

Rats selectively bred for low aerobic capacity have reduced hepatic mitochondrial oxidative capacity and susceptibility to hepatic steatosis and injury

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Fatty liver has been linked to low aerobic fitness, but the mechanisms are unknown. We previously reported a novel model in which rats were artificially selected to be high capacity runners (HCR) and low capacity runners (LCR) that in a sedentary condition have robustly different intrinsic aerobic capacities. We utilized sedentary HCR/LCR rats (generation 17; max running distance equalled 1514 ± 91 vs. 200 ± 12 m for HCR and LCR, respectively) to investigate if low aerobic capacity is associated with reduced hepatic mitochondrial oxidative capacity and increased susceptibility to hepatic steatosis. At 25 weeks of age, LCR livers displayed reduced mitochondrial content (reduced citrate synthase activity and cytochrome *c* protein) and reduced oxidative capacity (complete palmitate oxidation in hepatic mitochondria (1.15 ± 0.13 vs. 2.48 ± 1.1 nmol g⁻¹ h, $P < 0.0001$) and increased peroxisomal activity (acyl CoA oxidase and catalase activity) compared to the HCR. The LCR livers also displayed a lipogenic phenotype with higher protein content of both sterol regulatory element binding protein-1c and acetyl CoA carboxylase. These differences were associated with hepatic steatosis in the LCR including higher liver triglycerides (6.00 ± 0.71 vs. 4.20 ± 0.39 nmol g⁻¹, $P = 0.020$ value), >2-fold higher percentage of hepatocytes associated with lipid droplets (54.0 ± 9.2 vs. $22.0 \pm 3.5\%$, $P = 0.006$), and increased hepatic lipid peroxidation compared to the HCR. Additionally, in rats aged to natural death, LCR livers had significantly greater hepatic injury (fibrosis and apoptosis). We provide novel evidence that selection for low intrinsic aerobic capacity causes reduced hepatic mitochondrial oxidative capacity that increases susceptibility to both hepatic steatosis and liver injury.

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Abbreviations ACC, acetyl CoA carboxylase; ALT, serum alanine aminotransferase; CPS1, anti-carbamoylphosphate synthetase 1; FAS, fatty acid synthase; LCR, low capacity runner; β -HAD, β -hydroxyacyl-CoA dehydrogenase; HCR, high capacity runner; TAG, triglyceride; H&E, haematoxylin–eosin; 4-HNE, 4-hydroxynonenal; NAFLD, non-alcoholic fatty liver disease; PPAR α , peroxisome proliferator-activated receptor α ; SREBP-1c, sterol regulatory binding protein-1c; TGF- β , transforming growth factor β ; TUNEL, terminal desoxynucleotide transferase-mediated dUTP nick end labelling; PPAR γ , peroxisome proliferator-activated receptor γ .

Non-alcoholic fatty liver disease (NAFLD) encompasses a gamut of liver maladaptations and is a primary cause of chronic liver disease and liver-related morbidity and mortality. Fatty liver occurs at a higher rate in individuals with obesity and type 2 diabetes and is considered

the hepatic component of the metabolic syndrome (Schindhelm *et al.* 2006). Currently, 34% of the general population (Browning *et al.* 2004) and 75–100% of obese and extremely obese individuals are estimated to have fatty liver (Bellentani *et al.* 2000).

Low aerobic capacity, or low cardiorespiratory fitness, is the strongest predictor of early mortality (Myers *et al.* 2002). Aerobic capacity is dependent upon the ability of the cardiorespiratory system to deliver oxygen and the capacity of mitochondria to utilize oxygen for energy production. In the absence of exercise training, it is believed that genetic inheritance accounts for up to 70% of the variation in intrinsic aerobic capacity (Bouchard *et al.* 1986). In most cases, exercise training significantly improves aerobic capacity; however, exercise-induced responses are grossly heterogeneous (Kohrt *et al.* 1991; Bouchard & Rankinen, 2001). Recent evidence also links low aerobic capacity (Nguyen-Duy *et al.* 2003; Church *et al.* 2006; McMillan *et al.* 2007) to an increased deposition of fat in the liver; however, there is no mechanistic information detailing these connections.

Over several generations of selective breeding for high and low treadmill endurance running (performance on 3 graded exercise tests), we have developed two strains of rats with grossly different intrinsic endurance exercise capacities (max run distance during a graded exercise test to exhaustion is ~7-fold different) and aerobic capacities (30% different at generation 11) (Wisloff *et al.* 2005). This novel animal model is valuable to study the interaction between aerobic capacities and chronic disease for two main reasons: (1) the contrasting aerobic capacities occur in a sedentary, caged-activity-only condition (Hoydal *et al.* 2007) removing the conflicting effects of daily exercise training (Wisloff *et al.* 2005; Bernal-Mizrachi & Semenkovich, 2006); and (2) the strains are a polygenic model of disease that more accurately mimics the pathology of human chronic metabolic disease(s) than single gene mutations. At generation 11, the sedentary low capacity runners (LCR) displayed a higher incidence of both cardiovascular and metabolic syndrome risk factors than sedentary high capacity runners (HCR) (Wisloff *et al.* 2005). In addition, at generation 13, LCR rats were susceptible to high-fat diet induced obesity and insulin resistance while the HCR rats were largely unaffected (Noland *et al.* 2007a). In both reports, skeletal muscle mitochondrial oxidative capacity, which is high in the muscle of HCR, and low in the muscle of LCR, was hypothesized to be the primary cause for protection or susceptibility to metabolic dysfunction, respectively.

In the current study, we investigate the potential links between intrinsic aerobic capacity, hepatic mitochondrial oxidative capacity and susceptibility to hepatic steatosis in generation 17 HCR and LCR rats. Importantly, these studies occurred in sedentary rats which are genetically predisposed to high and low aerobic capacities allowing for the determination of hepatic metabolism independently of the effects of exercise training. We hypothesize that the phenotype of low aerobic capacity is associated with both reduced hepatic mitochondrial content and oxidative capacity which increases both susceptibility to early life

hepatic steatosis and significant hepatic injury at an older age.

