N-Acetylcysteine Attenuates Progression of Liver Pathology in a Rat Model of Nonalcoholic Steatohepatitis

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Abstract

A “2-hit” model for nonalcoholic steatohepatitis (NASH) has been proposed in which steatosis constitutes the “first hit” and sensitizes the liver to potential “second hits” resulting in NASH. Oxidative stress is considered a candidate for the second hit. N-Acetylcysteine (NAC), an antioxidant, has been suggested as a dietary therapy for NASH. We examined the effects of NAC in a rat total enteral nutrition (TEN) model where NASH develops as the result of overfeeding dietary polyunsaturated fat. Male Sprague-Dawley rats consumed pelleted AIN-93G diets ad libitum or were overfed a 9200 kJ·kg⁻⁰·⁷⁵·d⁻¹ liquid diet containing 70% corn oil with or without 2 g·kg⁻¹·d⁻¹ NAC i.g. for 65 d. Hepatic steatosis was not influenced by dietary supplementation with NAC; however, the liver pathology score was lower (P ≤ 0.05) and NAC provided partial protection against alanine aminotransferase release (P ≤ 0.05). NAC attenuated increased hepatic oxidative stress (TBARS; P ≤ 0.05) and prevented increases in cytochrome P450 2E1 apoprotein and mRNA and in tumor necrosis factor-α (TNFα) mRNA. Titers of auto-antibodies against proteins adducted to lipid peroxidation products were lower in serum of the NAC group than in the 70% corn oil group (P ≤ 0.05). NAC also decreased Picosirius red staining of collagen, a marker of fibrosis. However, markers of hepatic stellate cell activation were unaffected. Using NAC in a TEN model of NASH, we have demonstrated that NAC prevents many aspects of NASH progression by decreasing development of oxidative stress and subsequent increases in TNFα but does not block development of steatosis.

Introduction

Nonalcoholic fatty liver disease is the most common of liver pathologies and is closely associated with obesity and the metabolic syndrome. There is a wide spectrum of pathologies covered by nonalcoholic fatty liver disease, ranging from simple reversible steatosis to nonalcoholic steatohepatitis (NASH),¹ in which steatosis is accompanied by inflammation, necrosis, apoptosis, and fibrosis. The mechanisms leading to NASH are multifactorial. In patients with NASH, there is an initial development of steatosis, plus a second insult resulting in disease progression. The 2-hit model of NASH was proposed in 1998 (1) in which alterations in lipid homeostasis associated with obesity, insulin resistance, and adipokine disruption result in steatosis and constitute the first hit. This sensitizes the liver to potential second hits resulting in hepatocellular injury, inflammation, and fibrosis. Oxidative stress is frequently cited as a central mechanism of hepatocellular injury in NASH, correlating with the accumulation of lipid peroxidation products, appearance of mitochondrial dysfunction (2–4), and elevation of proinflammatory cytokines. It is considered one of the best candidates for the second hit, because it can explain all the recognized histological features of the disease (5–7). Glutathione (GSH) is a major endogenous antioxidant. N-Acetylcysteine (NAC), a precursor of GSH, has been in clinical use for more than 30 y as an antidote for acetaminophen overdose (8). Potential protective effects of NAC are being studied in chronic diseases characterized by decreased GSH or oxidative stress such as alcoholic liver disease and NASH (9–12). Most of the beneficial effects of orally administered NAC are theorized to be a result of its ability to...
either reduce extracellular cystine to cysteine or to be a source of sulphydryl metabolites. As a source of sulphydryl groups, NAC can stimulate GSH synthesis, enhance GSH-S-transferase activity, promote detoxification, and act as a scavenger of free radicals as it interacts with reactive oxygen species (ROS) (13,14). We have developed a unique rat model in which liver pathology closely resembles clinical NASH using overfeeding of a high polyunsaturated fat diet via total enteral nutrition (TEN) (15). NASH is histologically indistinguishable from alcoholic steatohepatitis and it is proposed they may also have many similarities in pathogenesis, including oxidative stress and increased cytochrome P450 2E1 (CYP2E1), proinflammatory cytokines, and tumor necrosis factor-α (TNFα) (1,15,16). We have previously shown NAC to be successful in prevention of alcohol-induced liver disease in rats fed via TEN (12). The aim of the present study was to determine the effects of NAC treatment on the development of liver pathology in our TEN NASH model.

