Feeding a corn oil/sucrose-enriched diet enhances steatohepatitis in sedentary rats


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Rivera, C. A., S. H. Abrams, M. H. Tcharmtchi, M. Allman, T. T. Ziba, M. J. Finegold, and C. W. Smith. Feeding a corn oil/sucrose-enriched diet enhances steatohepatitis in sedentary rats. Am J Physiol Gastrointest Liver Physiol 290: G386–G393, 2006.—The current study investigated the combined effects of feeding a high-fat/high-sucrose (HF/HS) diet to rodents rendered sedentary via hindlimb unloading (HU). For 3 wk before HU, male Wistar rats were fed chow or a diet in which 32% of calories were derived from corn oil fat and 48% of calories from sucrose. Feeding continued during an additional 3-wk period of HU. Subsequently, blood samples were collected for determination of circulating leukocyte counts, insulin levels, and portal vein endotoxin. Inflammation, necrosis, and steatosis were assessed in formalin-fixed liver sections. No biochemical or histological evidence of injury was observed in control rats fed chow or HF/HS. HU increased circulating neutrophils and resulted in hyperinsulinemia. Mild hepatic fat accumulation and minimal focal necro-inflammation were observed in this group. Feeding HF/HS during HU exacerbated hyperinsulinemia, hepatic steatosis, Kupffer cell content, and cytokine expression. Significant portal endotoxemia was noted in HU rats but was not influenced by HF/HS diet. On the other hand, feeding HF/HS significantly enhanced lipid peroxidation end products in liver of HU rats by approximately threefold compared with chow-fed rats. In summary, these findings demonstrate that feeding a high-calorie diet potentiates steatosis and injury in sedentary HU rats. Mechanisms underlying enhanced injury most likely involved lipid peroxidation. Importantly, these findings suggest that dietary manipulation combined with physical inactivity can be used to model steatohepatitis.

NOMALCOHOLIC STEATOHEPATITIS (NASH) is a slowly progressive disease characterized by fat accumulation in hepatocytes, mixed cell-type inflammation, focal necrosis, and occasional fibrosis. This disease has been observed worldwide, and the prevalence of NASH is estimated to be 2–3% of the general population (6). Although the incidence of NASH seems to be related to multiple factors, including obesity and type II diabetes, the exact trigger(s) for progression from a benign fatty accumulation to more severe injury has not been elucidated, partly because of the need for more suitable animal models. In the current models, disruption of homeostatic mechanisms governing fat synthesis, uptake, and export has been accomplished via chemical inhibition of mitochondrial fatty acid β-oxidation (8–10) and manipulation of obesity-associated genes (2, 5). In rodents, diets rich in monosaccharide stimulate de novo fatty acid synthesis via induction of lipid-generating enzymes (13, 17, 25). In a more recent study, Lieber et al. (20) fed a high-fat (71% energy) liquid diet to rats and found extensive steatosis, hepatic inflammation, and signs of insulin resistance. Although these methods reproduce some of the features of NASH, none of the present models have investigated the role of sedentary behavior in the genesis of steatohepatitis.

In the rodent hindlimb unloading (HU) model, sedentary behavior has been indicated by altered locomotor patterns and postures such as unstable and irregular alternating step cycles with long double feet stance phases (3, 4). Using the HU model, we recently reported hepatic injury and inflammation in endotoxin-sensitive rodents, as indicated by increased neutrophil content and elevated serum transaminase activity (38). Cytoplasmic accumulation of fat in hepatocytes was also observed, a phenomenon most likely resulting from altered lipogenesis. In fact, Vecchini et al. (46) demonstrated that just 2 wk of HU was sufficient to enhance the expression of acetyl-CoA synthase, acetyl-CoA carboxylase, fatty acid synthase, and 3-hydroxy-3-methylglutaryl-CoA reductase, key enzymes involved in fatty acid and cholesterol synthesis. There is also evidence of enhanced lipid storage mechanisms after HU (12). Despite elevated portal endotoxemia, endotoxin-resistant C3H/HeJ mice did not develop steatosis and injury in response to HU.

For the first time, the present study investigated the consequence of combined dietary and behavioral approaches to the induction of steatohepatitis. Based on previous studies, it was hypothesized that increased dietary fat and carbohydrate would exacerbate hepatic injury and steatosis in sedentary rodents. Indeed, injury, steatosis, and inflammation were markedly enhanced by the combined insults of feeding high fat/high sucrose to rats rendered sedentary via HU without the addition of exogenous chemicals or genetic manipulation. These findings suggest a potential for the use of dietary and lifestyle manipulations to model steatohepatitis.

