Novel mechanisms in alcohol neurodevelopmental disorders via BRCA1 depletion and BRCA1-dependent NADPH oxidase regulation

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ARTICLE INFO
Keywords: Breast cancer 1 susceptibility gene (Brca1) Catalase Neurodevelopmental disorders NADPH oxidase (NOX) Proteasomal activity

ABSTRACT
The breast cancer 1 protein (BRCA1) facilitates DNA repair, preventing embryolethality and protecting the fetus from reactive oxygen species (ROS)-induced developmental disorders mediated by oxidatively damaged DNA. Alcohol (ethanol, EtOH) exposure during pregnancy causes fetal alcohol spectrum disorders (FASD), characterized by aberrant behaviour and enhanced ROS formation and proteasomal protein degradation. Herein, ROS-producing NADPH oxidase (NOX) activity was higher in Brca1 +/− vs. +/+ fetal and adult brains, and further enhanced by a single EtOH exposure. EtOH also enhanced catalase and proteasomal activities, while conversely reducing BRCA1 protein levels without affecting Brca1 gene expression. EtOH-initiated adaptive postnatal freezing behaviour was lost in Brca1 +/− progeny. Pretreatment with the free radical spin trap and ROS inhibitor phenylbutylnitrone blocked all EtOH effects, suggesting ROS-dependent mechanisms. This is the first in vivo evidence of NOX regulation by BRCA1, and of EtOH-induced, ROS-mediated depletion of BRCA1, revealing novel mechanisms of BRCA1 protection in FASD.

1. Introduction
Breast cancer 1 (BRCA1) regulates DNA repair and protects against breast cancers arising in cells with homozygous (−/−) BRCA1 mutations or silenced BRCA1 expression [1,2]. Silencing/mutation of both Brca1 alleles also results in embryolethality [3], Brca1 CNS-specific −/− KO mice are viable but exhibit impaired development of brain structures, resulting in ataxia and a shortened lifespan [4]. Thus, tissue-specific or global homozygous deficiencies in BRCA1 can respectively cause serious developmental disorders or lethality.

We previously reported that heterozygous (+/−) Brca1 conditional knockout (KO) embryos exhibiting 28% lower BRCA1 develop normally, but when exposed in culture to ethanol (EtOH) exhibit enhanced DNA damage and embryopathies compared to wild-type (+/+) littermates [5]. In a standard KO model, saline-exposed +/− Brca1 KO embryos exhibiting 58% lower BRCA1 exhibited enhanced DNA damage in fetal brain and postnatal cognitive disorders, which were sustained or worsened by EtOH [6–8]. In human +/− BRCA1 subjects with varying mutations [9,10], DNA repair is slowed/halted [10–12]. Variable mutations in BRCA1 heterozygotes can result in different levels or functional efficacy of BRCA1 protein and DNA repair capacity [13].

During oxidative stress, proteins can be oxidized, enhancing their regeneration or removal mechanisms. Signaling proteins with essential roles in cell cycle progression, like BRCA1, contain intrinsically disordered sequences making them more susceptible to 20S proteasomal degradation [14]. Under oxidizing conditions, expression of the 20S proteasome is in part regulated by the ROS sensor nuclear factor erythroid 2-related factor 2 (NRF2) and constitutes the major protein degradation machinery [14–16]. EtOH-enhanced ROS formation has divergent concentration-dependent effects, with proteasomal activity being respectively enhanced and inhibited by low and high oxidative stress levels [17,18].