Materials and Methods

Reagents. All chemicals were purchased from Sigma-Aldrich unless otherwise specified. Potassium chloride, potassium phosphate, and potassium ferricyanide were purchased from Fisher Scientific. Enhanced chemiluminescence for chemiluminescent detection in western blotting was purchased from Amersham Biosciences. TRI Reagent used for RNA extraction was obtained from Molecular Research Center. Reagents for the assessment of RNA quality using the Agilent Bioanalyzer were acquired from Agilent Technologies.

Experimental animals and diets. Male Sprague-Dawley rats (175 g) were purchased from Harlan Sprague-Dawley. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility. Animal maintenance and experimental treatments were conducted in accordance with ethical guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. Animals were randomly assigned to diet groups. Control rats (C) consumed ad libitum water and a pelleted AIN-93G diet in which the protein source (partially hydrolyzed whey) matched that of the liquid diets utilized for NASH development (Table 1). Rats fed by TEN received 9200 kJ/kg-0.75 d-1 (which represents 17% greater energy intake than recommended by the NRC). TEN diets contained corn oil at a level equal to 70% of the total energy (15) with or without 2 g/kg d-1 NAC [high fat (HF) or HF+NAC] (Table 1). TEN-fed rats had an i.g. infusion commenced as described previously (12,15,18–21). Vitamin and mineral content was the same in all diets (Table 1) (17,21). All diets recommended levels as described previously. The energy density was 890 kJ/L/0.75 and the diet was fed at 9200 kJ/C10.75/C1 d-1 (21).

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Diet composition</th>
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<tr>
<td></td>
<td>Pelleted diet1,2</td>
</tr>
<tr>
<td></td>
<td>g/kg</td>
</tr>
<tr>
<td>Protein</td>
<td>178 g</td>
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<tr>
<td>Carbohydrate</td>
<td>751 g</td>
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<tr>
<td>Fat</td>
<td>70 g</td>
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</table>

1 Diet made according to the AIN-93G diet formula except that corn oil replaced soy bean oil and the protein source was whey protein (New Zealand Milk Products) as described previously (17).
2 Whey protein was supplemented with an amino acid mix to ensure that all essential amino acids were present at levels to meet or exceed NRC recommended levels as described previously (17).
3 TEN diets formulated with vitamins and minerals added to meet or exceed NRC recommended levels as described previously. The energy density was 880 kg/L-0.75 and the diet was fed at 9200 kJ/L-0.75 d-1 (21).
4 Carbohydrate in the TEN diet was 25% maltodextrin and 75% dextrose and fat in the TEN diet was corn oil as described previously (21).

Pathological evaluation. Liver pathology was assessed by hematoxylin-eosin (H&E) and Oil Red O staining of liver sections and scored by a board-certified pathologist (L. Hemnings). The pathology calculation was based on ballooning degeneration (0–2), presence or absence of serum markers of necrosis [alanine aminotransferase (ALT) score: cut-off was 55, based on data from Charles River Laboratories and our baseline values, <55 = 0, ≥55 = 1], the lipidosis score (based on evaluation of Oil Red O stained slides), and lobular inflammation/necrosis (0 = 0 foci, 1 = <2 foci, 2 = 2–4 foci, and 3 = >4 foci) as described previously (15). Portal fibrosis was detected by Picrosirius red staining of collagen (22).

