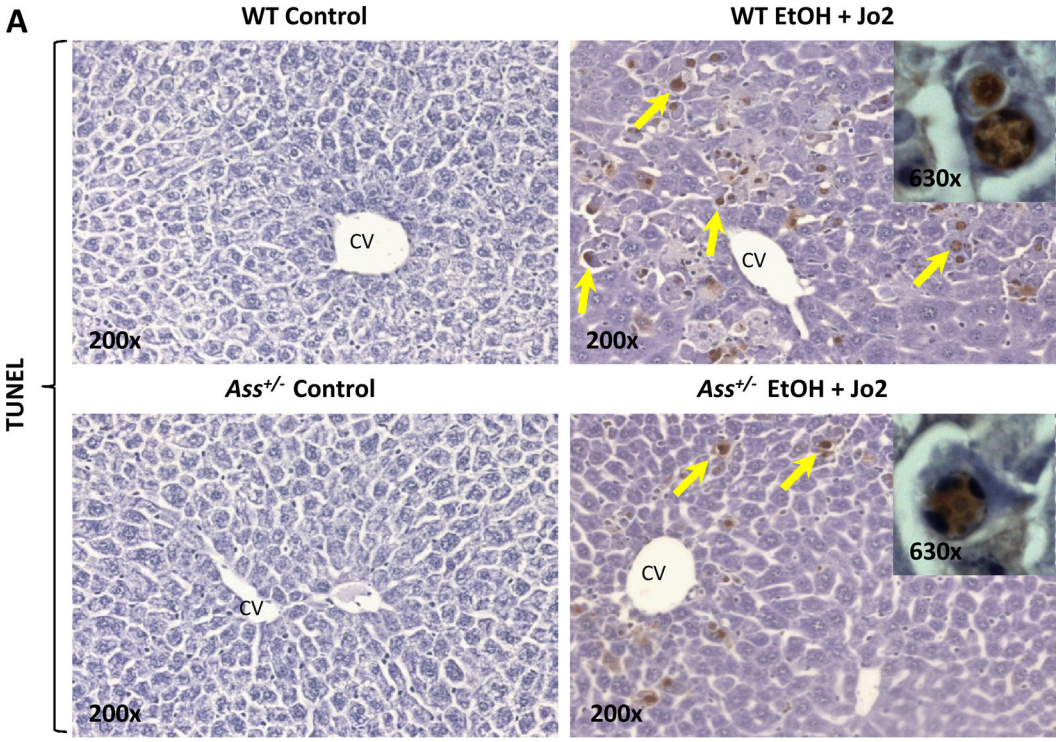
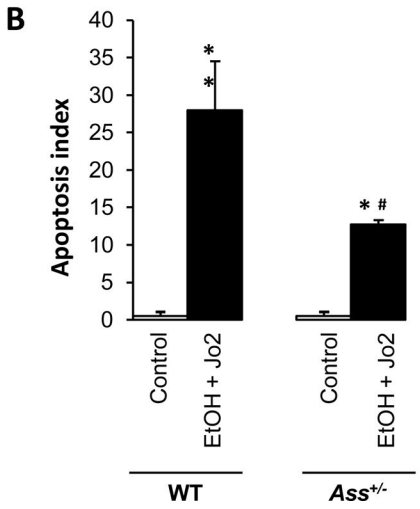


Figure 1. *Ass*$^{+/-}$ mice are protected from ethanol plus Jo2-induced liver injury

ASS expression was analyzed by Western blot. The quantification of the intensity of the bands corrected by that of Calnexin is indicated under the blots (**A**). Serum ALT and AST activity are increased in ethanol plus Jo2-treated WT mice compared to *Ass*$^{+/-}$ mice (**B–C**). H&E staining shows necrosis (yellow arrows) and apoptosis (yellow arrowheads) in ethanol plus Jo2-treated WT mice and in *Ass*$^{+/-}$ mice, CV: central vein (**D**). The necrosis score indicates more necrosis in ethanol plus Jo2-treated WT mice than in *Ass*$^{+/-}$ mice (**E**). Results are expressed as average values ± SEM; n=6, *p<0.05 and **p<0.01 for ethanol plus Jo2 *vs* control; $^{\#}p$<0.05 and $^{\#\#}p$<0.01 for *Ass*$^{+/-}$ *vs* WT.





