The In Vivo Effects of Alcohol in Lung and Liver are at Least Partially Mediated through the Alpha 4 Nicotinic Acetylcholine Receptor

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Abstract

Rational: Chronic alcohol abuse is a major risk factor for the development of acute lung injury, with 40% of annual cases in the U.S. linked to this disorder. Alcohol is not only associated with increased incidence of acute lung injury in at-risk individuals, but also increased mortality. The exact mechanisms by which alcohol abuse renders the host susceptible to acute lung injury remain poorly defined. We have reported that α4 nicotinic acetylcholine receptors (α4 nAChRs) may serve as potential sensors for alcohol in lung fibroblasts; however, we have not tested their role *in vivo*.

Methods: To test the role of α4 nAChRs in mediating alcohol-related events *in vivo*, we generated α4 nAChR knockout (KO) animals in C57Bl/6 using Crispr/Cas technology. Wildtype (WT) and α4 nAChR knockout (α4 KO) animals were used to harvest primary lung fibroblasts for study *in vitro*. *In vivo* experiments included exposure to Lieber DeCarli isocaloric or Maltose-Dextrin control diet for 6 weeks.

Results: Having ensured that the α4 KO animals indeed lacked the α4 nAChR, we isolated primary lung fibroblasts and evaluated their expression of the matrix glycoprotein fibronectin after exposure to nicotine (50 ug/ml) or alcohol (60 mM). As expected, nicotine induced fibronectin expression independent of the presence or absence of α4 nAChRs. In contrast, alcohol induced fibronectin mRNA expression in primary lung fibroblasts harvested from WT animals, but not from α4 KO animals. We then engaged in *in vivo* studies designed to examine the expression of specific genes in whole lung and liver; including the cysteine transporter Slc7a11 (which controls redox state), the pro-inflammatory cytokine TNFα (which has been implicated in alcohol-induced lung injury), and the protease inhibitor PAI-1, (which also appears involved in alcohol-related injury to lung and liver). No overt structural abnormalities were detected in the α4 KO animals. After 6 weeks of control or alcohol diets, lungs and livers were harvested and processed for mRNA evaluation. WT lungs and livers showed significant induction of all three mRNAs when exposed to alcohol, whereas the α4 KO animals showed little to no induction. Liver histology also showed evidence of increased steatosis in WT animals when compared to the α4 KO animals.

Results

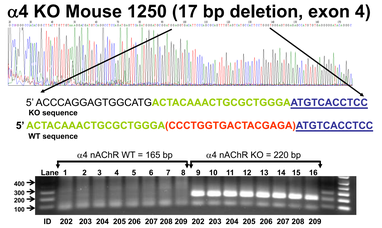

Fig. 1. Analysis of the exon splicing phase of transcript. Chrna4-QO1 showed that mutation of exon 4 will produce appropriate loss of function. The Crispr/Cas9 system using SgRNA (CCCATCTGAA CTCATCTGG GG) to specifically target the α4 nAChR gene and generate a Double Strand Break (DSB) in the genomic DNA of the desired gene was employed. The Refractory Oligonucleotide Mutational Allele Notification System (**ROMANS**) was developed to detect the α4 nAChR gene mutation.

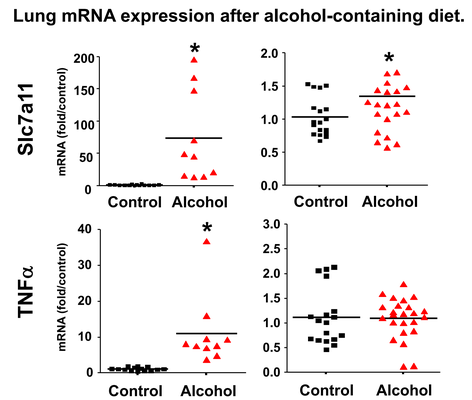

Fig. 3. The lungs of α4 KO animals fail to express certain genes in response to alcohol-containing diet. WT and α4 KO animals were exposed to control or alcohol-containing diet for 6 weeks. Afterwards, the animals were euthanized and the lungs were harvested and processed for mRNA isolation. **A**, Slc7a11 was significantly increased in WT lung, but much less in α4 KO lungs. **B**, TNFα was significantly induced in WT, but not in α4 KO lungs. **C,** PAI-1 was also significantly induced in WT, but not in α4 KO lungs. Note the differences in Y axis numbers.