MATERIALS AND METHODS

Animal model. Male Wistar rats (300–325 g) were divided into the following two dietary groups (n = 10 rats/group): 1) rats fed standard...
laboratory chow [PicoLab Rodent diet no. 5053 (Purina Mills); by weight, ~20% protein, ~5% fat, ~5% fiber, ~5% combined simple carbohydrates (glucose, fructose, and sucrose)]; and 2) rats fed a diet based on AIN-76A modified to contain high fat/high sucrose (HF/HS; catalog no. 101588; Dyets, Bethlehem, PA). The HF/HS diet contained (wt/wt) 20% protein, 5% fiber, 15% corn oil, and 50% sucrose, in addition to the standard vitamin and mineral nutrients as outlined in Table 1. Rats were fed the appropriate diet beginning 3 wk before HU. Because preliminary studies indicated no differences in food intake between chow- and HF/HS-fed rats, food was administered ad libitum. Thus the total daily caloric intake in rats fed HF/HS (4,151.8 kcal/kg) was likely higher than in the chow-fed rats (~3,080 kcal/kg).

Subsequently six rats from each dietary group were subjected to HU according to the method of Wronski and Morey-Holton (51). Briefly, a cast-like apparatus consisting of surgical tape over a coated wire frame was applied to the tail while rats were under ketamine-xylazine anesthesia. To facilitate free movement about the cage, the cast was attached to a swivel anchored to the cage top, allowing a 360° range of movement; food and water were freely accessible in this position. Rats were positioned in a 30° head-ward tilt orientation such that the hindlimbs were suspended slightly above the cage floor. In this position, animals were still able to maneuver about the cage and rest on the front limbs. The remaining four rats in each group were housed individually but were not subjected to HU (control group). Dietary treatment was continued throughout the entire experiment for a total of 6 wk. All animals were used in accordance with procedures approved by the Animal Care and Use committee of Baylor College of Medicine.

Circulating leukocyte counts. The total number of leukocytes in samples of whole blood was determined using a coulter particle counter (Beckman, Miami, FL). The proportion of neutrophils, lymphocytes, and monocytes were assessed in hematoxylin- and eosin-stained blood smears by light microscopic analysis of nuclear morphology.

Serum insulin. A commercially available enzyme immunosorbent assay kit was used to measure insulin levels in serum collected at death (Crystal Chemical, Downers Grove, IL).

Histology. Sections of liver preserved in zinc-buffered formalin were embedded in paraffin and stained with hematoxylin and eosin. To demonstrate hepatic lipid accumulation, additional sections of liver were processed for real-time PCR according to the manufacturer’s instructions (Applied Biosystems). In a separate tube, 18S ribosomal RNA was amplified as a reference.

Real-time PCR reactions were carried out in 20-μl reaction mixtures of TaqMan universal PCR master mix (Applied Biosystems), 50 nM TaqMan probe for the target gene, 20 nM forward primer, and 80 nM reverse primer, made up to volume with RNase-free H2O. Samples of cDNA (10 ng) derived from livers of each animal were assayed in triplicate using an ABI PRISM 7500 bioanalyzer under the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene expression was quantified using a comparative critical threshold (ΔΔCT) method according to the manufacturers' suggestions. The CT value reflects the cycle number at which the DNA amplification is first detected. For each sample, a ΔΔCT value was obtained by subtracting CT target values from those of each reference gene. The mean CT value of the chow control group was used as a reference and was subtracted from the remaining samples to derive a ΔΔCT value. Finally, expression was presented as 2−ΔΔCT. Thus expression of each target gene was normalized to 18S content and expressed relative to the chow control group.

Lipid peroxidation assay. Liver samples were rinsed in ice-cold saline and immediately frozen in liquid nitrogen. Just before assay, a 30% homogenate was prepared in 20 mM Tris buffer containing 5 mM butylated hydroxytoluene to prevent sample peroxidation. The tissue was homogenized on ice using a Potter-Ellicott tissue grinder, and the resulting homogenate was centrifuged at 3,000 g for 10 min at 4°C. Lipid peroxidation end products were measured in liver homogenates using a colorimetric assay (Oxis International, Portland, OR).

Endotoxin measurement. The platelet-rich plasma fraction was isolated from heparinized blood samples drawn from the portal vein just before death. Samples were prepared as described previously (37), and endotoxin was detected using a kinetic chromogenic assay (BioWhittaker, Walkersville, MD).

Statistical analysis. Data are presented as means ± SE of four (control) or six (HU) observations. Statistical analysis was performed using one-way or two-way ANOVA and Bonferroni’s multiple-comparisons test; *P < 0.05 was selected as the level of significance.