Biochemical analysis. Serum ALT activity levels were measured when the rats were killed using the Infinity ALT liquid stable reagent (Thermo Electron) according to the manufacturer’s protocols. Liver microsomes were prepared by differential centrifugation and stored at −70°C until analysis. Protein concentrations of the microsomes were determined by the Bradford method using the Bio-Rad Protein Assay. Liver lipid peroxidation was assessed as a measure of oxidative stress as described by Ohkawa et al. (23). Western immunoblot analysis of apoprotein expression for CYP2E1 was conducted as previously described (15,24). CYP2E1 was a gift from the laboratory of Dr. Magnus Ingelman-Sundberg (Karolinska Institute) (25). Western immunoblot analysis of protein expression for malondialdehyde (MDA) was conducted as described by Polavarupu et al. (26). Briefly, total proteins (30 μg) were loaded on SDS-PAGE and transferred to nitrocellulose membrane and incubated with 2% bovine serum albumin in Tris-buffered saline/Tween for blocking and subsequently incubated with the antibody (1 mg/L-1) (Cosmo Bio). This procedure was followed by the addition of horseradish peroxidase-linked goat antibody to mouse IgG and detected by enhanced chemiluminescence (27,28). The bands were visualized by autoradiography and entire lanes were quantified.

Measurement of auto-antibodies to proteins adducted with MDA and arachidonate hydroperoxide. Circulating auto-antibodies to proteins adducted with the lipid peroxidation products MDA or arachidonate hydroperoxide (LOOH) were measured as described previously by Mottaran et al. (29).

Real-time RT-PCR. Total RNA was extracted from livers using TRI Reagent and cleaned using RNaseasy mini columns (Qagen). RNA quality was ascertained spectrophotometrically (ratio of A260/A280) and also by checking ratio of 28s:18s ribosomal RNA using the RNA Nano Chip on a 2100 Bioanalyzer (Agilent Technologies). Total RNA (1 μg) was reverse transcribed using the iScript Reverse Transcription kit (Bio-Rad Laboratories) according to the manufacturer’s instructions. The reverse transcribed cDNA (10 ng) was utilized for real-time PCR using the 2× SYBR Green Master mix and monitored on a ABI Prism 7000 sequence detection system (Applied Biosystems). Gene-specific probes were designed using Primer Express Software (Applied Biosystems; Supplemental Table 1) and the relative amounts of gene expression were quantitated using a standard curve according to the manufacturer’s instructions.

Measurement of hepatic GSH concentrations. Hepatic GSH concentrations were quantified from C and NAC-treated rats using a commercially available kit (703002) from Cayman Chemical. The kit is based on enzymic recycling of GSH using GSH reductase followed by reaction of GSH with Ellman’s reagent. The colored product is measured at 405 nm and quantified using a standard curve.

Statistical analysis. Data are expressed as means ± SEM. Densitometric quantitation of Western blot autoradiograms was performed using Quantity One software (Bio-Rad). We used SigmaStat software package version 3.0 (SPSS) to perform all statistical tests. The data were tested using Levene’s test for equality of variance. We performed Pearson product moment correlation using SigmaStat software. Group differences were evaluated via 1-way ANOVA followed by Student-Newman-
Results