Methods

Animal strain and protocols

The development of LCR and HCR rats has previously been described (Wisloff *et al.* 2005; Noland *et al.* 2007a). Briefly, two-way artificial selective breeding was used to create low capacity runner (LCR) and high capacity runner (HCR) strains that were divergent for treadmill running capacity (run time to exhaustion on a graded exercise test). The founder population (N: NIH stock) and generation of the HCR and LCR strains has been previously reported (Wisloff *et al.* 2005). In brief, the 13 lowest and 13 highest running capacity rats of each sex were selected from the founder population and randomly paired for mating. At each subsequent generation, within-family selection from 13 mating pairs was practiced for each line, a number of families that maintains a relatively low coefficient of inbreeding (<0.01/generation) and maximizes the retention of genetic variation. After the rats were phenotyped for running capacity they were exposed to no further exercise training or testing and only underwent normal cage activity.

The animal protocols were approved by the Institutional Animal Care and Use Committees at the University of Missouri and the University of Michigan and the Subcommittee for Animal Safety at the Harry S. Truman Memorial VA Hospital.

Two separate sets of ~25-week-old male LCR ($n = 7$) and HCR ($n = 8$) rats from generation 17 were used for the investigation of hepatic steatosis and hepatic mitochondrial oxidative capacity. The rats arrived at ~14 weeks of age, were provided standard rat chow and water *ad libitum*, and were kept on a 12 h light–12 h dark time schedule until killed at ~25 weeks of age. On the morning of tissue collection, rats were intraperitoneally injected with sodium Nembutal (100 mg kg^{-1}), blood was collected, the animals were exsanguinated, and tissues were frozen with liquid nitrogen and stored at -80°C or fixed for immunohistochemistry. Tissue was collected in the same manner in a second group of 25-week-old, generation 17, HCR ($n = 8$) and LCR ($n = 8$) rats and ~500 mg of liver was quickly removed and placed in ice-cold buffer for the isolation of hepatic mitochondria or frozen for later measurement of peroxisomal enzyme activity. Additional generation 17 rats (HCR, $n = 4$; LCR, $n = 6$) were aged until natural death or until they were killed because of undue suffering at approximately 24–35 months of age at the University of Michigan where the rats were generated. After death animals underwent necropsy within 4 h and livers were fixed for immunohistochemistry only. Weekly food consumption and changes in body weight

were measured in an additional set of HCR/LCR rats ($n = 10$ in both groups) from 5 to 25 weeks of age. In addition, determinations of physical activity and oxygen consumption by indirect calorimetry were taken in the rats over a 2 day period at 25 weeks of age.

Intrahepatic lipid content

Hepatic triglycerides (TAG) were measured as referenced previously using a commercially available kit (Sigma, F6428) (Rector *et al.* 2008).

Histology analysis

Liver and epididymal adipose tissues were collected and fixed in 10% formalin for histological analysis. Routine hematoxylin–eosin (H&E) staining was performed in both liver and adipose and sirius red staining for collagen deposition was performed in paraffin liver sections as previously referenced (Wei *et al.* 2008). Hepatic steatosis and the percentage of hepatocytes associated with lipid droplets were determined as referenced previously (Ibdah *et al.* 2005) and morphometric determinations of adipocyte cell volume were carried out as previously referenced (Laye *et al.* 2006).

Immunohistochemistry

Liver paraffin sections were de-paraffinized and non-specific antibody binding was blocked with 5% normal goat or rabbit serum and 5% BSA in phosphate-buffered saline (PBS). These sections were incubated with anti-carbamoylphosphate synthetase 1 (CPS1), transforming growth factor β (TGF- β) or 4-hydroxynonenal (4-HNE) antibodies at 1 : 200 dilutions for overnight at 4°C. After washing, the sections were incubated with a second antibody conjugated with alexa-586 for 1 h at room temperature and mounted with DAPI (Vector) or biotin conjugated second antibody, following by 0.3% H₂O₂, ABC (Vector), NovaRed (Vector), and counterstained with hematoxylin. The primary antibody was omitted from this procedure for additional sections to ensure that results were not due to non-specific binding. Images were acquired with a light and fluorescence microscope (Nikon, Eclipse 50i).

Apoptosis by TUNEL staining

To evaluate apoptotic cell death, terminal deoxynucleotide transferase-mediated dUTP nick end labelling (TUNEL) was performed in liver sections using an *in situ* Apoptosis Detection Kit (Roche Applied Science, Indianapolis, IN, USA) as per the manufacturer's instructions. TUNEL-positive and -negative nuclei were

counted at five random fields for each section. Results are expressed as number of TUNEL-positive cell/total cells \times 100%.

Western blotting

Peroxisome proliferator-activated receptor (PPAR) γ , PPAR α , and sterol regulatory binding protein-1c (SREBP-1c) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against Acetyl CoA carboxylase (ACC) and cytochrome c were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA) and 4-HNE was purchased from Oxis International Inc. (Beverly Hills, CA, USA). Liver samples were homogenized in ice cold buffer, separated by SDS-PAGE gels, transferred to PVDF membranes and probed with primary antibodies. Protein bands were quantified using a densitometer and band densities were corrected for total protein loaded by staining with 1% amido-black (Sigma) as described previously (Rector *et al.* 2008).

Hepatic mitochondrial enzyme activities, isolation, and palmitate oxidation

Citrate synthase and β -hydroxyacyl-CoA dehydrogenase (β -HAD) activity were both measured as previously described in whole liver homogenates (Rector *et al.* 2008). Mitochondrial suspensions were prepared according to modified methods of Koves *et al.* (2005). A portion of the liver was quickly excised from unconscious rats and placed in 5 ml of fresh Buffer A (100 mM KCl, 50 mM Mops, 5 mM MgSO₄, 1 mM EGTA, 1 mM ATP, pH 7.4). The liver was then minced and suspended 7-fold (w/v) in Buffer A, and homogenized for 15 s. The homogenate was then centrifuged at 800 g for 10 min at 4°C and the supernatant was filtered through gauze and set aside. The remaining pellet was resuspended in Buffer A, homogenized, and the homogenate was then centrifuged at 800 g for 10 min at 4°C. The remaining supernatant was added to the previously set aside supernatant after filtering through gauze. The pooled supernatants were centrifuged at 9000 g to pellet the mitochondria. The pellet was resuspended in Buffer B (Buffer A, 0.2% BSA, pH 7.4), and centrifuged at 8000 g before the pellet was washed and resuspended in Buffer C (100 mM KCl, 50 mM Mops, 0.5 mM EGTA, pH 7.4) and centrifuged for 10 min at 7000 g. The final pellet was resuspended in 0.5 ml of sucrose EDTA (SET) buffer and protein content was determined. Palmitate oxidation was measured with radiolabelled [1-¹⁴C]palmitate (American Radiolabeled Chemicals, Inc., St Louis, MO, USA) in the freshly isolated liver mitochondria preparation using methods previously reported (Laye *et al.* 2008; Rector *et al.* 2008).