In utero EtOH exposure during pregnancy can cause fetal alcohol spectrum disorders (FASD) [19], including growth retardation, craniofacial defects and impairment of executive function, motor coordination, visual-spatial abilities and learning and memory [20–22]. EtOH exposure can enhance ROS production via induction of NADPH oxidases (NOXs) [23,24], its CYP2E1-catalyzed metabolism [25], and via the regeneration of NAD + for alcohol and aldehyde dehydrogenase activity [26–28]. NOXs produce superoxide anions [29], which participate in neural cellular signaling and in the immune response to pathogens [30,31]. EtOH-induced ROS enhancement can contribute to multiple cellular effects implicated in FASD mechanisms, including DNA damage [26],

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https://doi.org/10.1016/j.redox.2021.102148
Received 11 May 2021; Received in revised form 20 September 2021; Accepted 20 September 2021
Available online 23 September 2021
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epigenetic changes [32–34], altered protein levels and modifications affecting signal transduction pathways [35,36] and cellular processes (e.g. apoptosis) [32].

Herein, a single in utero EtOH exposure, equivalent to 4–6 drinks [37], enhanced fetal and adult ROS production and depleted protective BRCA1 protein levels in fetal and adult brains, revealing a new mechanism by which EtOH initiates developmental disorders. BRCA1 depletion resulted from EtOH-enhanced proteasomal activity, and Brca1 expression was unaltered. Proteasomal enhancement was blocked by pretreatment with a free radical spin trap and ROS inhibitor [38,39], phenylbutylnitrone (PBN). NOX and catalase activities also were enhanced by EtOH exposure over a time course congruent with the reduction in BRCA1 protein and prevented by PBN pretreatment. Wild-type (+/+) Brca1 progeny but not +/− littermates exposed in utero to EtOH exhibited postnatal freezing behaviour in a conditioned fear study, prevented by PBN pretreatment, suggesting ROS-mediation.

Even saline-exposed Brca1 +/− adults and progeny exhibited increased baseline brain NOX levels, revealing a new mechanism by which BRCA1 regulates ROS-dependent disorders, and enhances susceptibility to FASD.

2. Methods

2.1. Animals

Animal studies were conducted in accordance with Canadian Council on Animal Care standards and were approved by the institutional Animal Care Committee. Up to 4 +/− Brca1 KO mice (01XC4; RRID: IMSR_NCIMR:01XC4; National Institute of Health, Frederick, MD [40]) were housed per cage in light- and temperature-controlled rooms (14 h light/10 h dark cycle, 20 °C, 50% humidity) with corn bedding. Teklad 18% protein rodent chow (Harlan Laboratories, Mississauga, ON) and tap water were provided ad libitum. Mating and treatments are detailed in the supplementary data.

2.2. Genotyping

Brca1 [41] and sex [42] genotyping were performed as described in available protocols. DNA extraction and PCR details are indicated in the supplementary data.

2.3. NOX activity

NOX activity was assessed as previously described [23,43], with some modifications indicated in the supplementary data.

2.4. Catalase activity

Catalase activity was assessed as previously described [44,45], with some modifications indicated in the supplementary data.

2.5. 20S proteasomal activity

Activity of the 20S catalytic core was measured by fluorometric assay (MAK172, Millipore Sigma), where details are indicated in the supplementary data.

2.6. BRCA1 protein

BRCA1 protein levels were measured via western blotting performed as described in Drake et al. [46], and detailed in the supplementary data.

2.7. Brca1 expression

RNA extraction and RT-qPCR protocols were performed as described previously [47], using primers validated in +/+ Brca1 fetal brains. Details are outlined in the supplementary data.

2.8. Freezing behaviour

Learning & memory was assessed by passive avoidance as described previously [48], with additional observation of freezing behaviour. Details are outlined in the supplementary data.

2.9. Statistical analyses

Data for fetal brains from the same litter for each genotype and treatment group were averaged and analyzed using the number of litters per group as the experimental unit. Sex differences were not observed for any assessments, so male and female data were combined for statistical analysis using Prism, Version 7 (GraphPad Software, Inc., San Diego, CA). Differences in Brca1 expression and protein levels, and proteasomal, NOX and catalase activities, were evaluated by two-way ANOVA with Bonferroni post-tests at each timepoint. NOX and proteasomal activities over time were assessed by linear regression, where Pearson correlation coefficients (R²) values > 0.9 indicated a good fit and slopes were compared using the F-statistic (Supplementary data, Tables S1 and S2). Freezing behaviour was analyzed by the Wilcoxon Signed Rank Test, and which group displaying freezing behaviour was unknown until the data were analyzed. Group comparisons were evaluated at the 5%, 2-tailed probability level.

