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ORIGINAL RESEARCH ARTICLE

Mechanisms of Antioxidant Induction with High-Dose *N*-Acetylcysteine in Childhood Cerebral Adrenoleukodystrophy

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Abstract

Background Childhood cerebral adrenoleukodystrophy (CCALD), a progressive demyelinating disease affecting school-aged boys, causes death within a few years. Oxidative stress is an important contributing factor. *N*-acetylcysteine (NAC; 280 mg/kg/day) added as adjunctive therapy to reduced-intensity hematopoietic cell transplantation (HCT) improves survival in advanced cases. However, the mechanisms underlying the benefits of NAC are unclear.

Objective The aim of this study was to understand the mechanism of action of NAC in the setting of HCT in CCALD.

Methods Immunoassays were carried out to determine changes in heme oxygenase-1 (HO-1) and ferritin expression in plasma samples collected from boys with CCALD at three different timepoints during the course of transplantation. In addition, the induction of HO-1 was also confirmed in normal fibroblasts following incubation with 10–100 μmol/L NAC for 4 h.

Results Following NAC therapy we observed an increase in expression of the antioxidants HO-1 (∼4-fold) and its effector ferritin (∼160-fold) in patient samples as compared with baseline. We also observed that NAC exposure significantly increased HO-1 expression in fibroblasts.

Conclusion Our data suggest that HO-1 is a possible target protein of NAC and a mediator of its cytoprotective effects in these patients.

Key Points

Childhood cerebral adrenoleukodystrophy is an X-linked disorder causing progressive, debilitating effects on the central nervous system leading to death within a few years.

N-Acetylcysteine (NAC) administered intravenously as adjunctive therapy to reduced-intensity hematopoietic cell transplantation improves survival in advanced cases.

We report heme oxygenase-1, a cytoprotective protein, as a potential mediator contributing to the mechanism of action of NAC in adrenoleukodystrophy.

R. V. Kartha and J. Zhou contributed equally to this work.

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1 Introduction

Childhood cerebral adrenoleukodystrophy (CCALD) is a disorder that affects young boys between the ages of 4 and 10 years [1]. This manifestation of adrenoleukodystrophy (ALD), an X-linked disease, is a neuroinflammatory, demyelinating condition that is rapidly progressive, and is generally fatal within a few years after symptom onset. Oxidative stress has been shown to be increased in ALD, and presumably occurs due to the accumulation of very long-chain fatty acids (VLCFAs) as a result of defects in the peroxisomal membrane transporter protein, ABCD1 [2–6]. Saturated VLCFA, such as hexacosanoic acid (C26:0), accumulates in the brain and adrenal tissues of patients and is diagnostic for ALD [7].

There is no known effective therapy for late-stage CCALD, although hematopoietic cell transplantation (HCT) is offered at some centers [8–12]. HCT halts disease progression in early-stage CCALD and extends life, but requires intensive chemotherapy to ablate the bone marrow. This procedure has been associated with rapid advancement of disease during transplantation, often leading to death as a result of rapid progression. Our group investigated the use of high-dose intravenous *N*-acetylcysteine (NAC) as adjunctive therapy in association with a reduced-intensity preparative regimen to reduce oxidative damage and improve outcomes with HCT. This resulted in a statistically significant increase in 5-year survival [13, 14], although the underlying mechanism of NAC action is not understood. NAC is a thiol-containing antioxidant that scavenges free radicals, chelates metal ions, facilitates glutathione (GSH) biosynthesis, and regulates tissue-protective genes and proteins that reduce damage inflicted by reactive oxygen species (ROS) [15]. Antioxidants including NAC have been shown to confer cellular protection in in vitro and in vivo ALD models [4, 16–19]. In this study we examined whether NAC administered to ALD patients increases the expression of endogenous cytoprotective proteins such as heme oxygenase-1 (HO-1) and its downstream effector ferritin.