Both $^{14}\text{CO}_2$, representing complete fatty acid oxidation, and ^{14}C labelled acid soluble metabolites, representing incomplete fatty acid oxidation, were collected in the previously described trapping device and then counted on a liquid scintillation counter. In addition to [^{14}C]palmitate the reaction buffer contained a final concentration of $50\ \mu\text{M}$ cold palmitate and 0.5% BSA.

Hepatic peroxisomal enzyme activity

Acyl-CoA oxidase activity was determined using a modification of methods previously described (Small *et al.* 1985). Briefly, H_2O_2 production was measured spectrophotometrically (520 nm) through oxidative coupling with 3,5-dichloro-2-hydroxybenzenesulfonic acid to 4-aminoantipyrine in the presence of horseradish peroxidase. Reaction wells contained $20\ \mu\text{l}$ of liver homogenate and $200\ \mu\text{l}$ of reaction buffer consisting of 40 mM aminotriazole, 0.02% Triton X-100, 0.5 mM 4-aminoantipyrine, 4 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 8 U horseradish peroxidase, 11 mM potassium phosphate; pH 7.4. They were incubated for 5 min at 37°C . The reaction was initiated by the addition of acyl-CoA substrate ($50\ \mu\text{M}$) and absorbance was monitored continuously over 30 min at 37°C . Catalase activity was determined by the spectrophotometric (520 nm) oxidative coupling of 3,5-dichlorobenzene-sulfonic acid to 4-aminoantipyrine in the presence of HRP and H_2O_2 (Sigma) as previously described (Noland *et al.* 2007b).

Serum assays and blood pressure

Serum TAG (Sigma) and insulin (Linco Research, St Charles, MO, USA) were measured using commercially available kits. Serum alanine aminotransferase (ALT) concentrations were determined utilizing an Olympus AU400e Chemistry Immuno Analyzer (Olympus America, Inc., Center Valley, PA, USA). Systolic blood pressure was measured in triplicate using the tail-cuff method a week before sacrifice.

Food consumption, ambulatory physical activity, and oxygen consumption

Both *ad libitum* food consumption and total body weights were measured every week from the post-weaning period (~ 5 week) until 25 weeks of age. In addition, oxygen consumption (\dot{V}_{O_2}), carbon dioxide production (\dot{V}_{CO_2}), and spontaneous motor activity were measured using a Comprehensive Laboratory Monitoring System (CLAMS, Columbus Instruments, Columbus, OH, USA) over a 2 day period at 25 weeks of age at the University of Michigan Animal Phenotyping Core. The

Table 1. Characteristics LCR and HCR rats at 25 weeks of age

Variable	LCR	HCR
Body weight (g)	497 \pm 48*	350 \pm 28
Max run distance at 11 weeks (m)	188 \pm 15*	1486 \pm 86
Serum insulin (ng ml $^{-1}$)	3.03 \pm 0.44*	1.34 \pm 0.24
Serum TAG (mg dl $^{-1}$)	61.1 \pm 19.5*	16.7 \pm 1.8
Serum ALT (U l $^{-1}$)	61.1 \pm 14.1	57.4 \pm 38.4
Systolic blood pressure (mmHg)	160.2 \pm 2.3*	151.1 \pm 1.3

Means \pm S.E.M. *Significantly different from HCR, $P < 0.05$; m, metres.

measurements were carried out continuously for 48 h in an experimentation room set at $20\text{--}23^\circ\text{C}$ with 12–12 h (18.00 h–06.00 h) dark–light cycles. \dot{V}_{O_2} and \dot{V}_{CO_2} in each chamber were sampled sequentially for 5 s in a 5 min interval and the motor activity was simultaneously recorded every second in X and Z dimensions. The air flow rate through the chambers was adjusted at the level to keep the oxygen differential around 0.3% at resting conditions.

Statistics

For each outcome measure, a one-way analysis of variance was performed using SPSS 15.0 (Chicago, IL, USA). Pearson correlational analysis was also used on select data. Values are reported as means \pm S.E.M., and a P value < 0.05 was considered statistically significant.

Results

Animal characteristics

The HCR rats ran ~ 9 -fold further distance ($P < 0.001$) than LCR rats during the graded exercise test conducted at 11 weeks of age (Table 1). In the rats killed at 25 weeks, the LCR rats displayed overt characteristics of the metabolic syndrome compared to the HCR including heavier body weights, elevated serum TAG ($P = 0.045$) and insulin ($P = 0.005$) and higher systolic blood pressures ($P = 0.039$) (Table 1). Serum ALT levels, a marker of liver injury, were not significantly different between groups (Table 1).

Ambulatory physical activity, oxygen consumption and food consumption

At 25 weeks of age, the LCR rats had 40% lower ambulatory physical activity in the 12 h dark phase ($P < 0.001$) and a 22% lower activity in the light phase ($P = 0.008$) compared to the HCR (Table 3). This corresponded with a 16% and 11% lower total oxygen consumption in both the

light ($P = 0.005$) and dark phase ($P < 0.001$), respectively (Table 3). Average weekly food consumption (averaged from ~4 to 25 weeks of age) was also significantly lower in the LCR rats ($P = 0.03$) (Table 3). Collectively, these data indicate that the contrasting levels of ambulatory activity and oxygen consumption measured between the two strains corresponded with different rates of food consumption. Feeding efficiency (weight gain divided by food intake) was not different between the groups between 9 weeks and the time of kill at 25 weeks suggesting that overall energy balance was similar for 16 weeks prior to kill (data not shown).