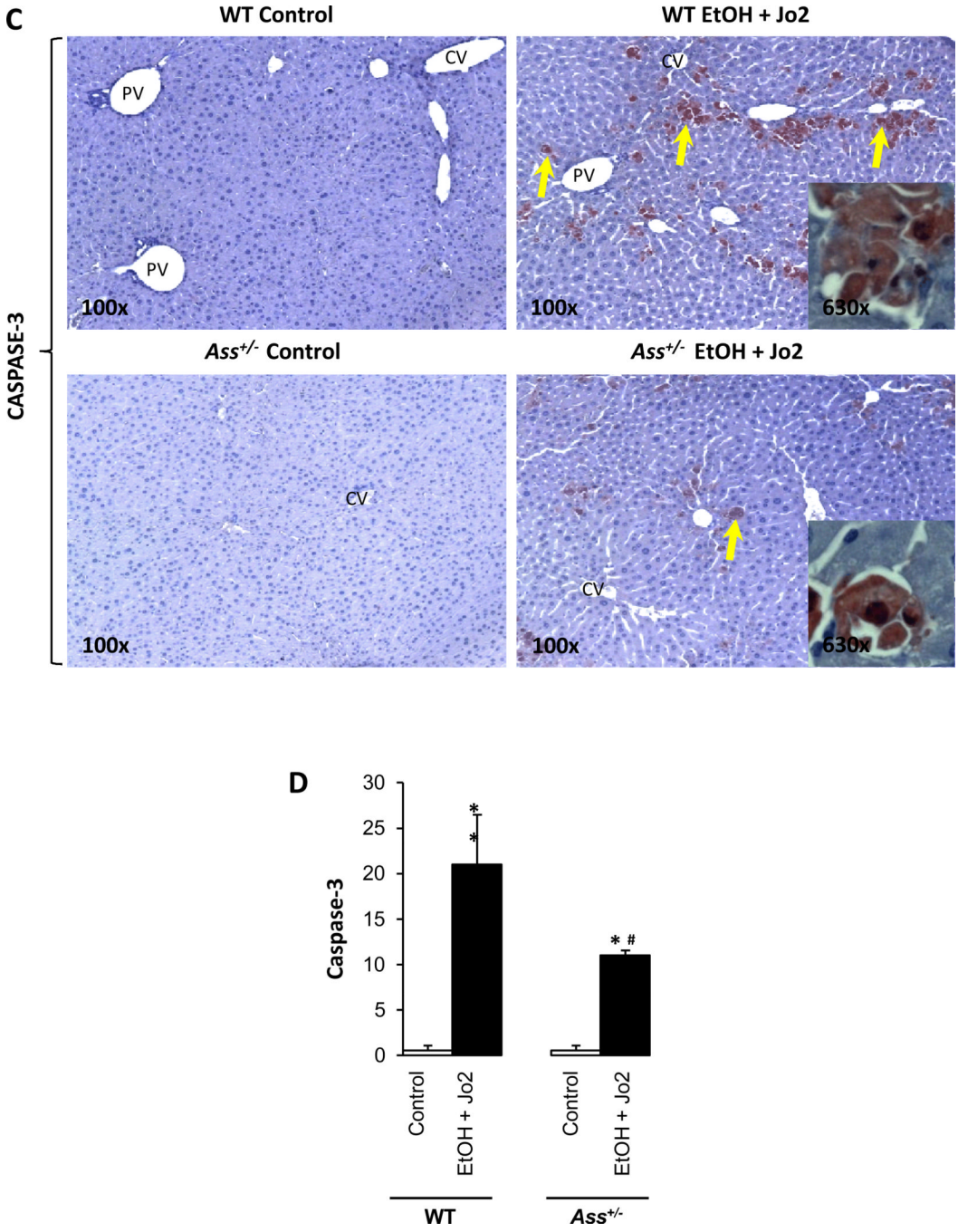


Figure 2. *Ass*$^{+/-}$ mice are protected from ethanol plus Jo2-induced apoptosis

TUNEL staining (yellow arrows and insets showing nuclear staining at 630×) (**A**), the apoptosis index (**B**), IHC for cleaved caspase-3 (yellow arrows and insets showing nuclear and cytosolic staining at 630×) (**C**) and morphometric analysis for caspase-3 (**D**) demonstrate more apoptosis in ethanol plus Jo2-treated WT mice than in *Ass*$^{+/-}$ mice. CV: central vein; PV: portal vein. Results are expressed as average values ± SEM; n=6, *p<0.05 and **p<0.01 for ethanol plus Jo2 *vs* control; #p<0.05 for *Ass*$^{+/-}$ *vs* WT.

