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A "Western Diet" promotes symptoms of hepatic steatosis in spontaneously hypertensive rats

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Summary

Systemic hypertension, characterized by elevated blood pressure ≥140/90 mm Hg, is a major modifiable risk factor for cardiovascular disease. Hypertension also associates with non-alcoholic fatty liver disease (NAFLD), which is becoming common due to a modern diet and lifestyle. The aim of the present study was to examine whether a high-fat "Western" diet had effects on hypertension and associated NAFLD. Normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) were placed on a normal chow or high-fat diet for 8 weeks; blood pressure was measured fortnightly and body weight recorded weekly. As expected, SHR had elevated blood pressure compared to WKY. Diet did not influence blood pressure. Compared to SHR, WKY rats gained more weight, associating with increased white adipose tissue weight. Normotensive rats also had higher plasma cholesterol and triglycerides in response to a "Western" diet, with no changes in plasma glucose levels. Neither strain developed atherosclerosis. Interestingly, high-fat diet-fed SHR had increased liver weight, associating with a significant level of hepatic lipid accumulation not observed in WKY. Further, they exhibited hepatocellular ballooning and increased hepatic inflammation, indicative of steatohepatitis. These findings suggest that a high-fat "Western" diet promotes features of NAFLD in SHR, but not WKY rats. Importantly, the high-fat diet had no effect on blood pressure.

KEYWORDS

hepatic steatosis, hypertension, liver

1 | INTRODUCTION

Systemic hypertension has become a major health concern globally and is attributed to nearly 13% of worldwide mortality (World Health Organization). It is defined as chronically elevated blood pressure, ≥140/90 mm Hg measured from the brachial artery. Cardiovascular disease (CVD) is twice as likely to occur in hypertensive patients compared to normotensive individuals,[1] with hypertension inducing several complications of CVD including myocardial infarction and stroke, as well as exacerbating atherosclerosis.[2,3] Independent of traditional cardiovascular risk factors, hypertension is also associated with non-alcoholic fatty liver disease (NAFLD), the most common liver disease in the world, occurring when lipid accumulates in the liver without excessive alcohol consumption.[4]

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A commonly used model of essential hypertension is the spontaneously hypertensive rat (SHR) and its normotensive counterpart, the Wistar-Kyoto (WKY) rat. The SHR was developed by selecting WKY rats with a systolic blood pressure >150 mm Hg over a 1-month period and breeding them over several generations.[5] Normotensive WKY rats have a systolic blood pressure ranging between 130 and 135 mm Hg from 10 weeks of age, whereas SHR are already hypertensive (systolic blood pressure ~180 mm Hg) and continue to experience increases in systolic blood pressure as they get older.[5] Importantly, the progression of hypertension in SHR mimics that of hypertensive humans—idiopathic and gradual—and they often experience similar CVD-related events, such as cardiac hypertrophy, cardiac failure and renal dysfunction.[6]

Here WKY and SHR rats were placed on a high-fat "Western" diet to interrogate the effects on blood pressure and cardiovascular profiles. We identified that SHR on a high-fat diet for 8 weeks gained less weight compared to WKY rats and had reduced plasma cholesterol and triglycerides. Importantly, fat-fed SHR stored more lipid in their liver, with altered expression of genes regulating lipogenesis, increased hepatic inflammation and hepatocellular ballooning, features of NAFLD.

2 | METHODS

2.1 | Animals

Eight-week-old male SHR and WKY rats (Animal Resource Centre) weighing between 180 and 190 g were euthanized for baseline studies or randomly placed on either normal chow (4.8% fat, 20% protein; Specialty Feeds) or a "Western"-style high-fat diet (22% fat and 0.15% cholesterol; Specialty Feeds SF00-219) for 8 weeks. All rats were housed in groups of 3 in standard-sized housing in an Optimice Hepa Filter System (Animal care Systems) with ad libitum access to food, water and environmental enrichment. Lighting was set to a 12-hour light/dark cycle, and temperature was maintained at 23°C. Rats were monitored daily and weighed weekly. Blood pressure was measured fortnightly using the non-invasive CODA™ mouse/rat tail-cuff system as previously described.[7] Prior to euthanasia, rats were fasted overnight. They were anaesthetised by isoflurane (3% induction and 2% for maintenance) and euthanized via cardiac exsanguination; the collected blood was immediately transferred to a BD Vacutainer blood collection tube containing ethylenediaminetetraacetic acid (EDTA) and placed on ice. Organs including the epididymal white adipose tissue (WAT), retroperitoneal WAT, kidney, spleen and liver were quickly weighed and immediately snap-frozen in liquid nitrogen for gene expression studies or fixed in 10% neutral-buffered formalin (Sigma-Aldrich) overnight for histological assessment.