Hepatic mitochondrial enzyme activity, palmitate oxidation and peroxisomal enzyme activity

β -HAD activity, the rate limiting enzyme for β -oxidation, was 65% higher ($P < 0.001$) in the HCR livers compared to LCR livers (Fig. 2A). Citrate synthase activity and cytochrome *c* protein content, markers of mitochondrial content, were 32–42% higher ($P < 0.001$) in the HCR livers compared to LCR (Fig. 2B and C). Immunofluorescence staining for CPS-1, a hepatic mitochondrial marker, qualitatively revealed a greater hepatic mitochondrial content in the HCR vs. the LCR (Fig. 2D). The HCR mitochondria also had higher rates of fatty acid oxidation than the LCR. Isolated hepatic mitochondria from the HCR rats had a 63% (Fig. 3A; $P < 0.0001$) higher rate of complete palmitate oxidation to CO_2 and a 22% higher (Fig. 3B; $P < 0.001$) rate of total palmitate oxidation (CO_2 + acid soluble metabolite production) than the LCR. The LCR rats also displayed significantly higher peroxisomal enzyme activity for both acyl CoA oxidase (Fig. 4A; $P = 0.039$) and catalase (Fig. 4B; $P = 0.02$) compared to the HCR rats, suggestive of a compensatory increase in peroxisomal activity due to reduced mitochondrial oxidative capacity. Collectively these data show that LCR livers have both a lower mitochondrial content and lower mitochondrial oxidative capacity (decreased fatty acid oxidation per mitochondria).

Hepatic lipogenesis

The content of SREBP-1c, a transcriptional regulator of fatty acid synthesis genes (Shimano *et al.* 1996; Horton *et al.* 2002), was significantly higher in the livers of LCR rats than HCR rats (Fig. 5A). Specifically, SREBP-1c is an upstream transcriptional regulator of both acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS). ACC protein content, the enzyme responsible for malonyl CoA production (a potent inhibitor of fatty acid oxidation and the initial substrate for FAS), was 3-fold higher ($P = 0.049$) in the LCR liver than the HCR liver (Fig. 5B). There

was no difference in serine⁷⁹ phosphorylation of ACC protein, a marker of ACC inhibition (data not shown), suggesting that ACC differences were due to total content of the enzyme and not due to differences in covalent modification. We found no difference in FAS content (Fig. 5C). Protein content of PPAR γ and PPAR α , vital transcriptional regulators of lipid synthesis and oxidation also were not different in the livers of LCR and HCR rats (Fig. 5C).

Hepatic and extrahepatic lipid storage

Total hepatic TAG concentrations were significantly higher in the LCR than the HCR rats ($P = 0.02$) (Fig. 1C). Within the LCR rats, hepatic TAG levels inversely correlated with maximum running distance ($r = -0.724$; $P = 0.034$), demonstrating an influence of endurance capacity within the LCR rat strain. The LCR livers also had a higher steatosis score ($P = 0.004$) and >2 fold higher number of hepatocytes associated with lipid droplets than the HCR (Table 2 and Fig. 1). The adipocyte cell volumes of both epididymal and omental fat pads were 35% ($P = 0.003$) and 50% ($P = 0.025$) larger in the LCR than the HCR rats (Table 2). Although not measured herein, a previous report has shown that total epididymal fat pad weight is significantly higher in the LCR than HCR rats (Noland *et al.* 2007a). Taken together, the LCR showed both increased ectopic lipid storage in liver and expansion of fat pads over the HCR.

Hepatic lipid peroxidation

LCR livers also displayed higher levels of lipid peroxidation (4-HNE), a marker of oxidative stress, than HCR livers (Fig. 1D ($P = 0.04$) (Western blot) and Fig. 1B (immunohistochemistry)) indicating that LCR livers possess both increased lipids and oxidative stress early in life.

Hepatic injury

Staining for apoptotic hepatocytes revealed that the LCR rats had a significantly higher percentage of apoptotic nuclei than their HCR counterparts both at 25 weeks of age ($P < 0.001$; data not shown) and after aging to natural death (age at death ranged between 24 and 35 months) (Fig. 6B; $P = 0.007$). Collagen deposition and fibrosis were assessed by sirius red staining in formalin-fixed liver sections. No visual fibrosis was detected from either group at 25 weeks of age (data not shown); however, the LCR livers display a greater percentage of fibrotic area than the HCR rats after aging to natural death (Fig. 6A; $P = 0.005$). These results suggest that early hepatic mitochondrial defects in animals with low intrinsic aerobic capacity are

associated with advanced disease development and hepatic injury later in life.

Discussion

The major findings of this study are that intrinsically low aerobic capacity in the LCR rats was associated with reduced hepatic mitochondrial content, decreased mitochondrial capacity to oxidize fatty acids, and an increased hepatic *de novo* lipogenic profile. Furthermore, the LCR rats developed hepatic steatosis by 25 weeks of age and greater hepatic fibrosis and apoptosis at death after natural aging compared to the HCR rats.

Low aerobic capacity, independent of physical activity levels, is the best predictor of early mortality (Blair *et al.* 1989; Myers *et al.* 2002) and is linked to type 2 diabetes (Mootha *et al.* 2003) and cardiovascular disease (Myers *et al.* 2002). Recently, Church *et al.* (2006) reported an inverse association between aerobic capacity assessed by a graded exercise test and the prevalence of NAFLD in a large cohort of male subjects, a finding that also has been replicated in other studies (Nguyen-Duy *et al.* 2003; McMillan *et al.* 2007). These studies suggest that low aerobic capacity either directly impacts hepatic metabolism or peripheral factors (insulin sensitivity or

visceral adiposity) that lead to the increased storage of fat in the liver. To our knowledge, there are no data describing the hepatic biochemical/molecular adaptations that occur in the livers of animals or humans with intrinsically low or high aerobic capacity. Early reports and editorials on the HCR/LCR model (Wisloff *et al.* 2005; Bernal-Mizrachi & Semenkovich, 2006; Hawley & Spargo, 2006; Noland *et al.* 2007a) postulated that LCR rats possessed low rates of mitochondrial oxidative capacity in skeletal muscle predisposing them to insulin resistance, obesity, and metabolic complications, a common hypothesis supported by studies in the skeletal muscle of humans (Mootha *et al.* 2003; Petersen *et al.* 2004). We report herein that intrinsically low aerobic capacity in sedentary animals is also linked to decreased mitochondrial content and mitochondrial oxidative capacity in liver, a phenotype that we postulate leads to an increased susceptibility to early life hepatic steatosis.