2 Methods

2.1 Study Population

Boys ($n = 17$) with a diagnosis of CCALD undergoing HCT at the University of Minnesota (Minneapolis, MN, USA) from 2009 to 2012 were enrolled in a study titled "Treatment of High Risk, Inherited Lysosomal and Peroxisomal Disorders by Reduced Intensity Hematopoietic Stem Cell Transplantation" (ClinicalTrials.gov identifier NCT00383448 [20]). The main objective of this open-label clinical trial was to test a novel transplant regimen for patients with advanced or high-risk, inherited, life-threatening leukodystrophies. Consecutive eligible patients were enrolled and are reported. A control patient with a different diagnosis (mucopolysaccharidosis type VI) was also transplanted on this high-risk protocol due to having received and rejected a prior transplant and received the identical chemotherapy regimen but without NAC. All protocols were approved by the University Institutional Review Board. Informed consent was obtained from all individual participants included in the study.

Patients included in this analysis were diagnosed with advanced radiographic disease (Loes score $\geq$10) and received NAC (Acetadote®; Cumberland Pharmaceuticals Inc., Nashville, TN, USA) as adjuvant therapy (70 mg/kg intravenously every 6 h from day −11 to day –8 prior to HCT, and days +1 to +100 post-HCT). The median age of ALD patients in this study was 8.2 years (range 4.4–14.5 years). The median weight of ALD patients at HCT was 24.4 kg (range 17.3–41.5 kg). The HCT regimen also included alemtuzumab, clofarabine, melphalan, total body irradiation, mycophenylate mofetil, and cyclosporine (ciclosporin) (Fig. 1) [13].

Baseline plasma samples were collected from patients prior to NAC therapy (day –12, Pre-NAC). Samples were also collected at three different timepoints following NAC therapy (day –8, Post-NAC1; day +7, Post-NAC2; day +21, Post-NAC3), as shown in Fig. 1. These samples were collected 1 h after NAC infusion. A few samples were not collected in some of the patients.

2.2 Determination of Plasma Heme Oxygenase-1 (HO-1) and Ferritin Levels

Plasma HO-1 levels were determined using enzyme-linked immunosorbent assay (ELISA) method (HO-1 [human] ELISA kit, Enzo Life Sciences, Farmingdale, NY, USA) per the manufacturer's protocol with the following modifications to improve linearity: plasma samples were diluted twofold using sample diluent (ADI-80-1587, Enzo Life Sciences) prior to the assay; and normal human plasma (Biological Specialty Corporation, Colmar, PA, USA) spiked with 10 ng/mL HO-1 was used to performed assay validation to establish linearity and reproducibility prior to sample analysis.

Plasma ferritin levels were determined using ELISA method (H-ferritin [human] ELISA kit, Abnova, Taiwan) per the manufacturer's protocol. Pre-NAC and Post-NAC1 samples were diluted fivefold while Post-NAC2 and Post-NAC3 samples were diluted 20-fold. After color development, the assay plates were read using a Synergy 2