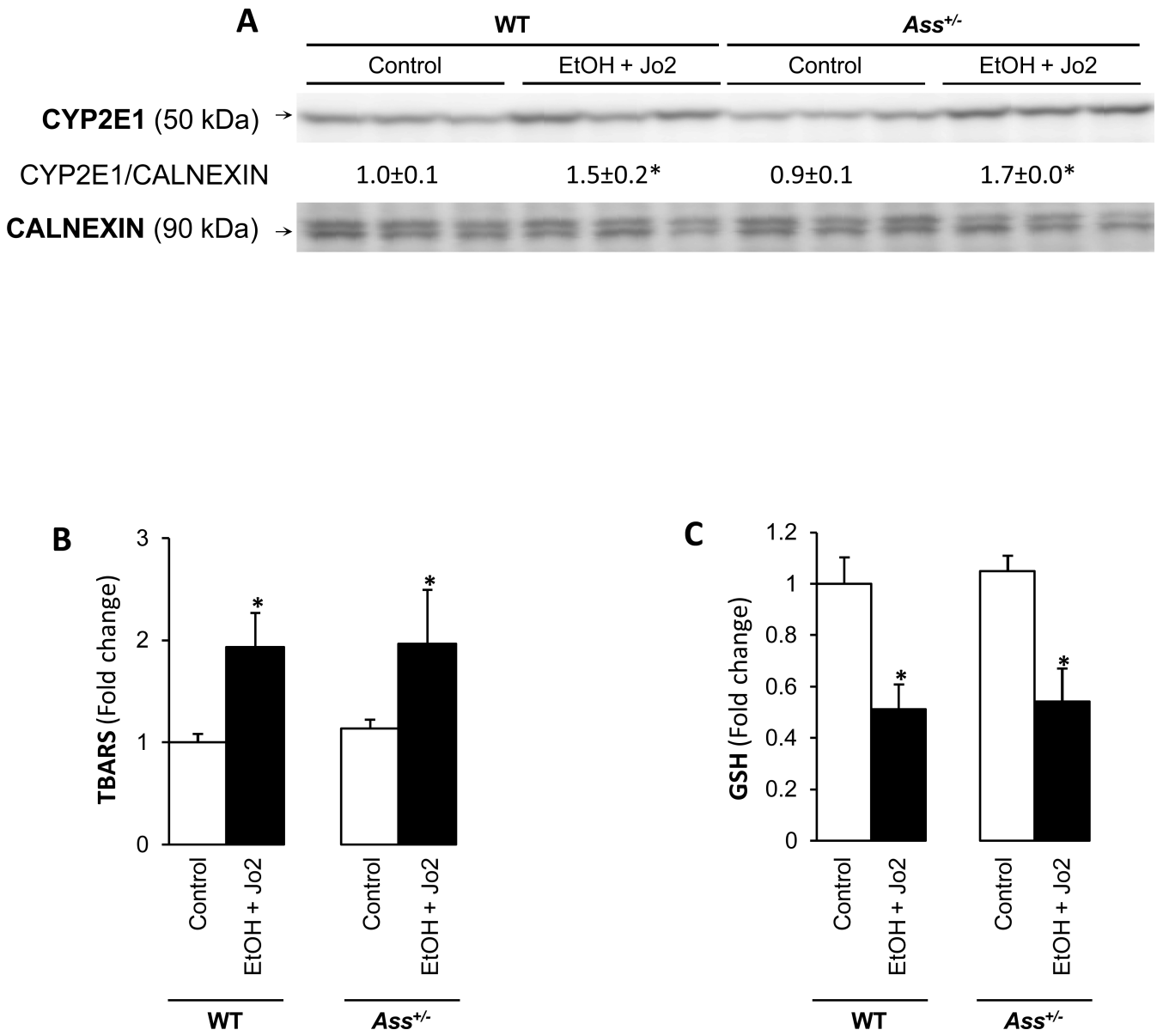


Figure 3. Ethanol plus Jo2 increase TBARS and decrease GSH both in WT mice and in *Ass*$^{+/-}$ mice

CYP2E1 expression was analyzed by Western blot. The quantification of the intensity of the bands corrected by that of Calnexin is indicated under the blots (**A**). The increased lipid peroxidation-end products measured by TBARS is comparable in ethanol plus Jo2-treated WT mice and in *Ass*$^{+/-}$ mice (**B**). GSH equally decreased in ethanol plus Jo2-treated WT mice and in *Ass*$^{+/-}$ mice (**C**). Results are expressed as average values ± SEM; n=6, *p<0.05 for ethanol plus Jo2 *vs* control.