Mobilized fatty acids produced from the lipolysis of visceral adipose are directly trafficked through the liver via portal circulation making it no surprise that increased visceral adiposity is strongly correlated to fatty liver (Nguyen-Duy *et al.* 2003; Church *et al.* 2006; McMillan *et al.* 2007). The LCR rats displayed increased expansion of visceral fat (increased adipocyte cell volume of omental fat

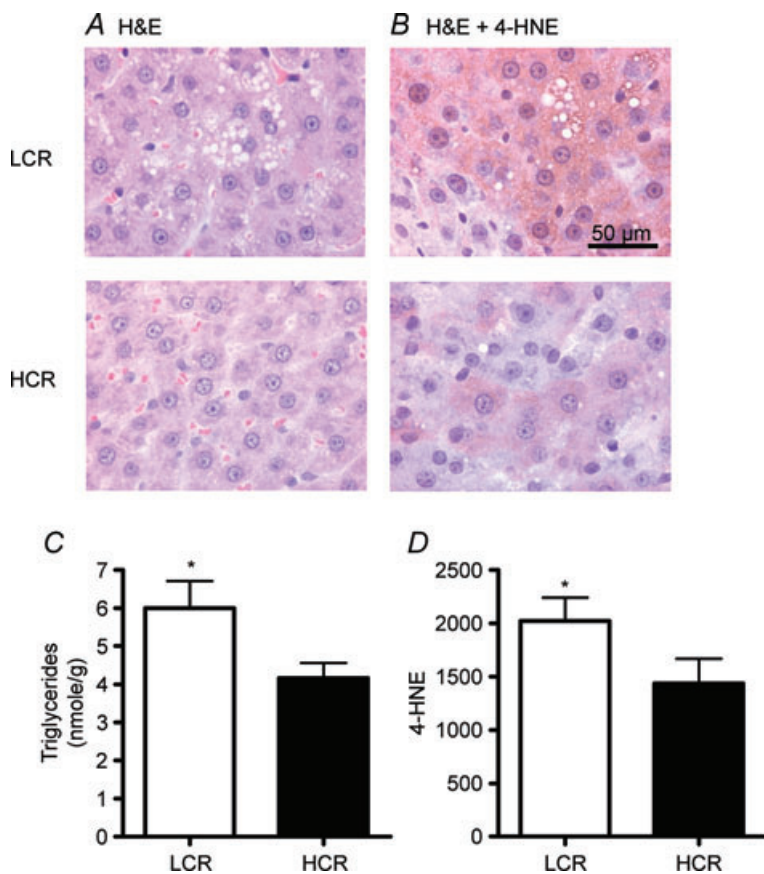


Figure 1. Hepatic lipids

A, representative liver sections with H&E staining reveal greater steatosis in the LCR livers than HCR. *B*, representative liver sections of 4-HNE staining also show greater lipid peroxidation in LCR livers. *C*, hepatic triglyceride content is significantly higher in the LCR than the HCR rats. *D*, lipid peroxidation quantified by western blotting with 4-HNE antibody is significantly higher in the LCR livers than HCR. Samples were obtained from 25-week-old rats. *Significant difference ($P < 0.05$); values are reported as means \pm S.E.M.; $n = 7-8$.

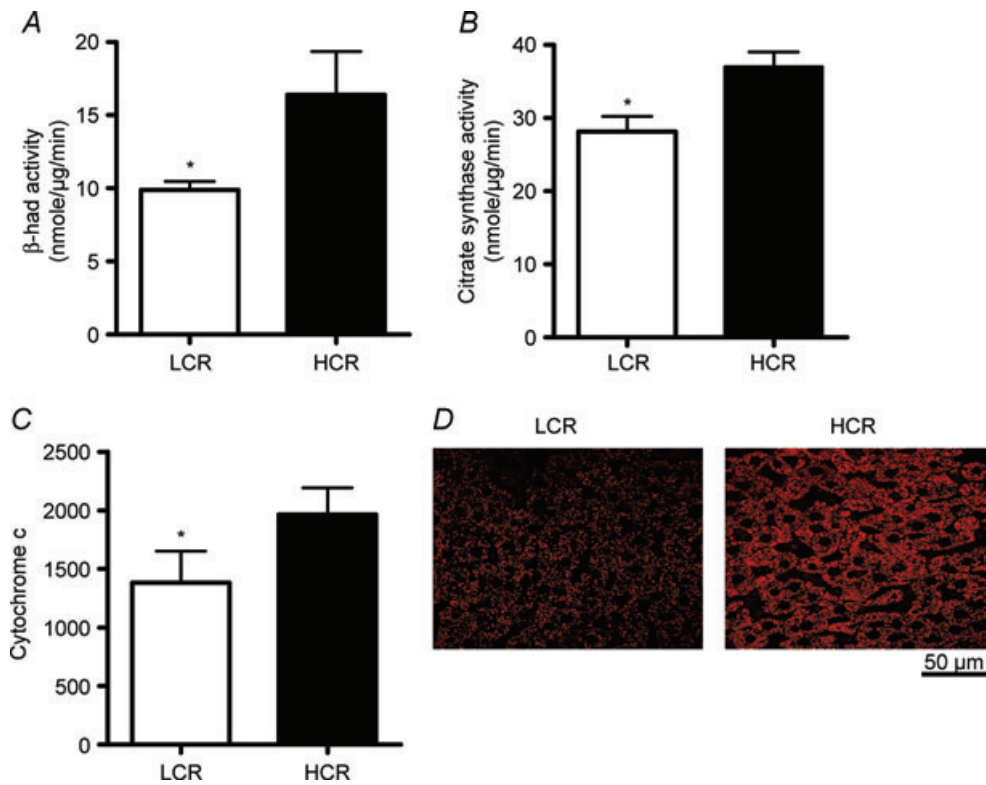


Figure 2. Hepatic mitochondrial content and enzyme activity

The activity of mitochondrial enzymes β -HAD (A) and citrate synthase (B) and the protein content of cytochrome c (C) were significantly lower in the livers of LCR rats than HCR rats at 25 weeks of age. D, representative immunofluorescence photomicrographs (magnification = 400 \times) for mitochondrial marker CPS1 (red) showed granular staining patterns in liver sections. The LCR livers visibly display decreased CPS-1 staining compared to the HCR at 25 weeks of age.