2.2 | Ethical approval

All studies were conducted at the Heart Research Institute (NSW, Australia) under the ethical approval of the Sydney Local Health District (Protocol 2013/049).

2.3 | Plasma chemistries

Glucose was measured in whole blood by glucometer (Accu-chek Performa). Plasma obtained at euthanasia was used to measure total cholesterol and triglycerides (Wako).

2.4 | Analysis of aortae

Formalin-fixed aortic sections were stained with Oil Red O solution to evaluate atherosclerotic plaque formation and staining area measured using ImageJ software (NIH) as previously described.[8,9]

2.5 | Histology and lipid accumulation

Formalin-fixed liver was stained with haematoxylin and eosin for general tissue architecture as previously described.[10] Brightfield images were captured using Zeiss Axio Imager Z2 microscope at 10× magnification for lipid droplet analysis, 20× magnification for hepatocellular ballooning, five images per sample. Lipid droplets were counted using the ImageJ software Cell Counter plugin (NIH). The number of lipid droplets were normalized to field of view and represented as ±SEM.

2.6 | Quantitative PCR (qPCR)

RNA was extracted from frozen liver tissue using TRI Reagent as previously described.[11] cDNA was synthesized as described.[11,12] Real-time qPCR was performed, and relative expression was determined using the $2^{-\Delta\Delta Ct}$ method using rat primers described in Table 1, normalized to GAPDH or β-actin house-keeping genes.

2.7 | Statistics

Results are expressed as mean ± SEM and analysed using GraphPad Prism Version 7.02 (GraphPad Software). Statistical comparisons were assessed with Student's *t* test,

TABLE 1 Primers

Gene	Forward primer 5′ to 3′	Reverse primer 5′ to 3′
HMG-CoAR	CATGCTGCCAACATCGTCA	CCCACATTCTGTGCTGCATC
SRB1	ATGGGACTTCCGGGCAGAT	CAGCGAGGATTCGGGTGC
SREBP1	CATGGATTGCACATTTGAAGAC	GCAGGAGAAGAGAAGCTCTC
MCP-1	ATGCAGTTAATGCCCCACTC	TTCCTTATTGGGGTCAGCAC
TNFα	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC
IL-6	TCCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC
IL-1β	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC
GAPDH	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT
β-actin	AACCGTGAAAGATGACCCAG	CACAGCCTGGATGGCTACGTA

or ANOVA (one- or two-way) with Dunnett's or Tukey's adjustment for multiple comparisons. A value of $P < .05$ was considered significant.

3 | RESULTS

3.1 | SHR rats have higher blood pressure, but gain less weight

Blood pressure was quantified fortnightly over the course of the study using a non-invasive tail-cuff system in conscious WKY and SHR rats in response to a normal chow or high-fat diet. As expected, compared to WKY, SHR had elevated blood pressure when maintained on normal chow or high-fat diet for 8 weeks (Figure 1A). No effect on blood pressure was observed in high-fat diet vs chow-fed animals at any time point in either strain (Figure 1B). While no age-related increase in systolic blood pressure was observed in WKY rats on normal chow compared to baseline (0 weeks), systolic blood pressure was significantly elevated in high-fat diet-fed WKY rats by 4 weeks (0 vs 4 weeks; 129.3 ± 4.4 vs 151.3 ± 3.8 mm Hg; $P < .05$) and remained elevated for the rest of the study. In contrast to WKY rats, the systolic blood pressure of chow-fed SHR was significantly elevated from baseline by 6 weeks (0 vs 6 weeks; 150.4 ± 4.3 vs 195.0 ± 3.0 mm Hg; $P < .05$) and by 4 weeks in high-fat diet-fed SHR (0 vs 4 weeks; 150.4 ± 4.3 vs 187.3 ± 6.0 mm Hg; $P < .05$), remaining elevated in both groups for the course of the diet. Therefore, these data show that while increasing age results in increased blood pressure in SHR, diet has no effect up to 8 weeks.