3. Results & discussion

Fetal and adult brains exhibited similar levels of superoxide production by NOX enzymes (Fig. 1A–C). However, Brca1 +/− fetal and adult brains exhibited enhanced NOX activity relative to +/+ littermates and adults, independent of treatment and time post-exposure. This corroborates reciprocal relationships between BRCA1 expression and (a) NOX subunits observed in tissues from patients with different cancers [49]; and, (b) in vitro ROS levels [50]. NOX activity was increased by EtOH in adult and fetal brains of both genotypes 6 h post-treatment (Fig. 1A&B), and returned to baseline within 24 h. EtOH was previously shown in the embryo to increase NOX activity [23], and enhanced NOX subunit expression in fetal brains [24]. PBN reduced NOX activity in both the saline and EtOH exposure groups (Fig. 1A&B). The ability of PBN to block the EtOH-initiated transient enhancement of NOX activity is consistent with its ROS inhibiting effects observed previously [38,39,51,52]. However, enhanced NOX activity in +/− vs. +/+ fetal brains was retained at 24 h post-treatment (Fig. 1C). The enhanced NOX activity in +/− Brca1 brains may contribute to their greater sensitivity to oxidative DNA damage and warrants further investigation in saline-exposed progeny to determine the pathogenic potential of physiological ROS levels when DNA repair is compromised. Full data sets over time are shown in Supplementary data Fig. S1.