Dietary supplementation with NAC partly protected against liver injury in NASH. The pellet-fed C group consumed 7800 kJ·kg\(^{-0.75}\)·d\(^{-1}\) and had a growth rate of 3.83 ± 0.03 g·d\(^{-1}\). In comparison, the HF TEN group infused with 9200 kJ·kg\(^{-0.75}\)·d\(^{-1}\) had a growth rate of 6.84 ± 0.1 g·d\(^{-1}\). The HF + NAC group infused with the same TEN diet + NAC had a growth rate of 5.89 ± 0.08 g·d\(^{-1}\). Growth rates for the HF groups were higher than that of the pellet-fed group (P ≤ 0.05). Pathological examination demonstrated no histological evidence of fat accumulation or inflammation in group C (Fig. 1D), but steatosis, hepatocellular ballooning, inflammation, necrosis, and portal/lobular fibrosis similar to that observed in Grade 3 clinical NASH was present in the HF group (Fig. 1E; Supplemental Fig. 1) consistent with previous observations for our laboratory (15). The total pathology score and pathology scores in excess of simple steatosis were lower in the HF + NAC group (Fig. 1F) than in the HF group (P ≤ 0.05) (Table 2). Development of NASH was accompanied by elevated serum ALT activity (P ≤ 0.05) (Table 3), indicating necrotic injury. The addition of NAC attenuated this increase; however, ALT values were still higher than in the C group (P ≤ 0.05) (Table 3). Picosirius red staining demonstrated an increase in portal/lobular fibrosis in the HF group (Fig. 1G,H) (P ≤ 0.05), which was attenuated by NAC administration (P ≤ 0.05) (Fig. 1I; Table 2). Oil red O staining and biochemical analysis of triacylglycerol showed an increase in hepatic steatosis in the HF group (Fig. 1A,B) (P ≤ 0.05) associated with an increase in relative expression of mRNA for the fatty acid transport-associated protein CD36 from 1.0 ± 0.08 to 12.1 ± 2.7 (P ≤ 0.05). NAC did not affect the overall level of fat accumulation in the liver or relative CD36 mRNA expression (14.1 ± 3.3) but did appear to attenuate progression of liver pathology beyond steatosis and result in formation of smaller lipid droplets (Fig. 1C; Table 3).

FIGURE 1 Representative Oil Red O (A–C), H&E (D–F), and Picosirius Red (G–I) stained liver sections from male rats overfed HF diets with or without NAC supplementation. Representative Oil Red O-stained liver sections: C (A); HF (B); and HF + NAC (C) (×20 magnification). Representative H&E-stained liver sections: C (D); HF (E); and HF + NAC (F) (×10 magnification). Representative Picosirius Red stained liver sections: C (G); HF (H); and HF + NAC (I) (×10 magnification).

Dietary NAC alleviated oxidative stress and inflammation. Hepatic lipid peroxidation increased (TBARS; \(P \leq 0.05\)) in the HF group (Fig. 2A), accompanied by decreased hepatic GSH concentrations (\(P \leq 0.05\)) (Fig. 2B). Although it did not reach statistical significance, as a result of inter-animal variability, the mean expression of MDA-adducted proteins also increased in Western blots from liver homogenates in the HF group (\(P = 0.1\)) (Table 3). An increase in CYP2E1 apoprotein and mRNA (\(P \leq 0.05\)) (Fig. 3A,B), a source of ROS, was also observed in the HF group as were increases in mRNA expression of the proinflammatory cytokine, TNF\(\alpha\) (\(P \leq 0.05\)) (Table 4). The level of mRNA for another proinflammatory cytokine, IL-1\(\beta\), tended to be greater in the HF group than in the C group (\(P = 0.08\)). Expression of CD14 mRNA, an indicator of Kupffer cell activation (12), did not differ between the groups (Fig. 4A). The ratio of CD68:CD45, an indicator of leukocyte activation and infiltration (30), tended to be greater in the HF group than in the C group (\(P = 0.077\)) (Fig. 4B). These indicators of oxidative stress and inflammation, except IL-1\(\beta\), were attenuated by NAC supplementation (\(P \leq 0.05\)).

NAC supplementation reduced immune responses to protein adducts of lipid peroxidation products. Titers of antibodies directed against proteins adducted to the lipid peroxidation products MDA and LOOH were higher in the HF group than in the C group (\(P \leq 0.05\)) and there were reduced by supplementation with NAC (Fig. 5).