Body weight was measured weekly in response to diet, and while SHR were heavier at baseline (Figure 1C), and all animals gained weight during the course of the study, no significant differences were observed between strains or diet at the end of the study (Figure 1D). Weight gain was assessed and calculated as an increase in body weight proportional to baseline. WKY gained ~20% more weight in response to 8-week high-fat diet vs normal chow, whereas weight gain observed in SHR on the high-fat diet vs normal chow was subtle (~10%) and did not reach statistical significance (Figure 1E). In fact, SHR demonstrated less weight gain compared to WKY on normal chow (~13%) and significantly less when fed a high-fat diet (~23%; SHR vs WKY; 74.8 ± 4.0% vs 97.3 ± 3.3%; $P < .05$). Collectively, the data illustrate that SHR rats have a reduced propensity for gaining weight.

3.2 | SHR rats have altered plasma chemistries

Next, glucose, total cholesterol and triglyceride levels in plasma were assessed. No differences were observed between strains in any plasma chemistries at baseline (Figure 2A-C). After 8 weeks of either normal chow or high-fat diet, WKY and SHR had significantly elevated blood glucose compared to baseline (Figure 2A). No difference in plasma glucose was observed between strains, indicating that age, but not diet or strain effected glucose levels (Figure 2A).

Interestingly, high-fat diet feeding did not increase plasma cholesterol or triglycerides above levels seen with normal chow for both strains (Figure 2B,C). However, WKY rats had significantly higher plasma cholesterol and triglyceride levels compared to SHR after 8 weeks of normal chow or high-fat diet (Figure 2B,C), suggesting that cholesterol and triglyceride metabolism may be altered in hypertensive rats.

Because hypertension and high plasma cholesterol exacerbate atherosclerosis, the predominant cause of CVD, we examined plaque formation in aortae. Oil Red O staining revealed no evidence of atherosclerosis in any group (Figure 2D), complementing other studies showing rats on a high-fat diet do not develop atherosclerotic plaque without genetic modification[13,14] or intervention such as stimulation of hyperuricemia.[15]

