Reviewer's report

Title: Dietary Diallyl Disulfide supplementation attenuates ethanol-mediated pulmonary vitamin D speciate depletion in C57Bl/6 mice.

Version: 3 Date: 14 February 2015

Reviewer: Adrian Gombart

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The authors propose a study to determine how chronic, excessive ethanol consumption reduces circulating vitamin D levels. They hypothesize that ethanol induces CYP2E1 which mediates the reduction of pulmonary 1,25(OH)2D3 and that the CYP2E1 inhibitor diallyl disulfide (naturally occurring compound in garlic) can attenuate this reduction.

To test this hypothesis, authors fed mice water or water containing 20% ethanol ad libitum for 8 weeks. In addition mice received either control chow or chow containing 0.15 microgram/day diallyl disulfide (DADS). They euthanized mice and collected whole blood, lung tissue and bronchial alveolar lavage fluid (BALF) and measured 25(OH)D3, 1,25(OH)2D3 vitamin D receptor (VDR), CYP27B1 and CYP2E1 levels. CYP2E1 protein levels increased about 35% and CYP27B1 levels decreased about 35% in lung tissue. These levels returned toward control levels with DADS treatment. They identified an approximately 35% decrease in 25(OH)D3 levels and a 50% decrease in 1,25(OH)2D3 levels in lung tissue and BALF with alcohol consumption, but levels were restored to levels similar to the control treated mice with the DADS diet. No change in serum levels of 25(OH)D3 were noted with feeding of alcohol. Alcohol consumption increased VDR levels by more 2-fold in the lung, but DADS feeding reduced the levels back to those of the controls.

Major Compulsory Revisions

1. The authors concluded that altered metabolism of vitamin D in pulmonary tissues by heavy alcohol consumption “can be ameliorated by dietary supplementation in C57BL/6NCRL mice”. They further speculated that this could lead to changes in vitamin D-target genes in their discussion, but target gene expression was not examined in any of the samples. Is the expression of VDR target genes altered in the lungs of alcohol-fed rats and reversed in alcohol/DADS-treated animals to levels observed in the controls? In fact, a very logical explanation, that they did not consider, for the results they observed (decreased 1,25(OH)2D3 levels) would be the induction of CYP24A1 (a very well-described target gene of the VDR and strongly induced by 1,25(OH)2D3) that catabolizes 1,25(OH)2D3. This was observed in chickens by Kent et al. (ref. 19 in this manuscript) and more recently by Shankar et al. in rats (Shankar K, Liu X, Singhal R, Chen JR, Nagarajan S, Badger TM, Ronis MJ. Chronic ethanol consumption leads to disruption of vitamin D3 homeostasis associated with induction of renal 1,25 dihydroxyvitamin D3-24-hydroxylase (CYP24A1).
In brief, Shankar et al. demonstrated that ethanol treatment reduced plasma 1,25(OH)2D3 concomitantly with a decrease in renal CYP27B1 and an increase in CYP24A1. Ethanol metabolizing enzymes alcohol dehydrogenase-1 (ADH-1) and CYP2E1 were induced in the kidney and blocking ethanol metabolism with 4-methylpyrazole (an ADH inhibitor) prevented the induction of CYP24A1. Further they demonstrated that MAPK signaling pathways were involved in ethanol-mediated induction of CYP24A1. Their major conclusion was that ethanol induces CYP24A1 via MAPK signaling that results from renal oxidative stress produced by local metabolism of ethanol via CYP2E1 and antidiuretic hormone-1. It seems that the study presented in this manuscript could be greatly informed by the Shankar et al. study. The authors of this current manuscript focused on lung tissue and BALF levels of vitamin D. As the kidney is a key organ in vitamin D synthesis and metabolism, they should examine renal expression of CYP27B1, CYP24A1 and CYP2E1 as well as expression of these in the lung. Does this study recapitulate what was observed in the kidney of rats? Is CYP24A1 increased in the lung by alcohol and reduced with DADS feeding? The authors must cite this work and determine if some or all of these pathways are conserved in the lung.

2. The manuscript would benefit greatly from a discussion that relates these findings to the human condition. There are papers that discuss association of dietary agents like garlic in protecting against alcohol-induced hepatotoxicity. There are studies regarding alcohol consumption and vitamin D that might be relevant. While it is great to treat mice, is there evidence from human studies (e.g. epidemiologic studies) that DADS treatment might protect humans from dysregulation of vitamin D metabolism due to excessive alcohol consumption?

3. To translate these findings to the human condition, a human lung cell line or human bronchial epithelial cells should be used to demonstrate that alcohol exposure increases CYP2E1 (and possibly CYP24A1) and decreases CYP27B1 expression to alter metabolism of 25(OH)D3 to 1,25(OH)2D3 and that DADS treatment prevents these changes. In addition, if these experiments were performed in the presence or absence of 25(OH)D3, then the authors could examine the effect of alcohol and DADS treatment on expression of cathelicidin as it is regulated by vitamin D in human cells and not mice. This would strongly support their hypothesis that immune responses regulated by vitamin D are impaired by the consumption of alcohol. The authors have used human lung cells in prior studies with alcohol and DADS (see Sapkota M, Hottor TK, DeVasure JM, Wyatt TA, McCaskill ML. Protective role of CYP2E1 inhibitor diallyl disulfide (DADS) on alcohol-induced malondialdehyde-deoxyguanosine (M1dG) adduct formation. Alcohol Clin Exp Res. 2014 Jun;38(6):1550-8. doi: 10.1111/acer.12439. Epub 2014 May 30. PubMed PMID:24891074; PubMed Central PMCID: PMC4049196.