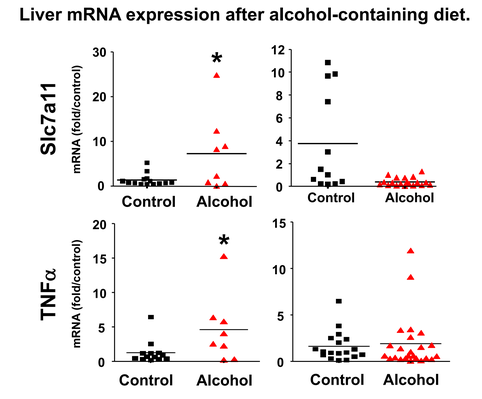

Fig. 4. The livers of α4 KO animals fail to express certain genes in response to alcohol-containing diet. WT and α4 KO animals were exposed to control or alcohol-containing diet for 6 weeks. Afterwards, the animals were euthanized and the livers were harvested and processed for mRNA isolation. **A**, Slc7a11 was significantly increased in WT livers but not in the α4 KO livers. **B**, TNFα was significantly induced in WT, but not in α4 KO livers. **C,** PAI-1 was also significantly induced in WT but not in α4 KO livers. Note the differences in Y axis numbers.

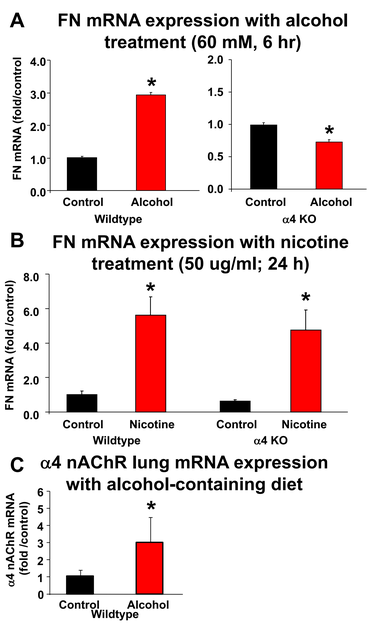

Fig. 2. Effects of alcohol in lung. Primary lung fibroblasts isolated from wildtype or α4 KO mouse lungs showed the alcohol-induced expression of the matrix glycoprotein fibronectin was inhibited in the absence of α4 nAChRs **(A)**, while nicotine (which typically works through α7 nAChRs) was able to induce fibronectin in the absence of α4 **(B)**. Lung tissue from wildtype animals exposed to control or alcohol-containing diet showed increased expression of α4 nAChR mRNA **(C)**.

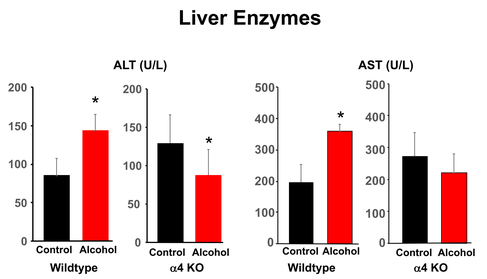

Fig. 5. Effects of alcohol in the livers of WT and α4 KO animals. WT and α4 KO animals were exposed to control or alcohol-containing diet for 6 weeks. Afterwards, the animals were euthanized and liver enzyme activity (Alanine Aminotransferase-ALT, Aspartate Aminotransferase-AST) was measured. Liver damage, as indicated by the increase in liver enzyme activity, was more prominent in the liver tissue of WT animals exposed to alcohol when compared to α4 nAChR KO mice.

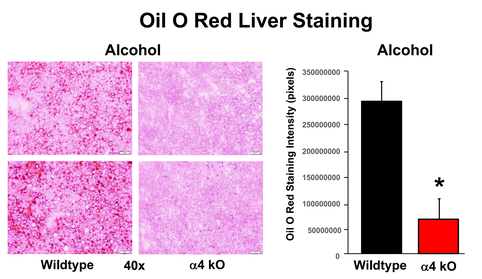

Fig. 6. Effects of alcohol in the livers of WT and α4 KO animals. WT and α4 KO animals were exposed to control or alcohol-containing diet for 6 weeks. Afterwards, the animals were euthanized and the livers were harvested and processed for histological analysis. Liver damage, as indicated by the presence of oil O Red stained fatty lipid droplets, was more prominent in the liver tissue of WT animals exposed to alcohol when compared to α4 nAChR KO mice.

Conclusions

1. α4 deficient animals do not demonstrate obvious pulmonary or liver structural abnormalities (not shown).
2. In primary lung fibroblasts, alcohol stimulates the expression of fibronectin via α4 nAChRs, while nicotine acts via other nAChRs (likely α7) (Fig. 2).
3. Alcohol not only acts via α4 nAChRs, it also enhances its expression (Fig. 2).
4. In vivo, alcohol stimulates the expression of several inflammatory markers and a cysteine transporter in lung via α4 nAChRs (Fig. 3).
5. By acting on α4 nAChRs, alcohol promotes inflammation in liver as highlighted by increased liver transaminases (Fig. 4), increased expression of inflammatory markers (Fig. 5), fat accumulation (Fig. 6).

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Acknowledgements

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