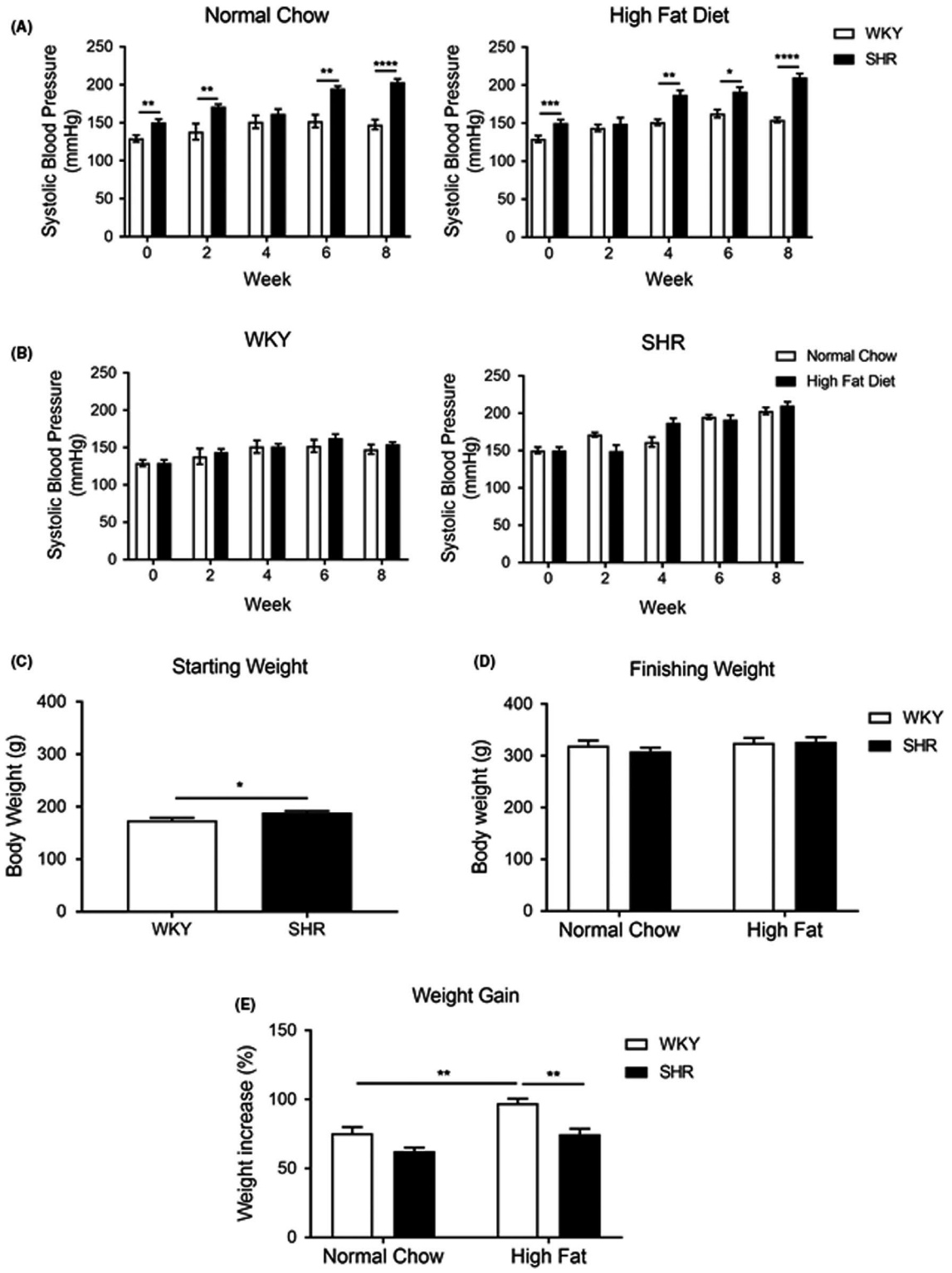

FIGURE 1 SHR rats have higher blood pressure but gain less weight than WKY. Systolic blood pressure over 8-wk feeding (A) WKY vs SHR on normal chow (left) and high-fat diet (right) or (B) effect of normal chow vs high-fat diet on WKY (left) and SHR (right) (0 wk, n = 18; 2-8 wk, n = 4-6). Body weights (C) at the beginning of the study (n = 11-12) and (D) after 8-wk feeding (n = 5-6), and (E) weight gain over the course of the study (n = 5-6). Results are mean ± SEM two-way ANOVA, *t* test or one-way ANOVA; **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001

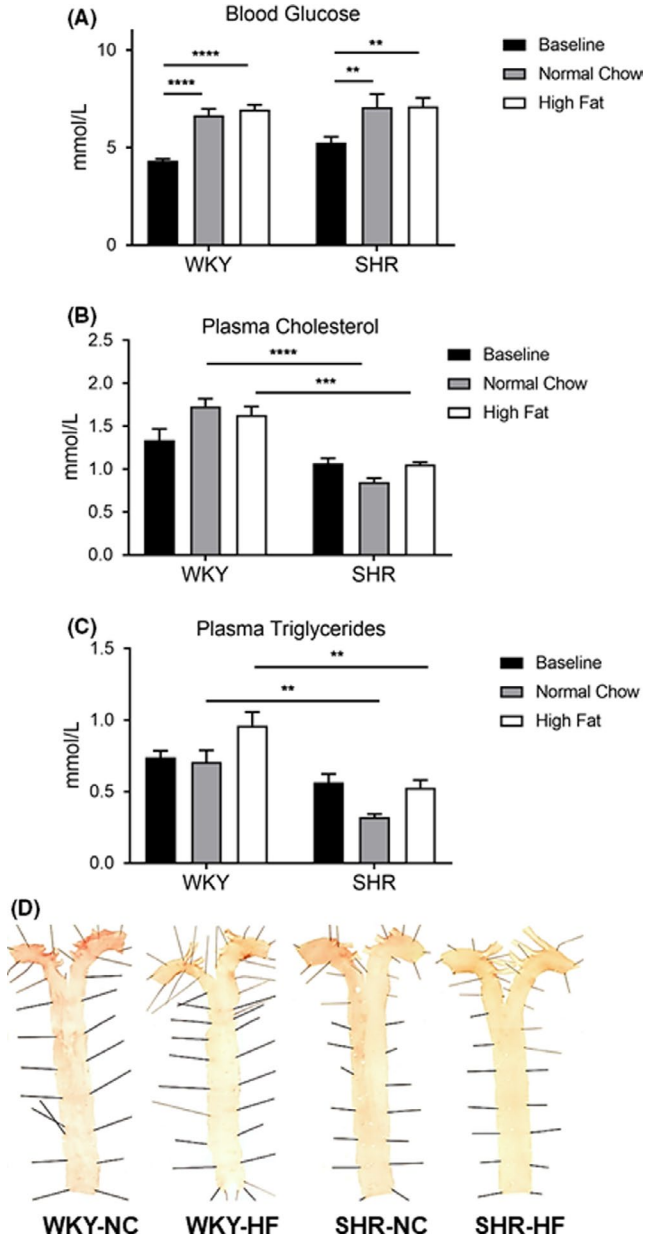

FIGURE 2 Plasma chemistries were measured at baseline and after 8-wk feeding. A, Blood glucose (baseline n = 9-14; normal chow and high-fat diet n = 5-6). Plasma (B) cholesterol and (C) triglycerides (n = 5-6). D, Aortae isolated and stained with Oil red O after 8-wk feeding; HF, high-fat diet; NC, normal chow. Results are mean ± SEM two-way ANOVA; $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