RESULTS

Systemic effects of HU and HF/HS diet. After a total of 6 wk of feeding, body weight measurements in chow-fed controls (479.3 ± 20.5 g) were not different from body weights of rats that consumed the HF/HS diet (451.0 ± 8.1 g). Rats in all

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<th>Component</th>
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<tr>
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Table 1. Components of the high-fat/high-sucrose diet
groups gained weight during the study. Despite similar daily food intake (data not shown), HU blunted weight gain in both dietary groups, with weight only reaching 385.8 ± 11.1 and 379.5 ± 18.8 g in chow- and HF/HS-fed rats, respectively. Blunted weight gain was likely related to loss of muscle and bone (24, 26, 27, 47, 50). At completion of the 6-wk study, the total number of circulating leukocytes in whole blood samples of chow- and HF/HS-fed control rats were similar (Fig. 1). Circulating leukocyte counts increased significantly in HU rats fed chow (17.5 ± 1.9 × 10⁶ cells/ml) or HF/HS diet (21.1 ± 1.9 × 10⁶ cells/ml). To determine which cell populations were affected, differential staining was performed, and the number of lymphocytes, neutrophils, and monocytes in systemic blood samples was calculated. Similar to total leukocyte counts, circulating neutrophils were increased by HU irrespective of diet; the number of circulating monocytes was only increased in the HU ± HF/HS group (Table 2).

Vecchinni et al. (46) reported previously that insulin levels were increased after 2 wk of HU. Here, insulin levels were measured in serum samples collected in the morning (fed state) just before death. In control rats, serum insulin levels were 0.30 ± 0.14 and 0.32 ± 0.22 ng/ml in chow and HF/HS rats, respectively. Three weeks of HU significantly increased serum insulin levels. Although HF/HS diet did not influence serum insulin in control rats, the additional insult of HU during the last 3 wk of feeding significantly enhanced insulin levels above HU alone (Fig. 2).

**Hepatic steatosis.** Figure 3 displays representative photomicrographs of liver sections stained with oil red-O. Livers of control rats fed a standard chow diet appeared normal. Occasional cytoplasmic lipid accumulation was observed in portal areas of livers from rats fed HF/HS diet. Mild perportal microsteatosis resulted from HU in chow-fed rats but was confined to zone 1 (score = 0.5 ± 0.2). The combination of HU and HF/HS diet markedly enhanced steatosis, with mixed-sized lipid droplets accumulating in zones 1–2, resulting in a steatosis score of 1.7 ± 0.2.

**Hepatic injury and inflammation.** Liver sections were evaluated by a pathologist blinded to the treatment groups. Occasional infiltrating leukocytes were observed in rats fed HF/HS diet; the extent of inflammatory infiltrate was not different from chow-fed controls. Exposure of chow-fed rats to HU resulted in patchy hepatocellular necrosis and occasional small clusters of inflammatory cells primarily in zone 1. Injury and inflammation were notably enhanced by HU + HF/HS. Necrotic hepatocytes and inflammatory foci were found consistently in livers of these rats. To characterize the inflammatory infiltrate, additional liver sections were subjected to immunohistochemical staining with an anti ED-1 antibody to visualize hepatic macrophage content. In all groups, ED-1-positive cells were observed (Fig. 4A). Necroinflammatory foci were intensely positive for ED-1 staining. As an index of the extent of macrophage accumulation, KCR expression was analyzed by real-time PCR (Fig. 4B). Although HF/HS or HU as individual insults had no effect, KCR expression in rats exposed to a combination of HU and HF/HS was elevated significantly, supporting histological findings of increased Kupffer cell content.

**Portal vein endotoxemia.** Endotoxin levels in the platelet-rich plasma fraction of blood samples collected from the portal vein were similar in chow-fed and HF/HS-fed controls (Fig. 5) but was increased by HU (126.7 ± 42.8 pg/ml). Portal endotoxin levels after feeding HF/HS diet during HU (213.7 ± 80.7) were approximately twofold higher than in the HU + chow group; however, no statistical differences were detected.

Endotoxin in the portal vein is cleared primarily by Kupffer cells, which results in the production of proinflammatory
mediators. Kupffer cell activation by endotoxin requires surface CD14/Toll-like receptor 4 expression and LBP. As markers of endotoxin-dependent signaling, hepatic mRNA expression of LBP and TNF-α were measured using real-time PCR. Significant elevations in LBP and TNF-α expression were only observed in HU rats fed HF/HS diet (Fig. 6).