pad) along with a greater storage of hepatic fat compared to the HCR, suggesting a connection between increased visceral adiposity and fatty liver in this model. Excessive daily food consumption and postprandial increases in circulating lipids also contribute to excessive fat storage in the liver and the expansion of visceral fat stores. However, it is unlikely that increased food consumption

is the primary cause of excessive fat storage in the LCR rats as the results presented here and in previous reports (Noland *et al.* 2007a) show that the LCR actually have a significantly lower (~12–25%) daily food consumption per body weight than the HCR. In summary, we postulate that reduced hepatic mitochondrial oxidation is the primary event mediating the increased fat deposition in

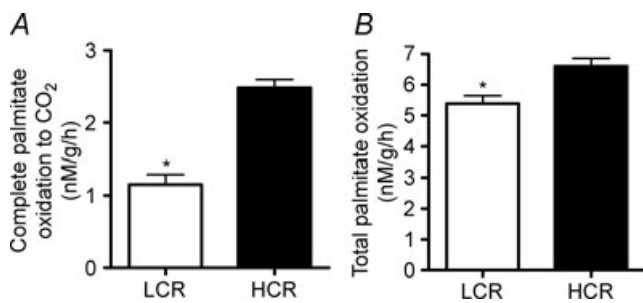


Figure 3. Hepatic mitochondrial fat oxidation

Both complete palmitate oxidation to CO₂ (A) and total palmitate oxidation (CO₂ + ASM) (B) were significantly lower in isolated mitochondria from the LCR rats than the HCR at 25 weeks of age. *Significant difference ($P < 0.05$); values are reported as means \pm S.E.M.; $n = 7$.

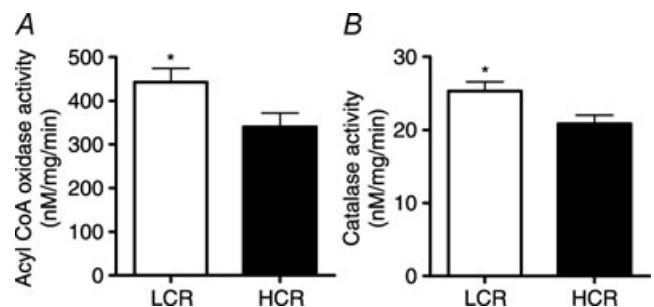


Figure 4. Hepatic peroxisomal activity

Hepatic peroxisomal enzyme activities of both acyl CoA oxidase (A) and catalase (B) were significantly greater in LCR than HCR rats at 25 weeks of age. *Significant difference ($P < 0.05$); values are reported as means \pm S.E.M.; $n = 7$.

the liver of the LCR although it is impossible to completely discount the contributing effects of increased adiposity or circulating insulin.

Aerobic capacity, hepatic mitochondrial oxidative capacity and steatosis

Our research group and other labs have shown that impaired hepatic fatty acid oxidation can be a primary cause or strongly associated with fatty liver (Ibdah *et al.* 2005; Rector *et al.* 2008); in contrast, others have shown increased rates of hepatic fatty acid oxidation in obese models of fatty liver disease (Brady *et al.* 1985; Sanyal *et al.* 2001; Miele *et al.* 2003). To explain these divergent findings, it is hypothesized that increased hepatic fatty acid oxidation is a secondary but insufficient adaptation to a chronic oversupply of lipids to the liver. Because of these conflicting results utilizing varied methods, it is difficult to determine if hepatic fatty acid oxidation differences are related to alterations in hepatic mitochondrial fatty acid oxidation or if they involve the extra-mitochondrial organelle, peroxisomes, that boost fatty acid catabolism (Noland *et al.* 2007a). PPAR α is a transcription regulator of genes involved in hepatic fatty acid oxidation, including mitochondrial β -oxidation proteins (Memon *et al.* 2000). However, hepatic PPAR α also increases peroxisomal proliferation and the capacity of peroxisomes to oxidize fatty acids

Table 2. Hepatic lipids and adipocyte cell volume

Variable	LCR	HCR
Steatosis score	2.20 \pm 0.28*	1.17 \pm 0.07
Hepatocytes with lipids (%)	54.0 \pm 9.2*	22.0 \pm 3.5
Epididymal fat pad cell volume (pl)	160.0 \pm 9.8*	118.4 \pm 3.4
Omental fat pad cell volume (pl)	122.3 \pm 13.3*	85.7 \pm 3.9

Means \pm S.E.M. *Significantly different than HCR, $P < 0.05$; pl, picolitres

(Reddy & Rao, 2006; Schoonjans *et al.* 1995; Schoonjans *et al.* 1996). Hepatic PPAR α protein content did not differ between groups in our study. However, complete hepatic mitochondrial fatty acid oxidation to CO₂ and total fatty acid oxidation (CO₂ + ASM) was suppressed in the LCR rats compared with the HCR, suggesting that oxidative capacity of hepatic mitochondria is reduced in LCR livers. In addition, the suppressed enzyme activity or protein content at all three critical steps of mitochondrial fat oxidation (β -oxidation, TCA cycle, and electron transport) in the whole liver homogenate combined with the reduced staining for CPS-1 strongly suggest that the LCR livers also have lower mitochondrial content. In agreement with Noland *et al.* (2007a), we also found no differences in rates of hepatic fatty acid oxidation between HCR and LCR rats when measurements were made in whole liver homogenate (data not shown), a technique that prevents the distinction between mitochondrial and peroxisomal contributions. The lack of a significant difference between groups using a liver whole homogenate suggested that LCR rats have a compensatory increase in peroxisomal activity. Indeed, we found that the LCR livers displayed higher peroxisomal activity (higher acyl-CoA oxidase and catalase activity) than their HCR counterparts. Increased peroxisomal activation can contribute to significant lipid peroxidation or oxidative stress, as acyl CoA oxidase produces hydrogen peroxide (Reddy & Hashimoto, 2001). Taken together these results clearly suggest that intrinsically low aerobic capacity is associated with a reduced hepatic mitochondrial quality and content and a compensatory increase in peroxisomal activity. Importantly, a pathogenesis of reduced mitochondrial oxidative capacity is documented in patients with NAFLD making these findings clinically relevant (Perez-Carreras *et al.* 2003).