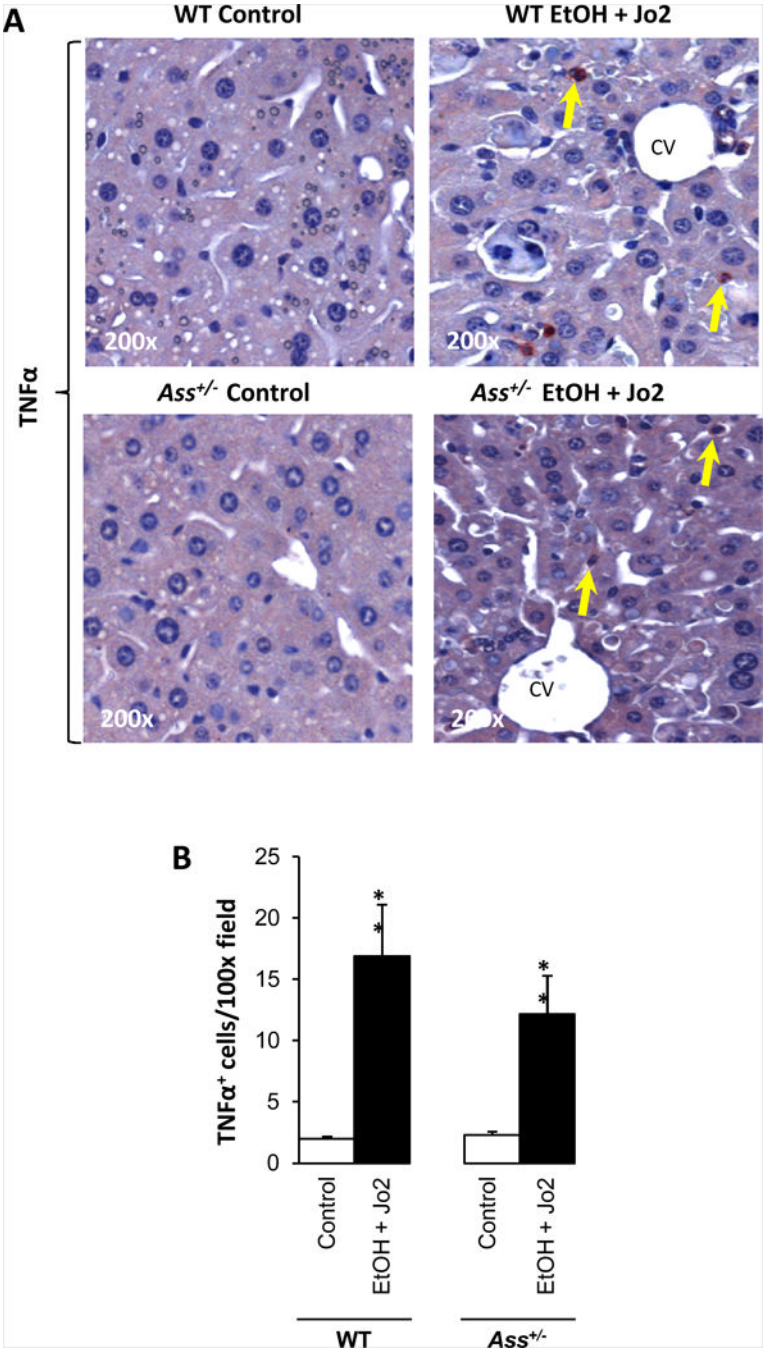