3.3 | WKY rats gain more retroperitoneal fat

We next measured weight of white adipose tissues (WAT), specifically focussing on epididymal and retroperitoneal WAT. As expected, weights of these were elevated from baseline in both WKY and SHR on normal chow and increased again in response to the high-fat diet (Figure 3A,B). Interestingly, retroperitoneal WAT weights were significantly

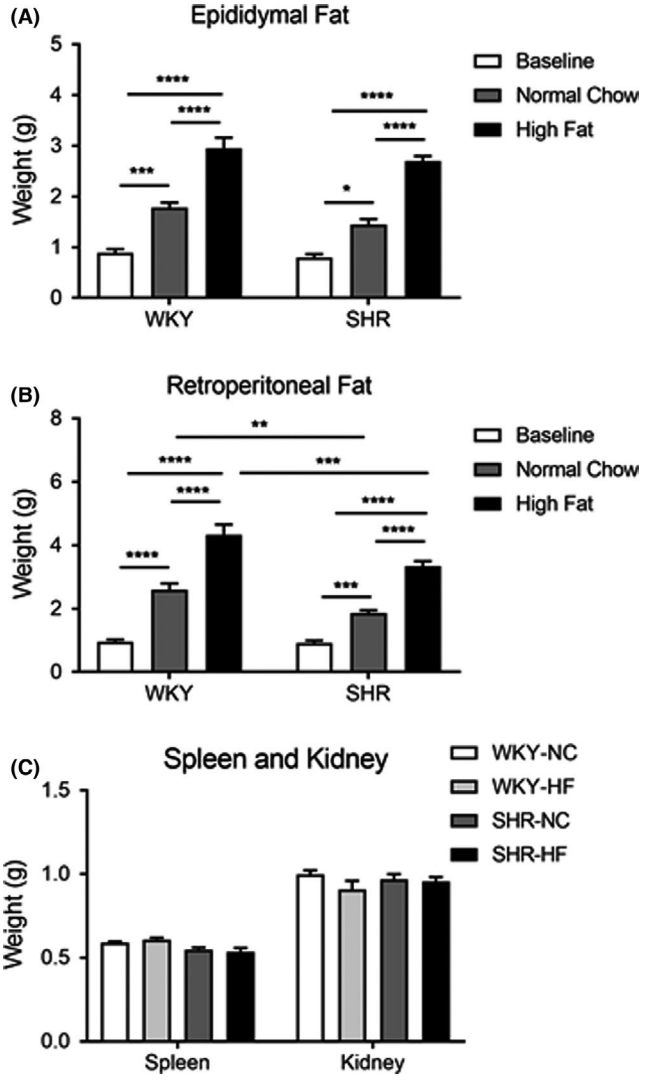

FIGURE 3 Organ weights. A, Epididymal and (B) retroperitoneal fat weight was measured at baseline and after 8-wk feeding. C, Spleen and kidney weights were measured after 8-wk feeding; HF, high-fat diet; NC, normal chow. n = 5-6 per group. Results are mean ± SEM two-way ANOVA or one-way ANOVA; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

reduced in SHR compared to WKY rats on both normal chow and high-fat diet (Figure 3B), consistent with weight increases observed in Figure 1E. Unlike WAT, spleen and kidney weights were unchanged between diets and strain (Figure 3C).