It is impossible to completely eliminate the influence of skeletal muscle mitochondrial function on the hepatic phenotypes observed in the HCR and LCR rats, as the model was created separating low and high running capacity, a selective pressure largely dependent on the function of skeletal muscle and the cardiorespiratory system. It is possible that the lower levels of ambulatory

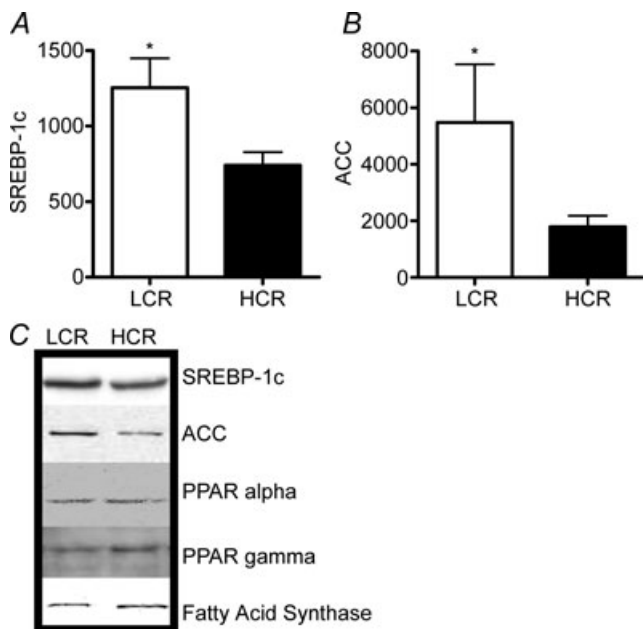


Figure 5. Hepatic lipid synthesis proteins

The protein content of SREBP-1c (A) and ACC (B) were significantly higher in the livers of LCR than HCR rats. C, no differences were observed for PPAR α , PPAR γ , or FAS protein contents between groups. Representative western blots are shown. *Significant difference ($P < 0.05$); values are reported as means \pm S.E.M.; $n = 7-8$.

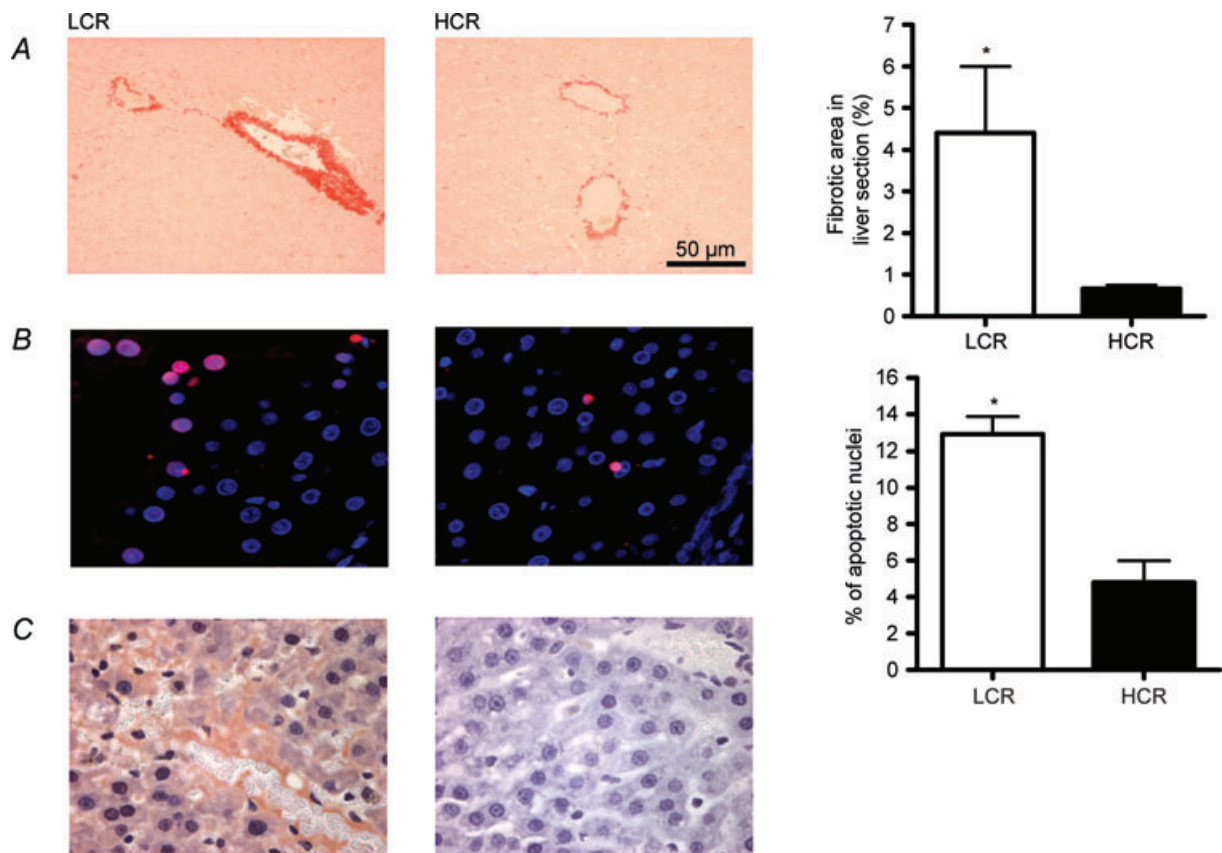
Table 3. Physical activity, oxygen consumption and weekly food consumption

Variable	LCR			HCR		
	Dark	Light	24 h	Dark	Light	24 h
Ambulatory activity (counts h ⁻¹)	345.5 ± 16.0*	78.7 ± 6.8*	216.7 ± 0.7*	573.4 ± 26.2	102.0 ± 4.7	345.8 ± 13.7
O ₂ consumption (ml kg ⁻¹ h ⁻¹)	1282.2 ± 36.4*	1042.1 ± 30.1*	1167.2 ± 34.8*	1532.7 ± 43.5	1176.0 ± 28.0	1362.7 ± 34.0
Food consumption (g wk ⁻¹ per 100 g BW)	52.05 ± 2.09*			58.79 ± 2.01		

