Differential Expression of TGFβ1 and ATF3 During Alcoholic Lung Oxidative Stress

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Abstract

Rationale: Our group previously showed that transforming growth factor beta-1 (TGFβ1) is implicated in the pathogenesis of chronic pulmonary oxidative stress in response to alcohol, and that this occurs via suppression of Nrf2-ARE function. The cellular defense against free radicals is handicapped, and in mouse models[2], susceptibility to acute lung injury is increased. The mechanism by which TGFβ1 is regulated under alcohol exposure is currently unknown.

In human airway smooth muscle cells[3], activating transcription factor-3 (ATF3) is induced by TGFβ1 and represses Nrf2 by interfering with CREB complex formation at the ARE. In a feedback loop, ATF3 expression appears to be dependent on Nrf2[4]. We hypothesized that this mechanism also occurs in response in the setting of acute and/or chronic alcohol exposure.

Methods: 3-month old C57bl/6 mice were given ethanol in their drinking water, increased by 5% (v/v) weekly until a final concentration of 20%. Lungs were harvested at 8 weeks. Both arms received standard chow.

Primary lung fibroblasts (PLF) were generated from wild-type mice by cell migration out of cultured thin lung slices in the method described previously[5]. Low passage cells were then subjected to treatment ± 60 mM ethanol for 24 hours.

NIH/3T3 fibroblasts were expanded in standard tissue culture to 80% confluent monolayer, then

(1) treated ± 60 mM ethanol for 6 hours; or
(2) transfected with 5 nM Nrf2 siRNA oligos versus scrambled siRNA as control, to achieve Nrf2 gene knockdown. The reaction was carried out in HiPerFect reagent (Qiagen) for 6 hours to mitigate cell death; or
(3) treated ± 5 ng/mL murine recombinant TGFβ1 (rTGFβ1) for 20 hours

Total RNA was purified from whole lung homogenate or trypsinized cell lysate respectively. Expression of ATF3 and TGFβ1 mRNA was quantified by RT-qPCR, normalized to 18s rRNA. Nrf2-ARE promoter activity was assayed by ARE/luciferace transfection, normalized to firefly/luciferace signal.

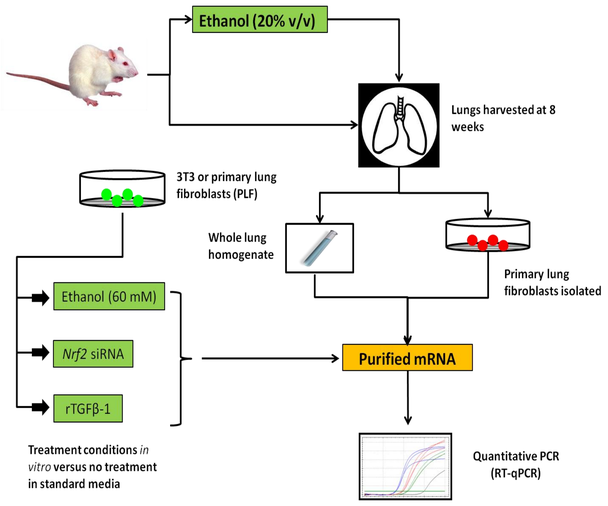
Results: Ethanol induces ATF3 expression by over 70-fold ($p < 0.01$) in primary lung fibroblasts when treated for a short duration (72 hrs). However, no change was observed in whole lung samples of alcohol-adulterated mice at 8 weeks.

3T3 fibroblasts treated with ethanol (72 hrs) show a 2.5-fold induction of TGFβ1 and mice in the alcohol treatment group demonstrated an 8-fold increase ($p < 0.01$).

Treatment of 3T3 fibroblasts with TGFβ1 results in 5-fold induction of ATF3 ($p < 0.01$) and suppresses Nrf2-ARE activity.

Conclusion: The stress-induced transcription factor ATF3 is upregulated in acute alcohol exposure. This is probably mediated by an upsurge in TGFβ1, which remains upregulated in both acute and chronic settings.

Methods Schematic



Results

Figure 1. Acute alcohol treatment results in early induction of ATF3 *in vitro*. (A) Primary lung fibroblasts treated with ethanol at 60 mM for 24 – 72 hours showed marked induction of ATF3. Data represented on $\log_{10}$ scale. ATF3 was hyperacutely repressed (> 32-fold, data not shown) at 6 hours. **(B) Chronic alcohol treatment** *in vivo* does not cause sustained induction of ATF3 expression. Whole lungs harvested from mice treated for 8 weeks with 20% ethanol did not show persistent induction of ATF3 ($n = 5$ in control and $n = 4$ in treatments groups).
* $P < 0.05$