**Lipid peroxidation.** End products of lipid peroxidation 4-hydroxyalkenals and malondialdehyde were measured in samples of homogenized liver. Lipid peroxidation was not detectable in livers of chow- or HF/HS-fed control rats (Fig. 7). After 3 wk of HU, lipid peroxidation end products were clearly detectable in the livers of rats fed chow. Consistent with enhanced injury, the hepatic content of 4-hydroxyalkenals and malondialdehyde was increased approximately threefold in HU rats fed HF/HS diet.

**DISCUSSION**

The innovative approach to the study of NASH employed here involved feeding a HF/HS diet to sedentary HU rats. Although there are several reports of the hepatic effects of diets enriched in fat and carbohydrate, much less is known about steatohepatitis in sedentary rodents. Moreover, the combined effects of diet and physical inactivity have not been elucidated. Findings reported herein demonstrate that steatosis and inflammation in sedentary rats was exacerbated by feeding a diet enriched in corn oil and sucrose (HF/HS). Hyperinsulinemia was also more severe in the HU + HF/HS group compared with HF/HS alone or HU rats fed chow, suggesting a state of insulin insensitivity. In support of the idea that HU induces insulin resistance, muscle and liver insulin receptor expression
was shown to be downregulated after 14 days of HU (41). Exposure of rats to HU for just 24 h was sufficient to induce a transient state of insulin resistance in muscle (34). Findings of hyperinsulinemia in the present study support the presence of an insulin resistance state during HU. Taken together, this is the first study to report exacerbated hepatic pathology in HU rats fed HF/HS that resembles some of the features of clinical NASH.

**Contribution of HU to steatohepatitis.** Several recent studies have provided evidence of the importance of diet and exercise in lipogenesis. Chronic exercise has been shown to counter the effects of high-carbohydrate diets. In fact, Fiebig et al. (11) demonstrated that exercise downregulated fatty acid synthase activity by 50% despite feeding a fructose-enriched diet. Because decreased exercise was shown to inversely correlate with circulating lipid levels (48), it is reasonable to expect that limited physical activity might promote storage of fat.

HU has been used to model the effects of hypokinesia/hypodynamia on bone and muscle (24, 26, 27, 47, 50), and decreased physical activity in this model has been well documented (3, 4). Results indicated that HU altered intermediary metabolism and induced lipid peroxidation in the immobilized areas (19, 21, 36, 40). In the liver, total lipid content was reportedly increased by 14–56% after HU (12). Our previous work and the present study (Fig. 3) demonstrated that lipid droplets accumulated in the cytoplasm of hepatocytes (38). Vecchini et al. (46) investigated mechanisms underlying hepatic steatosis resulting from HU and found that mRNA levels and activities of several proteins that modulate hepatic lipid content were altered in favor of lipid accumulation. Thus HU...
results in steatosis and injury due to inappropriate lipid trafficking, and feeding HF/HS during HU likely enhanced the presence of substrates for lipid storage and subsequent peroxidation.

Contribution of feeding a fat- and carbohydrate-enriched diet to steatohepatitis. Feeding animals a fat-enriched diet has been shown to induce hepatic steatosis by increasing triglycerides and lipogenic enzymes (7). In a more recent study, Lieber et al. (20) fed a high-fat (71% energy) liquid diet to rats and found extensive steatosis, hepatic inflammation, and signs of insulin resistance; induction of steatohepatitis was attributed to the inclusion of several types of fat in the diet. One control group in the present study was fed a hypercaloric diet; however, only mild steatosis without injury or lipid peroxidation was found in this group. Lower fat content (15%) from a single source (corn oil) may account for the absence of steatohepatitis in the HF/HS control group.

In addition to HU, rats in the present study were fed a diet enriched in corn oil and sucrose. Corn oil is an ω-6 polyunsaturated fatty acid. Although polyunsaturated fatty acids inhibit lipogenic pathways when administered in the absence of additional noxious stimuli, corn oil has been shown to exacerbate hepatic injury and steatosis in rats after chronic ethanol exposure (28, 29, 32, 44), a phenomenon mediated by endotoxin, lipid peroxidation, and arachidonate metabolites (1, 30, 31, 33). Sucrose is a disaccharide consisting of glucose and fructose consumed by humans in common table sugar. When fed individually at high concentrations (50–60% of total calories), both fructose and glucose have been shown to enhance hepatic lipogenesis via induction of pathways associated with the expression of lipogenic enzymes (13, 17, 25, 52). Although we did not find appreciable injury or fat accumulation in livers of HF/HS-fed control rats, significant pathology was noted in HU rats on the same diet (Figs. 3 and 4). The mechanism underlying the combined effects of HU and HF/HS on hepatic injury most likely involved leakage of endotoxin from the gut and lipid peroxidation, as discussed below.
Mechanism of injury involves endotoxia and lipid peroxidation. HU is used extensively in stress-evoked behavioral studies. Investigations monitoring the release of stress hormones in HU rats have reported significant elevations in corticosterone during up to 3 wk of suspension (15, 16, 18, 43). It has also been reported that feeding a purified diet high in corn oil/lard (39% energy) and sucrose (41% energy) to normal rodents similarly increased plasma corticosterone levels (22). Because rats in the present study were unloaded for 21 days in addition to being fed HF/HS diet, a causal role of stress-induced corticosterone in the observed pathology cannot be ruled out by the present experiments. Excessive levels of glucocorticoids are reported to be elevated in obese humans (23). In fact, the role of stress-related glucocorticoids in pathology of the entire metabolic syndrome that is secondary to obesity has been the attention of many recent reports (39), and findings suggest that these hormones exacerbate hepatic fat accumulation and dyslipidemia (22, 35). More experiments are needed to determine the relative importance of elevated corticosterone in animal models of steatohepatitis.