3.4 | SHR rats have altered lipid metabolism

Like WAT weight, liver weight also increased with age in both strains (Figure 4A). Because SHR rats had reduced plasma cholesterol and triglycerides compared to WKY on normal chow or high-fat diet, we predicted that SHR would also have reduced liver weights. Surprisingly, SHR liver

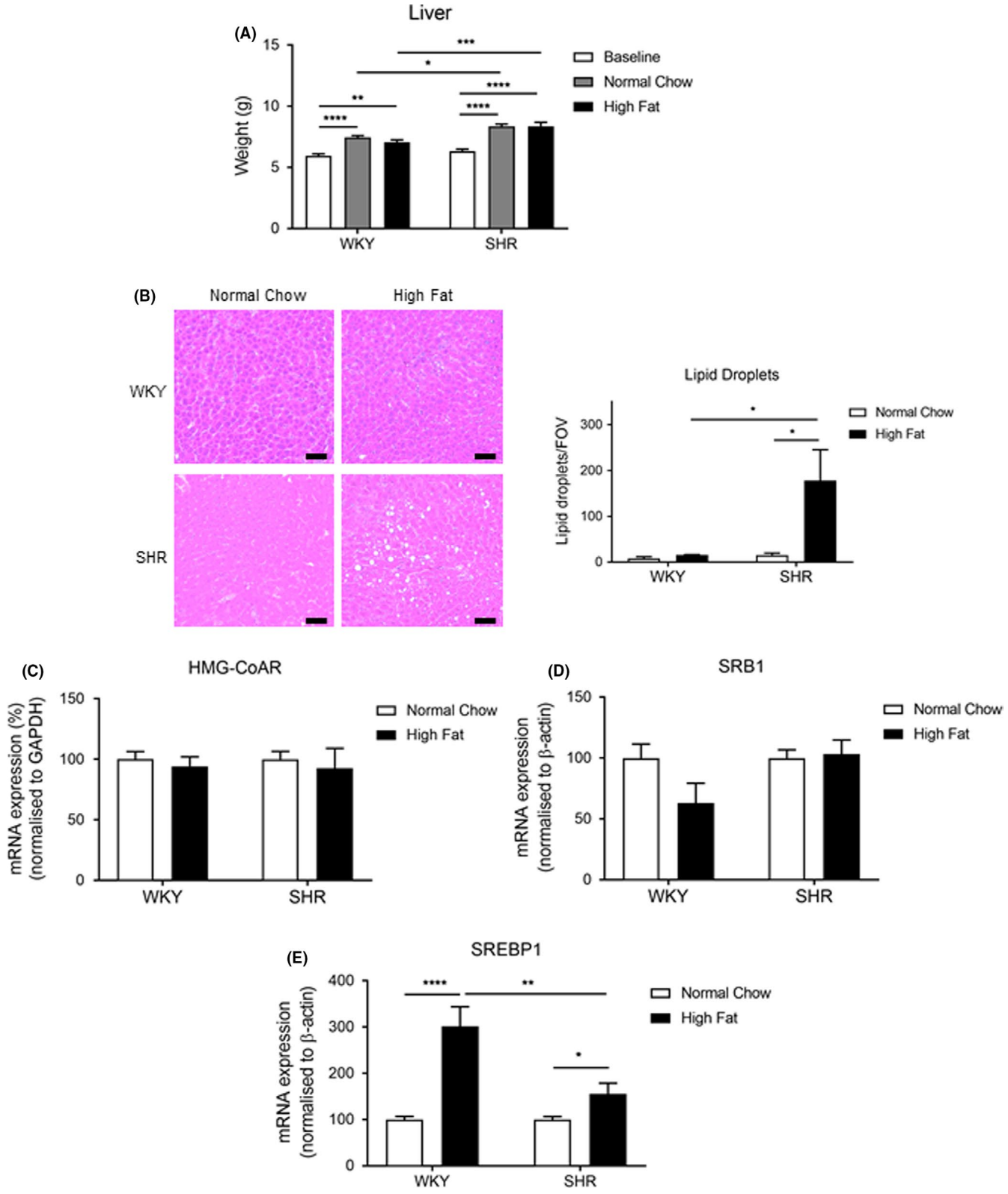

FIGURE 4 Spontaneous hypertension effects hepatic lipid metabolism. A, Liver weight at baseline and after 8-wk feeding. B, Accumulation of hepatic lipid droplets after 8-wk feeding. Left: H&E staining of liver, representative images; scale bar = 50 μm. Right: quantification, n = 5-6 per group. Hepatic gene expression measured by qPCR after 8-wk feeding; C, HMG-CoAR, D, SRB1 and E, SREBP1. mRNA was normalized to GAPDH or β-actin, n = 5-6 per group. Results are mean ± SEM two-way ANOVA; $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$

weight was significantly higher than WKY regardless of diet (Figure 4A), suggesting that cholesterol and triglycerides may be retained in the liver and not released into the circulation. We therefore next measured hepatic lipid storage by assessing the number of lipid droplets. WKY on normal chow or high-fat diet had low and unchanged numbers of lipid droplets (Figure 4B). In contrast, fat-fed SHR displayed an 11-fold increase in hepatic lipid accumulation compared to chow-fed SHR (Figure 4B). These striking differences were also observed comparing liver between high-fat diet-fed SHR and WKY (Figure 4B).

