Nonalcoholic fatty liver disease (NAFLD) is among the most common forms of chronic liver disease today. Current estimates suggest that approximately 20–30% of the population are affected by the condition (3). The pathogenesis of NAFLD is marked by excessive accumulation of intrahepatic lipid (IHL), increases in insulin resistance, and an elevation in profibrogenic activities such as inflammation, oxidative stress (12) and activation of hepatic stellate cells (7). Recently it has been shown that hepatocyte apoptosis contributes to this profibrogenic state (41). Apoptosis, as a form of programmed cell death, is an active ATP-dependent process that under normal physiological conditions contributes to the maintenance of tissue homeostasis. However, in certain pathophysiological conditions, such as NAFLD, apoptosis is upregulated, overwhelming the normal phagocytic engulfment of apoptotic cells, triggering a proinflammatory and profibrogenic response from hepatocytes (22).

Activation of apoptosis in hepatocytes initiates the caspase cleavage of the intermediate filament cytokeratin 18 (CK18) at two sites, Asp238 and Asp326 (8, 33). This cleavage is highly specific to apoptosis, and fragments (M30) can be detected by antibody specific ELISA (16). In a recent study, Wieckowska et al. (48) demonstrated that M30 fragments were elevated in NAFLD patients compared with controls and that the levels of these fragments were correlated with the presence of liver fibrosis. This finding has been confirmed in subsequent studies (49, 50), and it is thought that caspase-cleaved CK18 fragments may be a highly sensitive, noninvasive biomarker for determining the severity of NAFLD (46). Current evidence suggests that activation of the Fas apoptotic pathway may be involved in initiating hepatocyte apoptosis in NAFLD. Fas is a glycosylated protein that is widely expressed in the liver (2). It is activated by the binding of Fas ligand (FasL), leading to receptor trimerization and formation of the death-inducing signaling complex (DISC) (37). Data from Feldstein et al. (14) demonstrate elevations in Fas protein expression in liver samples from nonalcoholic steatohepatitis (NASH) patients. Additionally, Fas expression is upregulated in human HepG2 cells exposed to free fatty acids (FFA), and in a mouse model of NAFLD, resulting in increased sensitivity to Fas-mediated apoptosis (15).

Given its central role in the pathogenesis of NAFLD, identifying therapeutic treatments that minimize apoptosis in these patients may result in better outcomes for individuals with NAFLD. Due to its association with obesity, lifestyle interventions are recommended to patients presenting with NAFLD. As early as 1970, Drenick et al. (13) showed that diet-induced weight loss over 5 mo reduced hepatic steatosis, and when weight loss was maintained for an average of 17 mo, liver histology was either completely or almost completely normalized. More recently, Palmer and Schaffer (34) observed an improvement in alanine aminotransferase (ALT) and aspartate aminotransferase (AST), surrogate markers of liver damage, following 10% weight loss over an average of 16 mo. Lifestyle interventions utilizing a combination of diet and exercise have also been
shown to result in reductions in serum levels of ALT and AST, both in the presence and absence of weight loss (44). Furthermore, data from animal studies indicate that exercise training prevents the development of steatosis (4, 40), even in the presence of a high-fat diet (9). However, no study to date has investigated the effect of exercise training, independent of weight loss, on markers of apoptosis in individuals with NAFLD. We therefore examined the effect of a 7-day exercise program on plasma CK18 fragments in obese individuals with NAFLD. We hypothesized that exercise training would provide an antiapoptotic stimulus, resulting in reductions in CK18 fragments, and that this would be mediated, via reductions in the Fas signaling pathway.

**MATERIALS AND METHODS**

**Participants.** Thirteen obese, previously sedentary (individuals exercising for 20 min or more at least 2 times per week were excluded) adults (age 58 ± 3 yr; body mass index 35.2 ± 1.2 kg/m²; means ± SE) with NAFLD (>5% IHL assessed by 1H MR spectroscopy) were recruited from the local population to undergo a 7-day exercise training intervention. All volunteers underwent a medical history, physical exam, oral glucose tolerance test (OGTT), and complete blood profile (lipid profile, and hepatic/renal/hematological function tests). Medical screening excluded individuals with any disease, and/or taking medications known to affect our outcome variables. In addition, individuals consuming more than 5 units of alcohol per week were excluded. Screening also excluded those with any contraindications to physical activity that was detected during a resting 12-lead electrocardiogram and a submaximal exercise stress test. Female subjects were either postmenopausal and not using any hormone replacement therapy, or premenopausal and in the follicular phase of the menstrual cycle during the study period. The study was approved by the Institutional Review Board, and all subjects provided signed informed consent in accordance with our guidelines for the protection of human subjects.