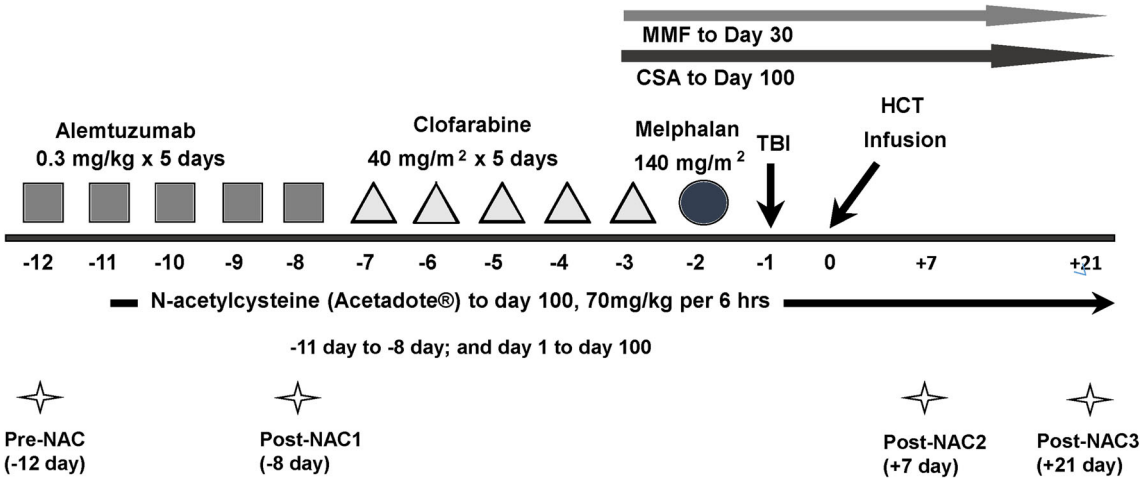

Fig. 1 A schematic representation of the transplant preparative regimen and the timepoints of sample acquisition. *N*-Acetylcysteine was administered intravenously for 4 days (from day –11 to day –8) prior to hematopoietic cell transplantation, and day +1 to +100 post-hematopoietic cell transplantation. Plasma samples were drawn at day –12 (Pre-NAC), day –8 (Post-NAC1), day +7 (Post-NAC2), and day +21 (Post-NAC3). *CSA* cyclosporine, *HCT* hematopoietic cell transplantation, *MMF* mycophenylate mofetil, *NAC* *N*-acetylcysteine, *TBI* total body irradiation

microplate reader (Biotek, Winooski, VT, USA) and the data processed using the four-parameter algorithm provided in the Gen5 Data Analysis Software (Biotek).

2.3 Cell Culture Conditions

Approximately 10^4 primary human non-transformed fibroblast cells (derived from control and CCALD subjects following consent) were seeded on 24-well plates (Corning®, Corning, NY, USA) in Dulbecco's Modified Eagle's Medium (DMEM) containing glucose supplemented with 10 % fetal bovine serum and 1 % antibacterials. Cells were incubated overnight in a 37 °C incubator with 5 % carbon dioxide. Cells were exposed to 10–100 µmol/L of NAC (Sigma-Aldrich, St Louis, MO, USA) for 4 h. HO-1 messenger RNA (mRNA) expression was quantified using real-time polymerase chain reaction (PCR) and protein expression by ELISA (HO-1 [human] ELISA kit, Enzo Life Sciences). Total protein levels were quantified using Bradford protein assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The amount of intracellular ROS was quantified using fluorescence-activated cell sorting (FACS) using fluorescent CM-H_2-DCFDA probes (details can be found in the Electronic Supplementary Material).

2.4 Statistical Analysis

Results are expressed as means ± standard deviation. One-way ANOVA and Dunnett's test were used for multiple comparisons. Student's *t* test was used to compare between two groups. A p value <0.05 was considered significant.

3 Results

3.1 *N*-Acetylcysteine (NAC) Increases Plasma Concentrations of HO-1 and Ferritin in Patients with Childhood Cerebral Adrenoleukodystrophy

We examined the levels of HO-1 in plasma obtained from 17 boys with CCALD who underwent HCT along with NAC therapy. Comparison of baseline (pre-NAC) samples with those collected following NAC infusion revealed sustained increases in HO-1 expression up to 21 days following HCT. The average Pre-NAC, Post-NAC1, Post-NAC2, and Post-NAC3 plasma HO-1 levels were 2.7 ± 0.3, 8.6 ± 0.9, 9.4 ± 1.9, and 10.3 ± 0.8 ng/mL, respectively (ANOVA $p < 0.0001$; Fig. 2a). In order to eliminate the interference of the HCT preparative regimen, we also compared Pre-NAC and Post-NAC1 samples that were collected prior to the regimen. HO-1 expression was observed to increase significantly following 4 days of NAC administration (*t* test $p < 0.0001$; Fig. 2a inset).