Genes important in cholesterol homeostasis include the cholesterol synthesis rate-limiting enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoAR), scavenger receptor, class B type 1 (SRB1) a facilitator in the transfer of cholesteryl esters from high-density lipoprotein (HDL) to the liver, and sterol regulatory element-binding protein 1 (SREBP1), a transcription factor that regulates lipogenesis in the liver. While no difference in hepatic HMG-CoAR or SRB1 gene expression was observed between strains or diet (Figure 4C-D), hepatic SREBP1 mRNA levels were significantly increased in response to the high-fat diet by ~3 and ~1.5-fold in WKY and SHR, respectively (Figure 4E). Further, SREBP1 mRNA expression was almost 50% lower in liver from high-fat diet SHR rats compared to their WKY counterparts. Taken together, these findings indicate that a high-fat "Western" diet increases hepatic lipid storage in SHR and that lipogenesis may be impaired in hypertension.

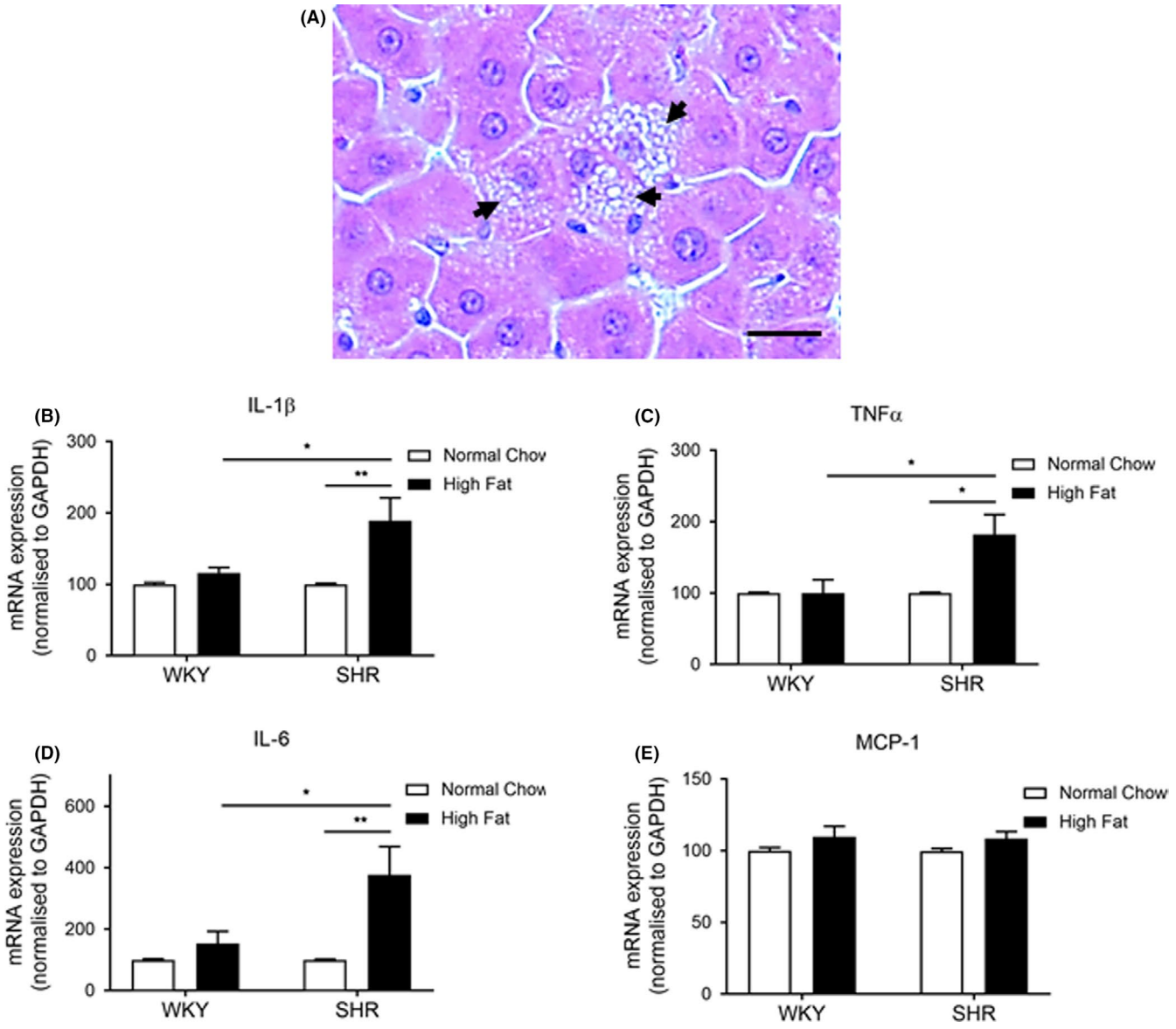

FIGURE 5 Fat-fed SHR are prone to features of NAFLD. A, Hepatic ballooning is evident in liver sections from fat-fed SHR. H&E staining of high-fat diet-fed SHR, enlarged cells with rarefied cytoplasm indicated by arrows; scale bar = 20 μm. Inflammatory genes in the liver measured by qPCR after 8-wk feeding; B, IL-1β, C, TNF-α, D, IL-6 and E, MCP-1. Results are mean ± SEM two-way ANOVA; *P < .05, **P < .01

3.5 | Liver from fat-fed SHR demonstrate hepatocellular ballooning and increased inflammation

Non-alcoholic steatohepatitis (NASH) is a more severe form of NAFLD,[16] characterized by an accumulation of lipid droplets, inflammation and ballooned hepatocytes. Given SHR fed a high-fat diet displayed increased hepatic lipid droplets (Figure 4B), liver from these animals was examined for features of NASH. High-fat diet-fed SHR displayed hepatocellular ballooning adjacent to areas with high lipid droplet numbers, where hepatocytes were enlarged with rarefied cytoplasm (Figure 5A). This was not observed in any other group (data not shown). Furthermore, high-fat diet-fed SHR had increased mRNA expression of inflammatory markers TNFα, IL-6 and IL-1β compared to chow-fed SHR (Figure 5B-D), while no changes in MCP-1 were observed (Figure 5E). Conversely, high-fat feeding of WKY rats had no effect on inflammatory markers when compared to their normal chow counterparts (Figure 5B-E), revealing that high-fat feeding in combination with hypertension accelerated the progression of NAFLD.