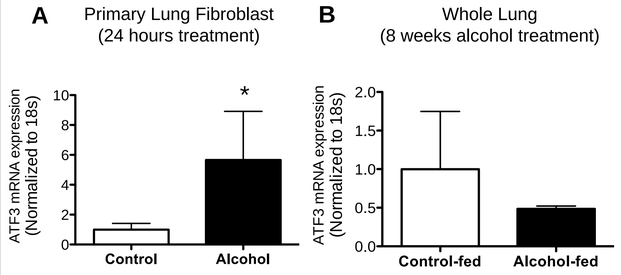
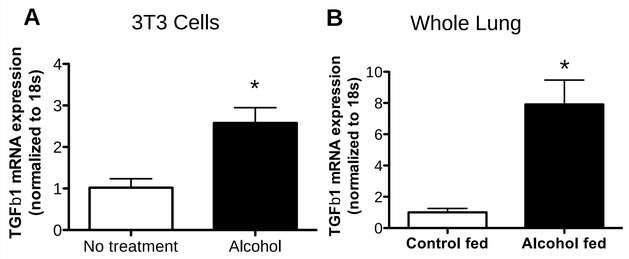


Figure 2. TGFβ1 remains upregulated in both acute and chronic alcohol exposure. (A) 3T3 fibroblasts treated with ethanol (60 mM) for 6 hr and **(B)** mouse whole lungs under the 8-week feeding regimen were analyzed for TGFβ1. **(C) TGFβ1 detected by ELISA tends to be enriched in 8 weeks alcohol-fed mouse broncheoalveolar lavage fluid (BAL)**($p = 0.38$). * $P < 0.05$. $n = 5$-8.



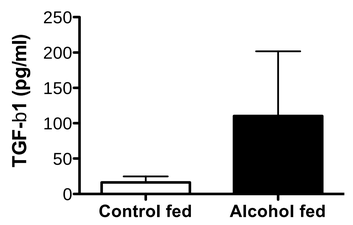


Figure 3. (A) Cell treatment with recombinant TGFβ1 results in inhibition of NRF2-ARE activity. 3T3 fibroblasts were treated with 5 ng/mL of rTGFβ1 and assayed using ARE-luciferace normalized to firefly. ($n = 8$) **(B) The same treatment results in acute induction of ATF3.** 3T3 cells ($n = 5$) showed 5-fold induction of ATF3 ($p < 0.01$), consistent with what has been reported in smooth myocytes.

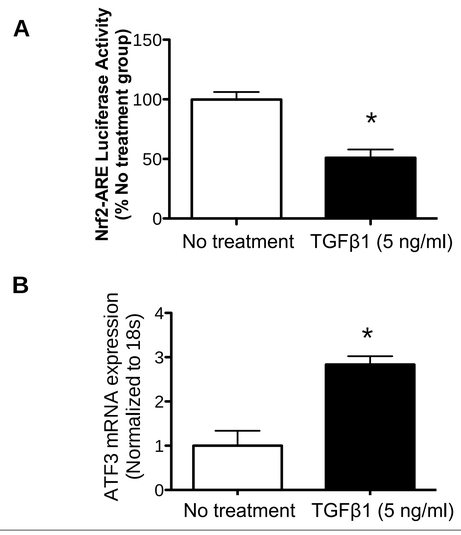
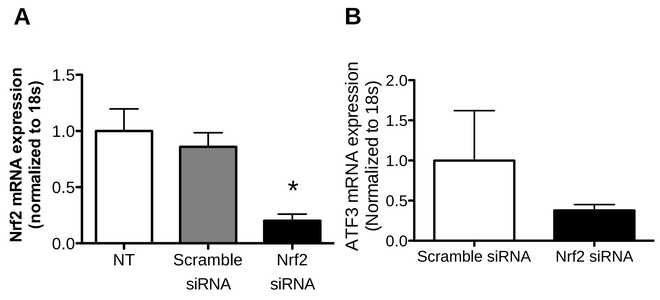


Figure 4. (A) RNA interference techniques resulted in effective knockdown of Nrf2 gene expression. 3T3 cells were transfected with 5 nM anti-Nrf2 siRNA or scrambled sequence, respectively, for 6 hours ($n = 6$). **(B) Transfection with anti-Nrf2 siRNA tended to repress ATF3** ($p = 0.35$, $n = 4$). NT = No treatment.



Discussion

The master redox signaling axis, Nrf2, is subject to multiple modes of regulation. At the transcriptional level, Nrf2 gene expression and ARE activity appear to be suppressed by TGFβ1, which we show here to be induced in 3T3 pulmonary fibroblasts treated acutely with ethanol *as well as* in the whole lungs of chronically alcohol-fed mice.

ATF3 is known to be controlled by TGFβ1 in several respiratory cell types, and acts as a direct repressor upstream of the ARE, offering a mechanism for how TGFβ1 suppresses Nrf2-ARE activity. We showed that in lung fibroblasts, ATF3 is acutely induced by alcohol and recombinant TGFβ1, as expected. What is surprising is the lack of sustained induction in alcohol-fed mice at 8 weeks.

These data suggest that acute regulation of ATF3 expression occurs in a TGFβ1-dependent manner in early alcohol exposure, but other mechanisms may prevail during the chronic phase. Further studies are needed to study the regulation of Nrf2-ARE activity during the temporal evolution of alcohol-induced oxidative stress.

References

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