Increased HO-1 activity can result in the increase of the downstream antioxidative protein, ferritin. Therefore, we examined ferritin levels in these samples. Ferritin levels significantly increased following NAC therapy and HCT. The average plasma levels of ferritin for Pre-NAC, Post-NAC1, Post-NAC2, and Post-NAC3 were 33.0 ± 8.6, 135.4 ± 21.7, 2305.0 ± 578.9, and 5557.0 ± 1181.0 ng/mL, respectively (ANOVA $p < 0.0001$; Fig. 2b). Comparison of Pre-NAC and Post-NAC1 samples also showed a significant increase in ferritin levels following 4 days of NAC therapy (*t* test $p < 0.001$; Fig. 2b inset). Notably,

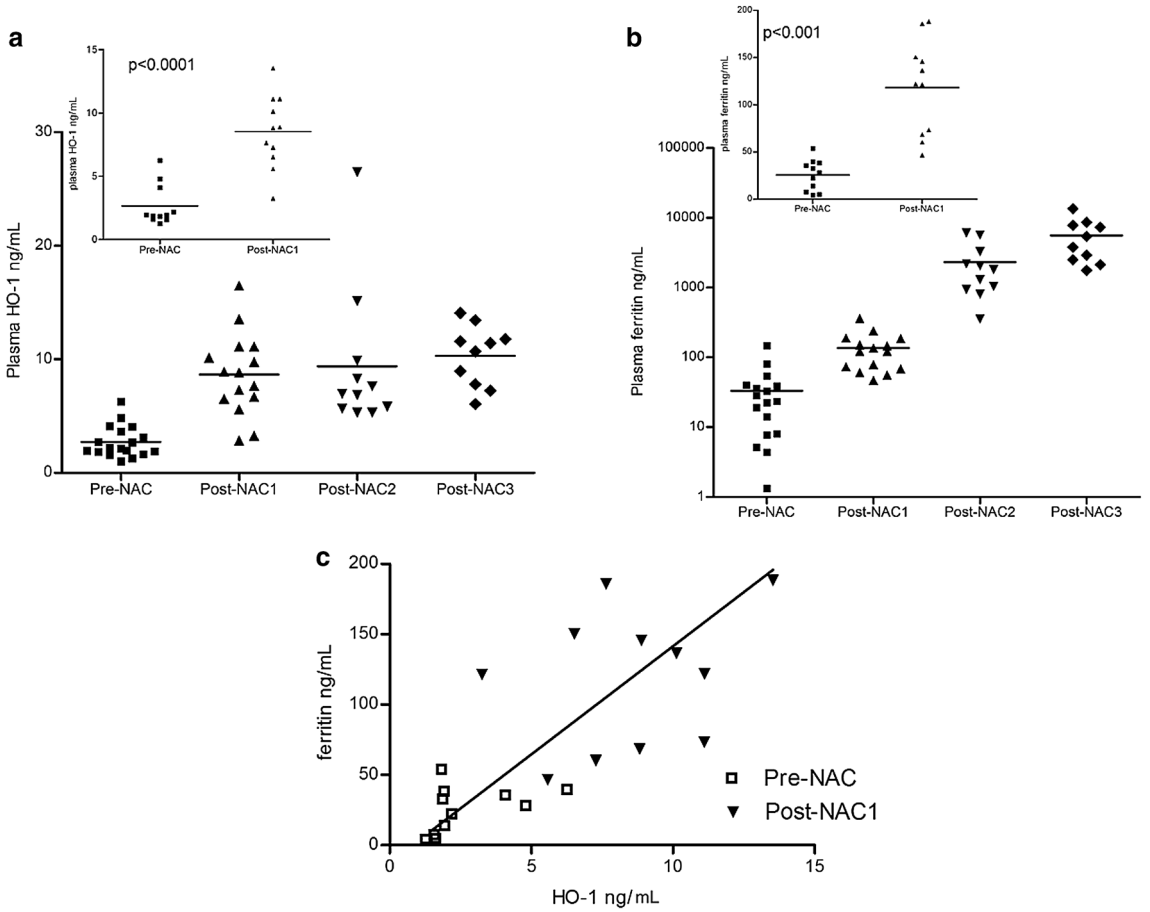

Fig. 2 Increase in plasma heme oxygenase-1 and ferritin levels after *N*-acetylcysteine administration. Compared with baseline, heme oxygenase-1 (**a**) and ferritin (**b**) levels increased significantly throughout the hematopoietic cell transplantation process ($n = 10$–17 subjects); ANOVA $p < 0.0001$ for both **a** and **b**. The mean values for each group are represented by *lines*; one-way ANOVA was used to compare heme oxygenase-1 and ferritin levels for all sampling timepoints. The *inset* shows the increase in plasma heme oxygenase-1 (**a**) and ferritin (**b**) levels ($n = 11$ subjects) following 4 days of *N*-acetylcysteine administration prior to hematopoietic cell transplantation compared with Pre-NAC samples. A paired *t* test was used for data analysis. (**c**) Correlation between plasma heme oxygenase-1 and ferritin levels in Pre-NAC and Post-NAC1 samples ($n = 11$ subjects). Pearson's correlation *r* is equal to 0.74 ($p < 0.0001$). *HO-1* heme oxygenase-1, *Pre-NAC* plasma samples drawn at day –12, *Post-NAC1* plasma samples drawn at day –8, *Post-NAC2* plasma samples drawn at day +7, *Post-NAC3* plasma samples drawn at day +21