4. Citations that do not support the authors’ statements adequately are being
used throughout the discussion. The authors should reread their discussion and scrutinize the statements and arguments being made and make sure that the citations are appropriate or sufficient. For example, on p. 13, line 369, the authors state, “This ethanol-mediated active vitamin D depletion could have wide-ranging effects on inflammation and innate immune response, which could partially explain statistically higher nosocomial infection incidence and mortality in hospitalized ethanol abusers [45,46].” These references have nothing to do with studies regarding infection and mortality of hospitalized ethanol abusers and vitamin D levels. They do address regulation of antimicrobial peptides by vitamin D and protection against infection and how inflammation contributes to mortality in pneumonia and sepsis, but nothing about alcohol. It seems that additional references are needed to support this statement. Also, on p. 13, line 390, the authors state, “This observation also leads us to infer that CYP2E1-mediated ethanol metabolism and/or metabolic intermediates are negative regulators of CYP27B1 function leading to reduced 1,25(OH)2D3 levels. This theory is supported by published work [52], as well as the observed lack of efficacy of DADS supplementation in attenuating ethanol-mediated reductions in lung tissue 25(OH)D3 as compared to the observed efficacy of DADS in recovering lung tissue levels of 1,25(OH)2D3.” Citation 52 only demonstrates that acetaldehyde is a substrate for CYP2E1 it does not show that metabolites are negative regulators of CYP27B1 so it is not clear how this citation supports their theory. Again citations 58 and 59 in the conclusion are focused on childhood deaths from respiratory infections and this reviewer does not understand the connection between excessive alcohol consumption and children suffering from infection. It seems that the work presented in this manuscript would be more relevant for a population that is consuming excessive alcohol, suffering from vitamin D deficiency and dying from lung infections. Citations demonstrating these links would be more appropriate.

Discretionary revisions

1. Although there are statistically significant changes in gene expression or protein levels are these biologically significant? Do they actually lead to increased or decreased enzyme activities? These activities could be measured in extracts made from treated cells and incubated with tritiated substrates. The resulting metabolites could be measured by HPLC. These experiments could be performed in the human lung cells in vitro if the findings identified in mice are conserved. This would strengthen the conclusions of the manuscript, otherwise a limitation of the study is that it is not known if CYP27B1 or CYP24A1 is responsible for the reduction in 1,25(OH)2D3 levels.

Minor essential revisions

1. The Introduction should explain briefly how alcohol is metabolized by the body and the role that CYP27E1 plays. This would clarify why its expression is being studied.

2. How many samples were included in the data represented in the bar graphs? Were all 8 animals for each arm analyzed?

3. Were the serum 25(OH)D3 levels measured using the same assay in lung and
blood? In other words, did the UNMC clinical analytical laboratory used the same assay (IDS, Inc.) as was used for the lung lysates?

4. p. 10, line 267 the authors should use the IDS abbreviation since it is already defined earlier on the page.

5. In the results it is stated that a 51% reduction in 25(OH)D3 levels was observed in lung tissue of ethanol-fed mice (p. 11, line 302); however, it is then stated that a 31% reduction in 25(OH)D3 was observed (p. 11, line 309). Which is correct or are these two different experiments with somewhat different results?

6. In Fig. 1B, the ethanol/DADS is not discussed in the Results section for lung tissue where it seems that the 25(OH)D3 levels are significantly lower than the control and similar to the ethanol only treatment.

7. How are Figs. 1B and and 2A different? Supplementation with DADS did not restore lung tissue levels of 25(OH)D3 and this is not described in the Results. Why is data presented in figures, but not described in the text? The titles on Fig. 2A and B mention “25(OH)D3 protein in lung...” or “1,25(OH)2D3 protein in lung...”. These are not proteins. The titles are confusing.

8. In Fig. 3A, it seems that data from Fig. 2B is being reused. This should not be done unless it is from another set of separate experiments. In both 3A and 3B it is not clear how the last two columns of data (ethanol/D3 and ethanol/D3/DADS) were generated. What were the experiments? No description in results, legends or M&M. The ~ sign above the ethanol/D3 column is not defined.

9. p. 13, line 383, should CYP7B1 be CYP27B1?

10. The use of “to within statistical indifference of control levels” is confusing. Why not say that levels were restored to those observed in the control treatment? This comment applies throughout the text of the manuscript.

11. In Fig. 5, the ~ sign above the ethanol/DADS column is not defined in panels A or B and in the figure legend a # is defined, but not present on the figure. The comparison of DADS treatment alone with control, ethanol or ethanol/DADS is not described.

12. Check references for correct spelling and capitalization, for example citation 45 has “Mortality” spelled as “MOrtality”.