Effects of NAC administration on early markers of hepatic stellate cell activation and the turnover of extracellular matrix. Transforming growth factor-\(\beta\) (TGF\(\beta\)) is a cytokine linked to the activation of hepatic stellate cells (HSC) and development of fibrosis. Platelet-derived growth factor receptor-\(\beta\) (PDGFr\(\beta\)) and \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) are also early markers associated with HSC activation (31). Hepatic mRNA for all 3 were higher in the HF group than in the C group (\(P \leq 0.05\)). However, elevation in expression of these mRNA in the HF group was not affected by the addition of NAC (Fig. 6A–C). mRNA expression was not affected for connective tissue...
TABLE 2 Pathology scores of rats fed C, HF, or HF + NAC diets for 65 d

<table>
<thead>
<tr>
<th>Variable</th>
<th>C</th>
<th>HF</th>
<th>HF + NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Cellular injury</td>
<td>0.0 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Hepatocellular ballooning</td>
<td>0.0 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lobular inflammation</td>
<td>0.6 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lipidosis (Oil Red O Staining)</td>
<td>0.8 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.7 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total score – lipidosis</td>
<td>1.4 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.1 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.0 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total score – fibrosis</td>
<td>0.6 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.6 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.3 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fibrosis (Picrosirius red staining)</td>
<td>3.6 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.2 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>1</sup> Values are means ± SEM. Means in a row with superscripts without a common letter differ, P ≤ 0.05.

Discussion

One area of general agreement between investigators studying mechanisms of NASH is that steatosis is a necessary prerequisite for any further damage. In the rat TEN NASH model, where pathology is driven by overfeeding of polyunsaturated fats, development of hepatic steatosis results at least in part from increased fatty acid uptake (15). Consistent with this, expression of the fatty acid transport associated molecule CD36 significantly increased. However, neither CD36 expression nor steatosis was reversed by NAC supplementation. The lack of effect on steatosis is similar to our previous findings with NAC in a rat model of alcoholic steatohepatitis (12).

The progression from steatosis to steatohepatitis and fibrosis (the second hit) is complex and not well defined. Oxidative stress is thought to play an important role in the second hit. Peroxidation of lipids accumulated within steatotic hepatocytes has been demonstrated (5–7). Mitochondrial dysfunction and induction of hepatic CYP2E1 (33) leading to the formation of ROS (34) promote lipid peroxidation and oxidative stress. Increased staining for lipid peroxidation products in steatotic livers, with added increases in those with steatohepatitis (35), has been confirmed by immunohistochemistry. Disease progression may also involve immune responses to proteins adducted by lipid peroxidation products (36). Increases in proinflammatory cytokines, such as TNFα and IL-1β, could also mediate hepatic inflammation.

TABLE 3 Serum ALT and triacylglycerols and hepatic triacylglycerols and MDA protein in rats fed C, HF, or HF + NAC diets for 65 d

<table>
<thead>
<tr>
<th>Variable</th>
<th>C</th>
<th>HF</th>
<th>HF + NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Serum ALT, U/L</td>
<td>38 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>104 ± 16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70 ± 6.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum triacylglycerol, µmol L&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>19 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver triacylglycerol, µmol·g&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>11 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatic MDA protein, %</td>
<td>8.3 ± 1.2</td>
<td>11 ± 2.9</td>
<td>54 ± 1.7</td>
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<sup>1</sup> Values are means ± SEM. Means in a row with superscripts without a common letter differ, P ≤ 0.05.