**Aerobic fitness.** Each participant performed an incremental graded treadmill exercise test to determine his or her maximal oxygen consumption (VO$_{2max}$), as previously described (32).Expired air was continuously sampled online with the use of an automated system (Jaeger Oxycon Pro; VIASYS, Yorba Linda, CA). VO$_{2max}$ testing was conducted on the pre- and posttesting days following an OGTT.

**Intervention.** All participants undertook 60 min of supervised aerobic exercise at ~80–85% of maximum heart rate each day for 7 consecutive days. Compliance to exercise intensity was monitored using a heart rate monitor (Polar Electro, Woodbury, NY). Participants were instructed to consume their normal diet and not make any dietary changes during the 7-day period of the intervention. Furthermore, subjects were instructed to avoid caffeine consumption for 12 h prior to and alcohol for 48 h prior to testing and to consume the same diet containing 250 g of carbohydrate on the day prior to the pre- and poststudy testing days. Posttesting occurred the morning of the day following the final exercise bout.

**Body composition.** Height and body weight were measured by standard techniques. Whole body adiposity was measured by dual-energy X-ray absorptiometry (model IDXA; Lunar, Madison, WI). IHL. Hepatic triglyceride content was measured by 1H MR spectroscopy using a 3T MR system (Siemens Sonata, Erlangen, Germany). Subjects arrived at the laboratory at 0600 following an overnight (8 h) fast. Briefly, a body array MRI coil was affixed to each subject’s back with velcro straps. The center of the body array coil was aligned with the subject’s spine and shoulders for accurate repositioning during longitudinal studies. Each subject was positioned face down and head first in the Siemens Verio 3T MRI scanner on a memory foam mattress to further minimize respiratory motion. After the completion of locator scans for positioning, an 8-cm$^3$ voxel was positioned within the right posterior lobe of the subject’s liver with guidance from the high-resolution localization images. Manual shimming was performed to a line width of ~40 Hz to ensure the high-quality spectra required to delineate water and the various lipid species. MR spectra with and without water suppression were acquired with a single-voxel PRESS acquisition with a long repetition time (TR = 5000 ms), and a short echo time (TE = 30 ms) to limit the effects of magnetic relaxation (20). The acquisition was acquired with 32 averages to obtain sufficient signal to accurately assess lipid components. The data were Fourier-transformed, filtered, baseline corrected, and phased. All NAFLD patients were confirmed to have greater than 5% IHL, which is the diagnostic criteria for hepatic steatosis (45).

**Insulin sensitivity and substrate metabolism.** Subjects arrived at the Clinical Research Unit following an overnight fast and lay supine in bed for 30 min followed by assessment of whole body fat oxidation (FOX) by indirect calorimetry using the following equation: FOX = 1.695VO$_2$ – 1.701VCO$_2$, where VO$_2$ is oxygen consumption and VCO$_2$ is carbon dioxide production. (35). Subsequently, a 75-g OGTT was administered. Baseline blood draws were obtained from an antecubital vein prior to ingestion of the glucose drink. Blood samples were drawn in EDTA tubes containing aprotinin at 30, 60, 90, 120, and 180 min after ingestion. Plasma glucose was determined using the YSI 2300 STAT Plus analyzer (Yellow Springs, OH), plasma insulin was determined via radioimmunoassay (Millipore, Billerica, MA) and plasma FFA were measured by colorimetric assay (NEFA C; Wako Pure Chemical Industries, Richmond, VA). Insulin sensitivity during the OGTT (ISI$_{OGTT}$) was calculated using the Matsuda index (30). We also calculated adipose insulin resistance (adipose IR = fasting plasma FFA × fasting plasma insulin) (28).