there was significant correlation between plasma levels of HO-1 and ferritin. In the 11 patients with paired plasma samples collected prior to and after NAC treatment, the Pearson's correlation coefficient *r* was found to be 0.74 ($p < 0.0001$; Fig. 2c).

3.2 NAC Increases HO-1 messenger RNA and Protein Levels in Human Fibroblasts

In an attempt to exclude confounding factors related to the HCT preparative regimen, we investigated the influence of NAC on HO-1 expression in vitro. Incubation of human control fibroblasts with increasing concentrations of NAC revealed significant increase in HO-1 levels over baseline (ANOVA $p < 0.01$ for both mRNA and protein; Fig. 3). However, CCALD fibroblasts showed higher baseline HO-1 levels than control, and did not show further significant changes in HO-1 expression with NAC exposure (Electronic Supplementary Material figure 1). Analysis of the intracellular ROS levels revealed that at baseline, CCALD fibroblasts have significantly higher ROS (>15-fold) than control fibroblasts ($p < 0.001$; Electronic Supplementary Material figure 2). Moreover, unlike control fibroblasts, which showed a NAC concentration-dependent increase in ROS levels, CCALD fibroblasts showed only minimal changes in ROS with NAC exposure.

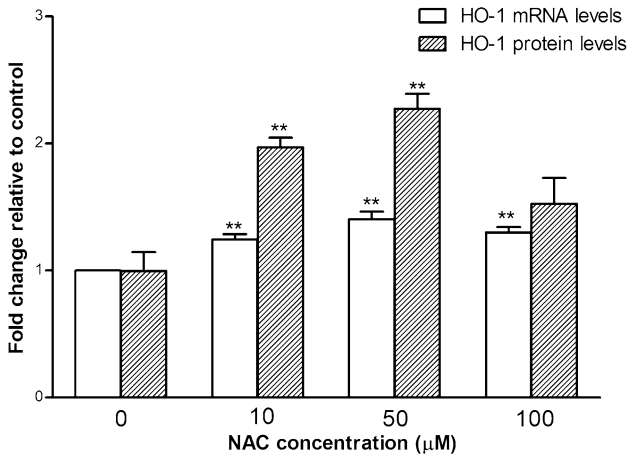

Fig. 3 Increased heme oxygenase-1 expression (messenger RNA and protein levels) in the normal human fibroblast cell line following *N*-acetylcysteine treatment ($n = 3$). ANOVA followed by a Dunnett's test was used to compare the treatments of different concentrations of *N*-acetylcysteine with control. *HO-1* heme oxygenase-1, *mRNA* messenger RNA, *NAC* *N*-acetylcysteine, $^{**}p < 0.01$