Interestingly, the hepatic accumulation of lipids in patients with NASH and in the current model is associated with increased mitochondrial β-oxidation of fatty acids (15,37,38), which has been suggested to be an adaptive response for limiting the accumulation of lipid. Respiratory chain complexes are reported to be poorly coupled in patients with NASH and increased mitochondrial FA oxidation may serve as a source of ROS (39). We observed hepatic lipid peroxidation, which correlates with development of steatosis, oxidative stress, increased TNFα, and increased necrosis (15). Bioactive aldehydes such as MDA are chemically reactive products of lipid peroxidation that result in chemotaxis and cytotoxicity (40,41) and the modulation of cell signaling cascades (21). Although the mechanisms whereby MDA and other aldehydes such as hydroxynonenal elicit these effects remain to be delineated, it is likely they occur as a consequence of protein modification. As has been described in NASH patients, proteins adducted with lipid peroxidation products such as MDA and hydroxynonenal and with LOOH also stimulate the host immune response, which could result in an autoimmune-like disease (39). The values for cellular injury and presence of auto-antibodies to LOOH-adducted proteins were weakly correlated (r² = 0.34). That NAC reduces the presence of antibodies to these protein adducts coincidental with attenuated progression of hepatic pathology suggests that immune mechanisms might be involved. Consistent with this are observations in humans showing that elevated titers of circulating IgG toward MDA and LOOH adducted proteins in a large fraction of hepatitis and/or cirrhosis patients but only in a few subjects with simple steatosis alone (39).

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Even though CD14 mRNA was not elevated in the HF group, indicating a lack of Kupffer cell activation, our data regarding the CD68:CD45 ratio and TNF-α is consistent with the ability of NAC to reduce inflammation in this model. TNF-α is a cytokine proposed to play a central role in the chronic inflammatory response in NASH (42,43). Kupffer cells are the major source of increased TNF-α secretion within the liver, with infiltrating monocytes and macrophages also contributing (42,43). TNF-α mRNA is increased in both the liver and adipose tissues in NASH patients (44). In both steatosis and steatohepatitis, soluble TNF receptor levels are elevated (45,46), and enhanced TNF-α secretion from circulating monocytes is seen in patients with NASH compared with healthy controls (47). TNF receptor knockout mice have less severe steatosis than wild-type littermates (48) and a significant reduction in liver injury in steatohepatitis after anti-TNFα therapy (49–51), supporting the pathogenic role of TNFα. In this study, the proinflammatory cytokine TNF-α mRNA was higher in the HF group but was attenuated by the addition of NAC to the diet. This suggests that TNF-α elevation in NASH is the result of increased oxidative stress, perhaps secondary to an increase in CYP2E1 expression. It has recently been shown that CYP2E1 located in the mitochondrial fraction is associated with mitochondrial lipid peroxidation and damage and is associated with TNFα production in alcohol-induced steatohepatitis (52). NASH patients have also been shown to have upregulation of CYP2E1, which may be one of the most important sources of ROS.

Regardless of etiology, oxidative stress plays a major role in activation of HSC and in hepatic fibrogenesis (53). Lipid peroxidation has also been shown to stimulate collagen production in fibroblasts and HSC (54). GSH precursors or antioxidant agents have been shown to exert protective effects against activation of HSC (55); however, the role of oxidative stress and the beneficial effects of GSH precursors or antioxidant agents during the initial phases of liver fibrosis have not been fully investigated. The antifibrotic effects of NAC have been demonstrated in other experimental models, especially in the lung (56,57). In the current study, we observed partial protection against development of hepatic fibrosis in the NAC-treated group; however, there was no reduction in TGFβ or its downstream targets, α-SMA and PDGFr-β mRNA, both markers of HSC activation, compared with the HF group. In addition, MMP13 or TIMP-1 were not significantly affected. However, CD44, the major cell surface HA receptor, was increased in the