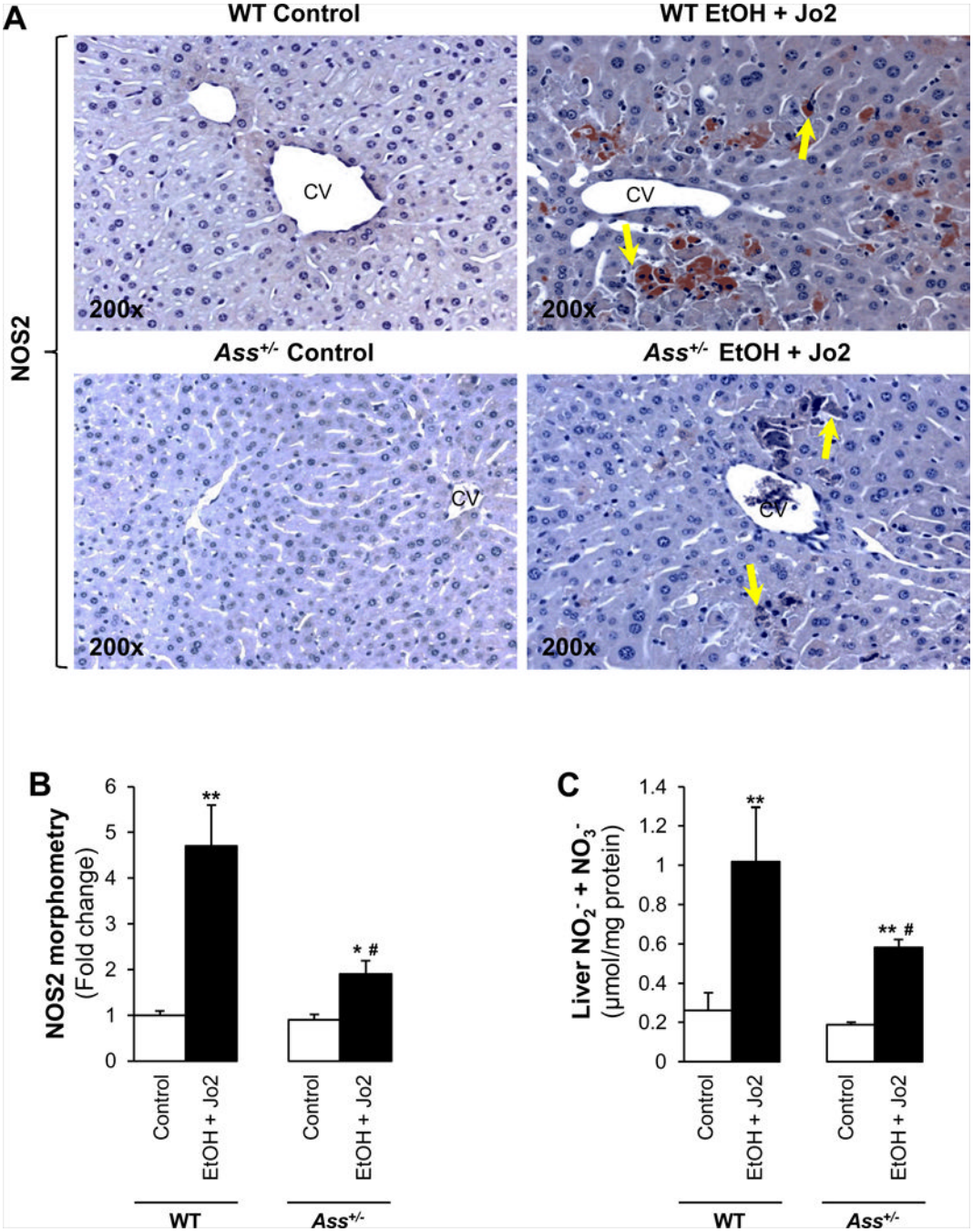