Mean ± S.E.M. *Significantly lower than HCR, $P < 0.05$; BW = body weight; ambulatory activity and O₂ consumption were measured over a 2 day period at 25 weeks of age. Average weekly food consumption was measured from 5 weeks of age (post-weaned) until 25 weeks of age.

physical activity witnessed in the LCR rats may have led to reductions in hepatic mitochondrial content compared to the HCR rats. This is unlikely as previous results from our group studying far larger contrasting levels

of physical activity (daily voluntary wheel running at a level of ~6 km a day for 6 weeks vs. sedentary cage only activity) caused no significant difference in hepatic mitochondrial content in healthy rats (Laye *et al.* 2008).

**Figure 6. Hepatic injury**

Hepatic injury was greater in the LCR than the HCR rats when measured at the time of natural death. *A*, sirius red staining for hepatic fibrosis (quantified by % of fibrotic area); *B*, apoptosis measured by TUNEL staining of apoptotic hepatocytes nuclei. *C*, TGF-1 β protein, a strong fibrogenic signal, was visually greater in livers from LCR than HCR rats at the time of natural death. *Significant difference ($P < 0.05$); values are reported as means ± S.E.M.; $n = 4-6$.

Our results clearly show that selective breeding for low aerobic capacity resulted in lower hepatic mitochondrial content and function in rats at 25 weeks of age, which we speculate causes the increased susceptibility for hepatic steatosis in the LCR rats. In support of this concept, preliminary results from our laboratory show that the phenotype of reduced lipid oxidation in the LCR livers is retained in primary cell culture (data not shown). Just as the selective breeding for low running capacity reduced skeletal muscle mitochondrial ATP production needed to fuel contractions, it also likely lowered the need for hepatic mitochondrial ATP production, which is needed to fuel the energy-costly process of gluconeogenesis needed to maintain glucose homeostasis during exercise.

Increased lipid peroxidation can lead to the production of reactive aldehydes that damage mitochondrial DNA (Demeilliers *et al.* 2002) or inactivate respiratory chain proteins (Chen *et al.* 2000), resulting in reduced mitochondrial content or function and hepatic injury. Despite the strong link between obesity or aging and reduced mitochondrial DNA in skeletal muscle, we were unable to find studies linking hepatic lipid peroxidation or oxidative stress to reduced liver mitochondrial DNA that do not involve liver damage induced by alcohol or pharmacological agents. Here we demonstrate that the aging LCR rats have increased hepatic levels of lipid peroxidation which is subsequent to reduced hepatic mitochondrial function compared with the HCR rats. This could be partially attributed to increased adiposity (Noland *et al.* 2007a) and potentially greater hepatic free fatty acid flux in the LCR animals. However, the LCR rats had lower relative food consumption and similar feed efficiencies compared to the HCR rats, indicating similar energy balance between the two strains. Collectively, these findings support our contention that hepatic mitochondrial dysfunction plays a primary role in non-alcoholic fatty liver disease development and progression in animals with low intrinsic aerobic capacity. Future studies are warranted to pair feed the two strains and/or attempt to match ambulatory activity levels across the lifespan.

Aerobic capacity, hepatic lipogenesis and steatosis

Both biochemical and histological measures showed increased hepatic fat deposition in the LCR compared to the HCR rats at 25 weeks of age. These findings contradict an earlier report (Noland *et al.* 2007a) which studied the effect of high fat diet on the metabolic phenotypes of the HCR and LCR strains. That study did not find a significant difference in hepatic fat storage between the LCR and HCR strains on a normal chow or high-fat diet; however, that study used the less sensitive and qualitative Oil Red O technique to stain neutral lipids in a notably

small subset ($n = 3$) of liver samples, while our report used the standard biochemical measure of hepatic triglycerides in a larger sample size. In addition, rats were studied from a different generation between the two studies (generation 13 in former report *vs.* generation 17 in this study) and it is difficult to discern what impact further selective breeding had upon metabolic or hepatic phenotypes. However, a comparison of reports from previous generations shows comparable values for both strains between generation 11 (Wisloff *et al.* 2005), 13 (Noland *et al.* 2007a), and 17 (this report) for measures of body weight, and serum insulin and triglycerides suggesting that methodological differences, and not generational differences, were the main reason that hepatic triglycerides were not previously reported to be higher in the LCR rats.

Insulin increases hepatic fatty acid synthesis, in part through increases in SREBP-1c transcriptional regulation of FAS and ACC (Shimomura *et al.* 1999). In the LCR rats, it could be speculated that the elevated SREBP-1c protein content was due to elevated serum insulin levels resulting from peripheral insulin resistance (Noland *et al.* 2007a). The elevated SREBP-1c content in liver of LCR rats was matched with a 2-fold higher protein content of ACC over the HCR rat, with no observed differences in FAS protein content or PPAR γ . ACC synthesizes malonyl-CoA, a metabolite that is both a substrate for *de novo* synthesis of fatty acids through FAS and a potent inhibitor of fatty acid oxidation (McGarry *et al.* 1977). Although malonyl CoA was not measured herein, the elevated hepatic ACC content also suggests that hepatic CPT-1 activity in the LCR could allosterically be reduced due to a presumed increase in hepatic malonyl-CoA levels.

In conclusion, these results show that the hepatic phenotype associated with reduced aerobic capacity is characterized by both reduced mitochondrial content and oxidative capacity, leading to early hepatic steatosis and lipid peroxidation and the later development of elevated markers of liver injury. These findings provide insight into the pathogenesis of hepatic steatosis and may have important clinical implications. Lower intrinsic aerobic capacity due to physical inactivity and/or genetic heritability may lead to reduced hepatic mitochondrial oxidative capacity and increased susceptibility to hepatic steatosis and injury. Therefore, the clinical measurement of aerobic capacity may serve as a valuable prognostic tool. More importantly, because exercise training and/or increased physical activity is the proven method to increase aerobic capacity, it should remain the cornerstone therapy for fatty liver disease.

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