**Plasma analyses.** Plasma analyses were performed on plasma which was stored at −80°C immediately following postdose processing. Plasma caspase-3-generated CK18 fragments were quantified by M30 apoptosense ELISA (PEVIVA; Alexis, Grunwald, Germany). The Human Fas/TNFRSF6 QuantiKine ELISA Kit and the Human Fas Ligand/TNFFSF6 QuantiKine ELISA Kit (R&D systems, Minneapolis, MN) were used for quantitative measurement of soluble Fas (sFas) and soluble Fas ligand (sFasL), respectively. ALT and AST were assessed using the Cobra Integra Alanine Aminotransferase (ALT) test, test ID 0-495, and the Aspartate Aminotransferase (ASTL) test, test ID 0–494 (Roche Diagnostics, Indianapolis, IN), respectively.

**Statistical analyses.** Values were tested for normality using the D’Agostino and Pearson omnibus normality test on GraphPad Prism 4.0 (Graphpad Software, San Diego CA). Pre to post intervention changes were assessed using a repeated-measures ANOVA for normally distributed samples. Pre to post changes that were not normally distributed were log transformed [area under the curve (AUC)] insulin. Data that were not normalized by log transformation [fasting plasma insulin (FPI) and ISI$_{OGTT}$] were assessed using a Wilcoxon signed rank test. Linear regression analysis was used to determine associations between normally distributed data. In addition, Spearman’s rank correlation analyses were used to identify relationships between variables that failed the test for normality ($\Delta$FOX and $\Delta$FasL). Statistical significance was accepted when $P < 0.05$. These analyses were carried out using StatView for Windows 5.0.1 (SAS Institute, NC), and all data are expressed as means ± SE.

**RESULTS**

**Participant characteristics.** Anthropometric data for the group are summarized in Table 1. Seven of the participants presented at screening with elevated ALT and AST values. Seven days of exercise did not alter body weight or body composition; however, aerobic fitness, as measured by VO$_{2max}$, did increase following exercise training. In addition, total IHL.

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assessed by $^1$H MR Spectroscopy remained unchanged following the intervention (Table 1).

**Blood glucose and insulin sensitivity.** The plasma glucose and insulin responses to glucose ingestion were significantly reduced (Table 1), and insulin sensitivity, measured by the Matsuda index, increased following exercise (Fig. 1A). Fasting glucose, FFA, and insulin did not change after the 7-day program; neither was adipose-IR altered by the intervention (Table 1).

**Apoptotic biomarkers.** Plasma CK18 (Fig. 1B; 558.4 ± 106.8 vs. 323.4 ± 72.5 U/l, $P < 0.01$) and ALT (Fig. 1C; 30.2 ± 5.1 vs. 24.3 ± 4.8 U/l, $P < 0.05$) were significantly reduced by the intervention, while the reduction in sFasL approached significance (Fig. 1D; 66.5 ± 6.0 vs. 63.0 ± 5.7 pg/ml, $P = 0.06$). However, there was no change in plasma AST (35.1 ± 6.2 vs. 34.5 ± 5.8 U/l, NS) or sFas (6,483.2 ± 358.0 vs. 6,284.9 ± 315.7 pg/ml, NS). There was a trend toward an association between the intervention-induced change in CK18 and ALT (Fig. 2; $r = 0.55$, $P = 0.05$). We found no correlation between the change in insulin sensitivity or adipose insulin resistance and markers of cell death.

**Substrate oxidation.** Resting FOX was increased following the exercise intervention (Table 1). In addition, changes in FOX were significantly correlated with changes in sFasL (Fig. 3; $\rho = 0.65$, $P < 0.05$).