Figure 4. The increase in TNF-α is similar in ethanol plus Jo2-treated WT mice and in *Ass*$^{+/-}$ mice

IHC analysis demonstrated an increase in TNF-α expression (yellow arrows) in ethanol plus Jo2-treated WT mice and in *Ass*$^{+/-}$ mice but there was no significant difference (**A**). The number of TNF-α^{+} cells is quantified in (**B**). CV: central vein. Results are expressed as average values ± SEM; n=6, $^{**}p<0.01$ for ethanol plus Jo2 *vs* control.



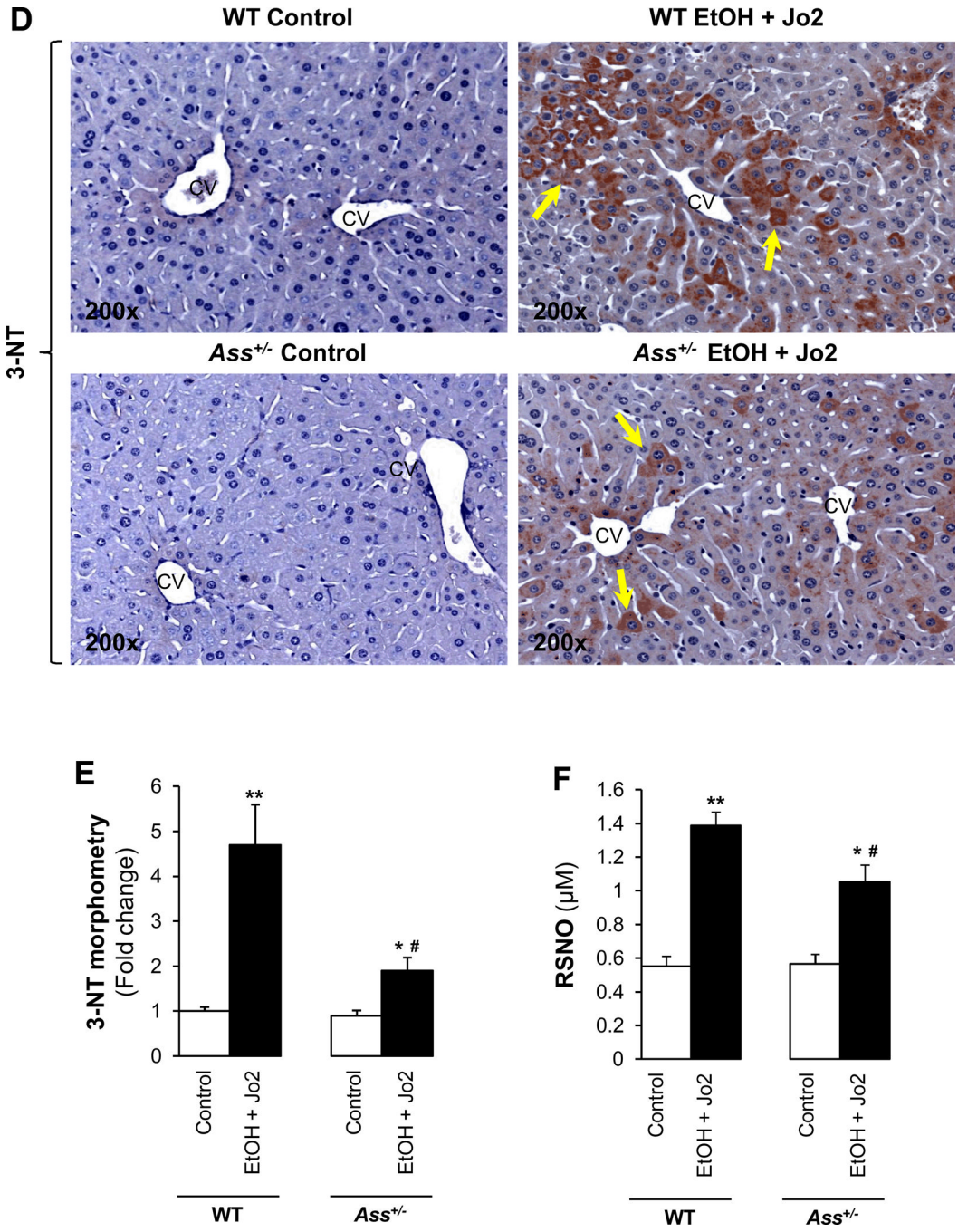


Figure 5. *Ass*$^{+/-}$ mice are protected from ethanol plus Jo2-induced nitrosative stress

IHC analysis demonstrated greater induction of NOS2 protein (yellow arrows) in ethanol plus Jo2-treated WT mice compared to *Ass*$^{+/-}$ mice (**A**). The morphometric analysis is shown in (**B**). Liver nitrites plus nitrates were higher in WT mice than in *Ass*$^{+/-}$ mice injected with ethanol plus Jo2 (**C**). IHC analysis depicting an increase in 3-NT protein residues in ethanol plus Jo2-treated WT mice compared to *Ass*$^{+/-}$ mice (**D**). The morphometric analysis is shown in (**E**). Hepatic RSNO (R is any molecule and *S*-NO is *S*-nitrosothiols) (**F**). CV: central vein. Results are expressed as average values ± SEM; n=6, *p<0.05 and **p<0.01 for ethanol plus Jo2 *vs* control; #p<0.05 for *Ass*$^{+/-}$ *vs* WT.