4 Discussion

In this study, we demonstrate for the first time the induction of HO-1 and ferritin in boys with CCALD following intravenous administration of high-dose NAC in conjunction with HCT. The plasma HO-1 concentration is measured clinically as a surrogate biomarker of disease [21–23], but the pharmacological induction of HO-1 is not well-documented. Bharucha et al. [24], in a first-in-human study, reported the activation of HO-1 following hemin administration in healthy volunteers. In our study, we observed an increase in plasma HO-1 levels following NAC administration prior to HCT, which was maintained at high levels during HCT with continuous NAC infusion. However, during the HCT preparative regimen (day –11 to day –8), patients received both NAC and alemtuzumab. Thus, the influence of alemtuzumab on plasma HO-1 cannot be ruled out, although to the best of our knowledge there are no reports showing relationship between HO-1 and alemtuzumab. Importantly, our in vitro data demonstrate the direct relationship between NAC and HO-1 expression, without involvement of free radicals, suggesting substantial contribution of NAC to the activation of HO-1 signaling. Moreover, analysis of day –12 and day –8 samples from one patient who underwent the same HCT preparative regimen but without NAC prior to HCT did not show HO-1 induction (data not shown).

NAC is believed to increase intracellular GSH, a potent endogenous antioxidant. Through its de-acetylation, NAC provides the rate-limiting substrate cysteine for GSH synthesis [25]. However, recent stable isotope-labeled studies from our group suggest that NAC is not a direct precursor of GSH [26]. This is consistent with a previous study that demonstrated that NAC can release protein-bound cysteine, suggesting that it may act indirectly to increase cysteine in the plasma [27]. The unbound plasma cysteine is then available for transportation into cells, enhancing GSH synthesis, or to be eliminated from the body. Here we demonstrate that in addition to increased biosynthesis of GSH, the cytoprotective action of NAC is mediated by the induction of tissue-protective proteins. The induction of HO-1 could potentially be beneficial for patients undergoing HCT. Preclinical studies have shown that HO-1 induction in liver/bowel tissues improved overall survival and reduced acute graft-versus-host disease rates in mouse bone marrow transplantation models [28, 29]. Further HO-1 was found to be important for tolerance induction during transplantation, thereby reducing the need for ongoing immunosuppression [30]. A recent study has also demonstrated the beneficial effects of HCT on oxidative stress in ALD [31].

In our study, we observed consistent high levels of HO-1 throughout the entire post-NAC period. This implies that the induced HO-1 levels reached steady state after the first few days of therapy. This suggests one of two scenarios: either (i) the induction of HO-1 by NAC reached steady state after the first few days of therapy; or (ii) the turnover rate of HO-1 protein is such that we were not able to observe any apparent increase in HO-1 between the two timepoints following HCT. Excessive HO-1 induction is harmful and has been associated with tissue iron sequestration and mitochondrial insufficiency in vitro [32], highlighting that modulation of HO-1 levels is crucial for mitigating oxidative stress. Our observation of stable HO-1 induction by NAC may therefore be beneficial, although the optimal level is not clear. Prior to HCT, ferritin levels increased in proportion to increases in HO-1 following NAC administration, which indicates consistent induction of both HO-1 and ferritin by NAC. However, following HCT, ferritin increased almost exponentially, which is in line with the observation that ferritin can be induced by bone marrow transplantation [33]. Ferritin can also be an acute-phase reactant, and in a setting of transplantation some or all of the elevation could be related to this. Further experiments involving patients who undergo transplantation without NAC in their regimen are necessary in order to delineate the effect of NAC on ferritin in the context of HCT.

5 Conclusion

Our results offer evidence that among NAC's mechanisms of antioxidant activity is the induction of HO-1, a possible target protein of NAC and mediator of its cytoprotective

effects in patients with CCALD. Further studies that control for age and other confounding chemotherapeutic agents are needed to confirm this finding.

Author contributions Designed the experiments: RVK, JZ, LB, HS, PJO, JC. Performed the experiments: JZ, RVK, LB. Analyzed the data: JZ, RVK, LB, HS. Contributed essential reagents/tools: PJO, JC, HS. Wrote the paper: RVK, JZ, LB, HS, PJO, JC.

Compliance with Ethical Standards

Funding The study was funded by a University of Minnesota Academic Health Center Faculty Development Grant to JC and PJO.

Conflict of interest RVK, JZ, LB, HS, PJO, and JC declare that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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