**DISCUSSION**

Exercise currently forms a major component of the treatment recommended by the American Gastroenterological Association (1) for NAFLD, despite a lack of published evidence on the effectiveness of exercise in treating this disease (26). Here we show, for the first time, that exercise in the absence of weight loss significantly reduces plasma levels of the apoptotic marker, caspase-cleaved CK18 fragments, in previously sedentary obese individuals with NAFLD. These data collectively indicate a reduction in the profibrogenic apoptotic state present

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre</th>
<th>Post</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>58 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>100.3 ± 3.8</td>
<td>100.4 ± 3.7</td>
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<tr>
<td>BMI, kg/m$^2$</td>
<td>35.2 ± 1.2</td>
<td>35.3 ± 1.1</td>
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<tr>
<td>FM, kg</td>
<td>45.8 ± 2.4</td>
<td>45.6 ± 2.3</td>
<td>0.298</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>54.4 ± 3.1</td>
<td>54.9 ± 3.1</td>
<td>0.065</td>
</tr>
<tr>
<td>IHL, %</td>
<td>18.2 ± 2.5</td>
<td>17.5 ± 2.1</td>
<td>0.285</td>
</tr>
<tr>
<td>$V_{0_2}$max, ml·kg$^{-1}·min^{-1}$</td>
<td>22.0 ± 1.4</td>
<td>23.6 ± 1.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Fasting glucose, mg/dl</td>
<td>112.9 ± 5.7</td>
<td>108.5 ± 3.7</td>
<td>0.285</td>
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<tr>
<td>Fasting insulin, μU/ml</td>
<td>25.3 ± 3.1</td>
<td>22.8 ± 2.7</td>
<td>0.249</td>
</tr>
<tr>
<td>Glucose AUC, log pg·ml$^{-1}·3$</td>
<td>13,663.6 ± 1,423.4</td>
<td>11,528.1 ± 1,517.1</td>
<td>&lt;0.01</td>
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<tr>
<td>Log insulin AUC, log pg·ml$^{-1}·3$</td>
<td>4.3 ± 0.1</td>
<td>4.16 ± 0.1</td>
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<tr>
<td>Fasting FFA, μmol/l</td>
<td>598 ± 51</td>
<td>605 ± 39</td>
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<td>Adipose-IR, AU</td>
<td>14.6 ± 1.4</td>
<td>13.2 ± 1.4</td>
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<tr>
<td>Resting FOX, mg/min</td>
<td>49.3 ± 6.1</td>
<td>69.4 ± 7.1</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. Pre, before exercise training; Post, after exercise training; BMI, body mass index; FM, fat mass; FFM, fat-free mass; IHL, intrahepatic lipid; $V_{0_2}$max, maximal oxygen consumption; AUC, incremental area under the curve; FFA, free fatty acids; IR, insulin resistance; AU, arbitrary units; FOX, fat oxidation.

Fig. 1. Short-term aerobic exercise training increases insulin sensitivity (A) and reduces circulating cytokeratin 18 (CK18) fragments (B); alanine aminotransferase (ALT) (C), and circulating soluble Fas ligand (sFasL) (D). Data are presented as means ± SE. *$P < 0.05$. OGTT, oral glucose tolerance test; ISLOGTT, insulin sensitivity (Matsuda) index; AU, arbitrary units.
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in NAFLD. This finding is important as hepatocyte apoptosis contributes significantly to the pathogenesis of NAFLD and progression to NASH and liver fibrosis (41). Recently Kistler et al. (26) examined the relationship between self-reported physical activity and the severity of liver fibrosis. They reported that individuals who met the vigorous activity levels recommended by the US Department of Health and Human Services had significantly lower odds of having advanced fibrosis. Our data support the view that exercise serves a protective function against the pathogenesis of NAFLD.

The changes in plasma CK18 fragments in our data were mirrored by changes in ALT levels, suggesting that the reductions we observed in plasma CK18 indeed reflect decreases in hepatocyte apoptosis. CK18 is expressed in epithelial cells, and therefore the reductions could theoretically be derived from decreased apoptosis in almost any tissue. However, ALT is an enzyme of the alanine cycle and is predominantly expressed in the liver. For this reason, it is used as a surrogate biomarker for liver injury. Several previous studies have demonstrated a positive effect of longer term exercise on ALT levels in serum (10–11, 44). It has also been recently reported that administration of a caspase inhibitor may reduce ALT and CK18 in NAFLD patients in a dose-dependent manner (39). Here, we confirm the beneficial effect of exercise on ALT levels and show that these effects become apparent within 1 wk of moderate- to high-intensity exercise. Furthermore, the positive correlation between percent changes in plasma CK18 levels and ALT suggests that improvements in CK18 may be a result of reduced hepatocyte apoptosis.