Activity of the antioxidative enzyme catalase is regulated by the ROS sensor NRF2 [53]. In addition to detoxifying hydrogen peroxide,
Fig. 1. (Panels A-C) NADPH oxidase (NOX) activity is enhanced in Brca1 +/- fetal and adult brains and is further induced by ethanol (EtOH). Relative luminescent units (RLU) emitted by lucigenin reaction with superoxide produced by NOX enzymes normalized to time and total protein loaded, measures taken every 2 min over 14 min shown in Fig. S1. Measured in fetal and adult brains from Brca1 +/- vs. +/+ mice post-saline or EtOH administration with and without pretreatment with the ROS blocking agent phenylbutylnitrone (PBN). Data from both sexes were combined because no sex-dependent differences were observed. (Panel A) Brca1 +/- adult brains exhibited 13–60% more superoxide formation relative to +/+ littermates independent of treatment (**p < 0.01, ****p < 0.0001). EtOH enhanced NOX activity by 14–44% relative to saline-exposed controls, 6 h post-exposure (ααα p < 0.001, αααα p < 0.0001). PBN reduced superoxide production by 9–36%, independent of genotype and treatment (β p < 0.05, ββββ p < 0.0001). The number of mice assessed is shown in parentheses above each bar, where samples were split by sex. (Panel B) Brca1 +/- fetal brains exhibited 17–43% more superoxide formation compared to +/+ littermates independent of treatment (***p < 0.001). EtOH enhanced NOX activity by 19–42% relative to saline-exposed controls, 6 h post-exposure (αααα p < 0.0001). PBN reduced superoxide formation by 15–20% in +/- fetal brains independent of treatment (ββββ p < 0.0001), but only by 13% in EtOH-exposed +/- fetal brains (ββββ p < 0.0001). (Panel C) Brca1 +/- fetal brains exhibited 42–46% more superoxide formation relative to +/- littermates, independent of treatment (****p < 0.0001). Effects of EtOH and PBN are no longer evident 24 h post-exposure. Eight fetal brains were split by sex and assessed from the number of litters shown in parentheses above each bar. (Panels D-F) Catalase activity is enhanced in Brca1 +/- adult and fetal brains and is further induced in +/- fetal brains by EtOH. Activity was measured in fetal and adult brains from Brca1 +/- vs. +/- mice post-saline or EtOH administration with and without PBN pretreatment. Data from both sexes were combined because no sex-dependent differences were observed. (Panel D) Brca1 +/- adult brains exhibit 13% enhanced catalase activity compared to +/- littermates (**p < 0.01). Similar 14 and 16% enhancements are observed in the PBN-pretreated saline and EtOH-exposed +/- adult brains compared to +/- littermates, respectively (****p < 0.0001). A similar genotype trend is observed for the saline-EtOH-exposed group. The number of mice assessed are shown in parentheses above each bar, where samples were split by sex. (Panel E) Brca1 +/- fetal brains exhibit 50–79% more catalase activity relative to +/- littermates, independent of treatment (****p < 0.0001). EtOH enhanced catalase activity by 36% in +/- fetal brains, 6 h post-exposure (αααα p < 0.0001). PBN pretreatment blocked the EtOH effects (ββββ p < 0.0001). (Panel F) Any treatment or genotype differences were no longer present by 24 h post-exposure in the fetal brain. Eight fetal brains were split by sex and assessed from the number of litters shown in parentheses above each bar.
catalase can participate in the metabolism of EtOH in peroxisomes [54], although demonstrating a negligible effect on maternal plasma alcohol concentrations [55]. In fetal and adult brains 6 h post-exposure, +/- Brca1 progeny demonstrated elevated catalase activity compared to +/- littermates and adults (Fig. 1D). This enhancement of antioxidative protection in Brca1 +/- brains follows a similar pattern to NOX activity. However, EtOH exposure did not upregulate catalase activity in the adult brains, and it abolished the genotype difference observed in saline-exposed adult mice. Catalase activity in fetal brains was only 38% of that in adult brains (Fig. 1D &E) which, along with fetal NOX activity being similar to adult levels, likely contributes to the greater susceptibility of fetal brain to ROS. Unlike in adult brains, catalase activity was enhanced in +/- Brca1 fetal brains by in utero EtOH exposure 6 h following exposure. However, the enhancement of this antioxidative enzyme in fetal brains was not sufficient to block the ROS-mediated effects on proteasomal activity and BRCA1 protein level, nor ethanol-induced freezing behaviour observed herein. The EtOH-induced enhancement of catalase activity was also blocked by PBN pretreatment, suggesting ROS-mediation of catalase induction (Fig. 1E). All elevated levels of catalase activity observed 6 h post-EtOH exposure returned to baseline (saline +/- levels) by 24 h post-exposure, revealing a transient treatment effect (Fig. 1F).

EtOH enhanced proteasomal activity in both Brca1 +/- and +/- fetal brains. This EtOH-induced enhancement was blocked by PBN pretreatment, implicating a ROS-mediated mechanism (Fig. 2B). The EtOH-initiated enhancement in proteasomal activity evident at 6 h was not observed at 24 h post-exposure (Fig. 2C). The 6 h treatment effects observed in the fetal brain were also observed in the adult (Fig. 2A), where proteasomal activity was enhanced following EtOH exposure and blocked by PBN pretreatment. Proteasomal activity was not assessed in adult brains 24 h post-exposure. Full data sets over time are shown in Supplementary data Fig. S2. The time course for this EtOH-enhanced activity coincided with that for the reduction in BRCA1 protein levels, which are typically maintained by expression and proteasomal degradation [56]. The levels of other proteins likely would also be reduced in response to this EtOH-induced enhancement of proteasomal activity. Furthermore, proteasomal activity can be differentially influenced by the level of oxidative stress, where small amounts may enhance activity to remove oxidatively damaged proteins, and large amounts may damage the proteasome itself, impairing function [17,18].