Previous studies suggest that leakage of bacteria or bacterial products from the gut may have profound effects on the pathogenesis of steatohepatitis. Endotoxin (lipopolysaccharide) is a polymer in the outer membrane of gram-negative bacteria found predominantly in the ileum and colon. Normally, the gut wall provides a protective barrier against the release of large amounts of endotoxin in the systemic circulation. However, under pathological conditions, intestinal permeability can increase significantly, allowing bacteria and bacterial components to leak into the circulation. In a previous study, we demonstrated that low-grade portal endotoxia was associated with hepatic injury in rats and C57BL/6 mice after HU (38). In contrast, no histological grade portal endotoxemia was associated with hepatic injury in the circulation. In a previous study, we demonstrated that low-grade portal endotoxia was associated with hepatic injury in rats and C57BL/6 mice after HU (38). In contrast, no histological grade portal endotoxemia was associated with hepatic injury in the circulation. In a previous study, we demonstrated that low-grade portal endotoxia was associated with hepatic injury in rats and C57BL/6 mice after HU (38). In contrast, no histological grade portal endotoxemia was associated with hepatic injury in the circulation. In a previous study, we demonstrated that low-grade portal endotoxia was associated with hepatic injury in rats and C57BL/6 mice after HU (38). In contrast, no histological grade portal endotoxemia was associated with hepatic injury in the circulation.

It is well known that endotoxin leads to the generation of reactive oxygen intermediates, including superoxide, hydrogen peroxide, and hydroxyl radicals. Polyunsaturated lipid components in the cell membrane are highly vulnerable to peroxidation by reactive oxygen; thus, lipid peroxidation is a common mechanism of cellular injury during endotoxin exposure (42). Indeed, the hepatic content of lipid peroxidation end products was elevated significantly after HU and was exacerbated by feeding HF/HS (Fig. 6). As expected during conditions of endotoxia and lipid peroxidation, the greatest extent of hepatocellular damage was noted in the HU + HF/HS group, as determined from histological evidence. Injury was accompanied by systemic inflammation indexed by the presence of inflammatory cells in the liver and increased circulating leukocyte counts.

It has been hypothesized that endotoxin plays a causal role in the development of steatohepatitis in humans. In support of this idea in mice that are genetically overweight, obesity and diabetes were accompanied by extensive hepatic fat accumulation; however, hepatocellular necrosis was only observed after systemic endotoxin administration in this model (53). In humans, severe steatohepatitis often occurs after jejunointestinal bypass and in patients placed on total parenteral nutrition, both of which are situations believed to cause bacterial overgrowth and endotoxia. A study by Wigg et al. (49) evaluated 22 patients with NASH for small bowel bacterial content, and it was found that bacterial overgrowth was prevalent among NASH patients compared with healthy age-matched controls. However, endotoxia was not detected in the blood of these patients, most likely because of the inadequate method of detection used by these authors. In a study by Vanderhoof et al. (45), liver injury and steatosis in a rodent model of jejunointestinal bypass was prevented by therapies that minimized bacterial load and improved the hepatocellular antioxidant capacity. Thus it is likely that endotoxin is necessary for the progression of hepatic steatosis to more severe pathology that includes necrosis and inflammation. The combined insults of HF/HS and HU mimicked this potentially important feature of NASH. In summary, the combined insults of dietary manipulation and chronic immobilization because of HU exaggerated a range of parameters (i.e., steatosis, injury, and inflammation) commonly associated with steatohepatitis in humans. The successful induction of steatohepatitis was likely related to multiple factors, including stress, endotoxia, and oxidative damage.

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