The exact mechanism by which exercise may reduce apoptosis in NAFLD remains to be elucidated. Insulin resistance is a key component in the pathogenesis of NAFLD (12). Our exercise program resulted in an increase in insulin sensitivity of ~25% in this subject population. Exercise-mediated improvements in insulin sensitivity are well documented (5, 18, 19, 24, 25). Exercise elicits favorable alterations in lipid uptake and metabolism in skeletal muscle (31), and it has been proposed that exercise-induced improvements in insulin sensitivity may decrease de novo lipogenesis and hepatic triglyceride synthesis (38, 40). We did observe an increase in whole body fat oxidation. Moreover, the alterations in FOX were negatively correlated with changes in sFasL. Collectively, these data support the hypothesis that improved whole body insulin sensitivity and greater fat oxidation may act to reduce lipotoxicity in the liver and thereby reduce the proapoptotic stimulus to hepatocytes.

Apoptosis is mediated via two pathways, the extrinsic and intrinsic pathway. In this study we also investigated the effect of exercise on biomarkers of the Fas death-inducing pathway, a component of the extrinsic pathway. We found that exercise resulted in a modest reduction in sFasL but not sFas. Previous reports have linked exercise training to reductions in oxidative stress (42). Induction of high levels of reactive oxygen species (ROS) subjects cells to oxidative stress, which is believed to play a pivotal role in the pathogenesis of NAFLD (27, 29, 47). While hepatocytes express Fas in large numbers, they do not normally express large numbers of Fas ligand. However, increased ROS production in hepatocytes can cause increased Fas ligand expression, resulting in fratricidal apoptosis (36). Mitochondrial dysfunction is believed to be a major contributor to ROS production in NAFLD (17). Exercise increases mitochondrial function and content principally through high-molecular weight (HMW) adiponectin (21). We have previously demonstrated increases in HMW adiponectin in insulin-resistant obese individuals following 7 days of moderate- to high-intensity exercise. Furthermore, the changes in HMW adiponectin were positively correlated with changes in FOX (23). Therefore, improved oxidative capacity and reduced oxidative stress following exercise training may cause a reduction in the generation of Fas ligand within hepatocytes and consequently attenuate the apoptotic signal. However, the relationship between improvements in FOX and ROS production in a NAFLD population following exercise remains to be confirmed.

There is also evidence that ROS increases TNF-α production, which interacts with and activates caspase-8, resulting in permeabilization of the mitochondrial membrane via truncated Bid and induction of cytochrome c release (36). Therefore exercise may also reduce apoptosis via reductions in intrinsic pathway signaling. Upregulation of the antioxidant defense system can also reduce oxidative stress-mediated apoptosis via the intrinsic pathway. Indeed, Sinha-Hikim et al. (43) recently demonstrated that a high-fat diet upregulated proapoptotic and downregulated antiapoptotic enzymes BAX and BCL-2, respectively, in a mouse model of NAFLD. However, supplementation with a glutathione precursor significantly reduced the high-fat-feeding upregulation of BAX and suppressed activation of caspase 3 in hepatocytes. Cakir et al. (6) recently demonstrated that exercise protects against glutathione depletion in hepatocytes of rats with alcoholic liver disease; how-
ever, data from human or NAFLD studies are currently lacking. The possibility that reductions in apoptosis are mediated through downregulation of the intrinsic pathway, on the basis of these data, cannot be excluded.

Conclusions. Empirical data on the effect of exercise on hepatocyte apoptosis in NAFLD are currently lacking; however, here for the first time we demonstrate that short-term exercise, in the absence of weight loss and changes in intrahepatic lipid, reduces a circulating marker of hepatocyte apoptosis in previously sedentary obese individuals with NAFLD. The exact mechanism by which this occurs is currently under investigation and for now our interpretation of the data is limited by the use of whole body indexes. Nonetheless, we provide evidence that reductions in apoptosis may be related to reductions in FasL, possibly resulting from improvements in oxidative capacity. These findings have significant clinical implications for the prevention of disease progression in NAFLD patients and support the use of exercise as an effective treatment for NAFLD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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