BRCA1 protein levels in fetal and adult brains of saline-exposed Brca1 +/- mice were lower than in +/- littermates and adults (Fig. 3A&B). In vivo exposure to a single administration of EtOH reduced BRCA1 protein in fetal and adult brains of both Brca1 genotypes at 6 h, and levels returned to baseline within 24 h. In Brca1 +/- fetuses and adult mice at 6 h post-exposure compared to 0 h (untreated mice), EtOH reduced BRCA1 protein to approximately +/- Brca1 brain levels. Similarly, in Brca1 +/- fetal and adult brains 6 h post-exposure, EtOH reduced BRCA1 by 79% and 88% compared to 0 h BRCA1 levels in Brca1 +/- fetal and adult brains, respectively. This BRCA1 reduction may significantly compromise BRCA1-dependent processes including the repair of ROS-initiated DNA damage [6-8]. The developmental consequences of an increased BRCA1 deficiency may include altered division, differentiation [57], migration [58], function and survival of neural progenitor cells [4,59]. The BRCA1 reductions caused by EtOH in fetal and adult brains were blocked by PBN pretreatment, suggesting a ROS-mediated mechanism (Fig. 3A&B). Our results implicate NOX-mediated ROS production in the mechanism through which BRCA1 is degraded following EtOH exposure, where both were reduced by PBN pretreatment.

Herein, Brca1 expression was lower as expected in saline-exposed +/- vs. +/- fetal brains at 6 and 24 h. In immortalized cancer cell lines, BRCA1 was reported to be decreased by the effect of EtOH on Brca1 transcription [60]. In contrast, herein in vivo, EtOH did not alter Brca1 gene expression compared to saline controls in either genotype or at either timepoint following EtOH exposure (Fig. 3C&D). Accordingly, the substantial decrease in BRCA1 protein levels in both fetal and adult brains caused by a single administration of EtOH is consistent with a mechanism involving ROS-enhanced proteasomal degradation rather than reduced Brca1 gene expression.

Transient postnatal freezing, a form of behavioural inhibition [61] was exhibited for up to at least 3 months postnatally by both male and...
female Brca1 +/- progeny exposed once in utero to EtOH, but not by +/- littermates (Fig. 4). During the first trial at both 9 and 12 weeks of age, all Brca1 +/- progeny exposed in utero to EtOH remained frozen for over 100 s (Supplementary data Fig. S3). The EtOH-initiated postnatal freezing behaviour in +/- progeny was blocked by maternal PBN pretreatment, suggesting a ROS-mediated mechanism (Supplementary data Fig. S4). Similar blocking by PBN of molecular damage and postnatal behavioural disorders is observed in other DNA repair-deficient models [62], and may be due to increased DNA damage regulating signaling via epigenetic or direct mechanisms at physiological levels of ROS [63,64].

A relationship between anxiety-related disorders and ROS/oxidative stress has previously been established [65,66], where lowered antioxidative protection and enhanced oxidative macromolecular damage may characterize anxiety disorders [67]. These observations are consistent with the enhanced proteasomal activity and reduced BRCA1 protein observed in EtOH-treated mice herein. In previous fear conditioning studies, increasing shock strength was dose-dependently associated with increased freezing behaviour [68]. While the Brca1 +/- mice might be uniquely sensitive to the shock, it seems more likely that the lack of freezing behaviour in the +/- Brca1 progeny constitutes a neurodevelopmental disorder that is detrimental to their survival. Recognition of danger induced by an electrical shock, and displaying