### Table 4

<table>
<thead>
<tr>
<th>Gene</th>
<th>C, n = 4</th>
<th>HF, n = 7</th>
<th>HF + NAC, n = 8</th>
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<tbody>
<tr>
<td>TNFα</td>
<td>1.0 ± 0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.3 ± 0.98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.2 ± 1.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.0 ± 0.17</td>
<td>2.6 ± 0.76</td>
<td>2.5 ± 0.26</td>
</tr>
<tr>
<td>IL-12</td>
<td>1.0 ± 0.29</td>
<td>1.0 ± 0.24</td>
<td>0.7 ± 0.32</td>
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<sup>1</sup> Values are means ± SEM, relative expression of target gene mRNA normalized to cyclophilin A mRNA as a housekeeping gene. Means in a row with superscripts without a common letter differ, P ≤ 0.05.
HF group and this was prevented by NAC. CD44 plays a role in clearing fragmented HA, which accumulates during tissue injury (32). However, HA may also play a role in promoting tissue repair as the result of interactions with Toll-like receptors. In an acute lung injury model, overexpression of high molecular mass HA resulted in protection against injury in part through Toll-like receptor-mediated activation of nuclear factor \( \kappa B \) (58). It is possible that increased HA, as the result of reduced CD44 expression in NAC-treated rats, contributed to the partial protection against fibrosis in the current model. There is a possibility that NAC was also working downstream of TGF\( \beta \) to diminish the progression of fibrosis via other signaling pathways. Dietary NAC is rapidly cleared from the body (13,59). Assuming a constant infusion of NAC at 2000 mg kg\(^{-1}\)d\(^{-1}\), steady-state plasma concentrations of NAC are calculated to be ~300 \( \mu \)mol L\(^{-1}\). NAC has provided protection in our model, despite this relatively low predicted in vivo concentration, compared with in vitro studies where antioxidant and antifibrotic activities of NAC have been reported at levels in excess of 5 mmol L\(^{-1}\) (41). The observed antifibrotic effect may be secondary to decreases in lipid peroxidation and proinflammatory cytokines, such as TNF\( \alpha \), both of which have been implicated in the development of fibrosis. Lipid peroxidation products can directly enhance collagen production by activated HSC (60). Consistent with a role of lipid peroxidation in steatosis-triggered fibrosis, vitamin E and C supplementation in NASH patients has been reported to improve fibrosis (61). The effects on the CD68:CD45 ratio and TNF\( \alpha \) suggest that NAC’s protective effect against liver fibrosis may be associated with its ability to inhibit infiltrating macrophages. To some degree, our data parallels in vivo studies of antifibrotic effects of other antioxidants such as curcumin, the major polyphenolic compound in turmeric. Curcumin has been shown to inhibit the development of liver fibrosis mainly due to its antiinflammatory activities and not by a direct antifibrotic effect (62).

We have shown that dietary NAC is an effective hepatic antioxidant that abolished NASH-induced lipid peroxidation, attenuated reductions in hepatic GSH, blocked NASH-associated autoimmune responses, inhibited the production of TNF\( \alpha \), and attenuated inflammation, leading to significant reduction in cellular damage, hepatocyte injury, and fibrosis. These effects are similar to those we have previously reported for NAC treatment in a rat model of alcoholic steatohepatitis (12). To our knowledge, this is the first report that administration of NAC partially protects against progression of liver injury, including fibrosis, in this type of dietary model of NASH. These data suggest that NAC supplementation in conjunction with other

**FIGURE 5** NAC supplementation reduces immune responses to protein adducts of MDA (A) and lipid hydroperoxides (B) in rats overfed a HF diet. Values are means ± SEM; \( n = 4 \) (C), 7 (HF), or 8 (HF + NAC). Means without a common letter differ, \( P \leq 0.05. \)

**FIGURE 6** Effects of overfeeding HF with or without NAC supplementation on relative mRNA expression of early markers of HSC activation: TGF\( \beta \) (A), \( \alpha \)-SMA (B), and PDGFr\( \beta \) (C). Values are means ± SEM; \( n = 4 \) (C), 7 (HF), or 8 (HF + NAC). Means without a common letter differ, \( P \leq 0.05. \)
treatments may be a valuable adjuvant therapy in NASH patients. Indeed, a clinical study has recently been published in which NASH patients treated for 12 mo with 1.2 g d−1 NAC in conjunction with metformin demonstrated significantly improved fibrosis (63).

Acknowledgments
We thank the following people for their technical assistance: Matt Ferguson, Jamie Badeaux, Tammy Dallari, and Michele Perry.

Literature Cited
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