**Fig. 3. Ethanol (EtOH) reduces breast cancer 1 (BRCA1) protein levels.** (Panels A & B) BRCA1 protein levels in untreated (0 h) fetal and adult Brca1 +/- and +/- mouse brains, and following exposure to EtOH or vehicle, with or without PBN pretreatment (6 or 24 h). Fold changes in BRCA1 protein levels are relative to +/- saline-saline samples 6 h post-exposure. Data from both sexes were combined because no sex-dependent differences were observed. (Panel A) Adult and (Panel B) fetal brains from +/- Brca1 progeny were 30–70% deficient in BRCA1 protein compared to +/- littermates, independent of treatment group or timepoint (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). (Panel A) At 6 h post-EtOH exposure, EtOH reduced BRCA1 protein levels by 51 and 79% in +/- and +/- adult brains, respectively (αααp < 0.001), which was blocked by PBN pretreatment (βββp < 0.001). By 24 h post-EtOH exposure, the BRCA1 protein levels were similar to those at 0 h post-EtOH. The number of mice assessed is shown in parentheses within the legend above the graph, where samples were split by sex. (Panel B) At 6 h post-EtOH exposure, BRCA1 protein levels were reduced by 56 and 70% in +/- and +/- fetal brains, respectively (αααp < 0.001), which was blocked by PBN pretreatment (βββp < 0.001). By 24 h post-EtOH exposure, the BRCA1 protein levels were like those at 0 h post-EtOH. Eight fetal brains were split by sex and assessed from the number of litters shown in parentheses within the legend above the graph. (Panels C & D) No EtOH effect in fetal brain Brca1 gene expression in vivo. Measured in Brca1 +/- vs. +/- saline samples at each timepoint. (Panel C) At 6 h following EtOH or saline exposure, +/- fetal brains had 81 and 68% reductions in Brca1 expression, respectively, compared to +/- littermates (p < 0.01). (Panel D) At 24 h following EtOH or vehicle exposure, respective 37 and 51% Brca1 expression reductions were observed for +/- fetal brains compared to +/- littermates (p < 0.001, p < 0.0001). Six fetal brains were split by sex and assessed from the number of litters shown in parentheses above each bar.
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anxious freezing behaviour, may be an appropriate response for the EtOH-exposed +/+ progeny. This freezing behaviour has previously observed in fear conditioning assessments [69,70], and is a typical rodent response to predators like cats.

4. Conclusion

This is the first in vivo study to demonstrate that EtOH induces a reduction in the level of BRCA1, and first report of enhanced baseline ROS-producing NOX activity in Brca1 +/+ progeny. These two discoveries expand the known mechanisms by which BRCA1 regulates ROS-dependent disorders. The mechanism of BRCA1 depletion by EtOH involved enhanced global ROS-initiated proteasomal degradation, with no apparent involvement of altered Brca1 gene expression previously reported in cell culture [60]. In utero EtOH exposure caused a long-lasting ROS-dependent adaptive postnatal freezing behaviour in all Brca1 +/+ progeny that was lost in Brca1 +/- littermates, possibly exposing +/- progeny to enhanced predation. The ROS-mediated increase in proteasomal activity and BRCA1 reduction in both fetal and adult brains, compounded by the increased NOX activity revealed in BRCA1-deficient progeny, provide new insights into the mechanisms and risk factors underlying EtOH-initiated neurodevelopmental disorders.

Declaration of competing interests

The authors declare no competing interests.

Acknowledgements

The authors’ research was supported by grants from the Canadian Institutes of Health Research (PJT-156023, MOP-115108, MOP-82812) and the University of Toronto Faculty of Pharmacy. DMD was supported in part by a scholarship from the University of Toronto Centre for Pharmaceutical Oncology.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102148.

Authors’ contributions

DMD designed the studies, and performed the measures and data analyses. PGW contributed to the study design, secured research funding, supervised the project, aided in data interpretation and co-wrote the manuscript.

Fig. 4. In utero exposure to EtOH causes transient postnatal freezing behaviour months later in Brca1 +/+ but not +/- progeny. During passive avoidance testing for learning and memory, all Brca1 +/- progeny of both sexes exposed in utero to EtOH exhibited transient freezing behaviour in the light chamber during the first of 3 trials at both 9 weeks (Panel A & B) and 12 weeks (Panel C & D) of age (p < 0.0001). Freezing and exploratory behaviour shown in Figs. S3 and S4, respectively. (Panels A & C) This freezing behaviour was not observed in +/- littermates exposed to EtOH, nor in saline-exposed progeny of either Brca1 genotype. (Panels B & D) No sex-dependent differences in time frozen were observed between 15 male and 15 female progeny and the data were combined. Significant differences were determined using the Wilcoxon Signed Rank Test. The number of litters assessed is shown in parentheses.