4 | DISCUSSION

This study compared changes in SHR and WKY rats fed a normal chow or a "Western" high-fat diet over 8 weeks. The key findings were (a) while blood pressure increased with age in SHR, 8-week high-fat diet feeding had no added effect on this, (b) high-fat diet-fed hypertensive SHR gained less weight associating with reduced retroperitoneal fat weight and (c) high-fat diet-fed SHR had increased hepatic lipid stores combined with hepatocellular ballooning and increased hepatic inflammation. Collectively, these findings demonstrate that spontaneous hypertension coupled with a "Western" diet modulates processes important in weight gain, hepatic lipogenesis and the development of NASH.

Spontaneously hypertensive rats are a model of primary hypertension and were developed by specifically breeding WKY rats with high blood pressure.[5] Ageing is associated with hypertension,[17,18] and as expected, blood pressure was increased in SHR over time. The high-fat diet increased blood pressure in WKY rats over 8 weeks, compared to baseline, whereas there were no significant changes in blood pressure between age-matched animals in either strain in response to the diet. There are a few reasons that may explain why we did not observe changes in blood pressure. In this report, we used a high-fat rodent diet consisting of 21% fat, 0.15% cholesterol and 43% carbohydrate for 8 weeks, mimicking a "Western" fast food style diet. Consistent with our findings, SHR fed a high-fat diet containing up to 40% fat for 10 weeks showed no differences in blood pressure compared to those on a chow diet,[19,20] whereas SHR on a high-fat (60%), low-carbohydrate (20%) diet for 10 weeks had attenuated blood pressure compared to controls.[21] In contrast, SHR fed a high-fat diet for 12 weeks (45% fat, 35% carbohydrate) showed elevated blood pressure.[22] Further, SHR fed a high-fat diet containing 58% fat from lard (36% carbohydrate) had ~25% increase in blood pressure by 4 weeks, which remained elevated for the 15 week study.[23] These suggest that the length of fat feeding and composition of the diet, including the source of fat are crucial factors modulating blood pressure.

Obesity has been identified as a risk factor for hypertension and cardiovascular disease,[24] with increased body mass index (BMI; kg/m^2) associated with hypertension in people.[25] While studies in rats and mice have shown that the "Western"-style high-fat diet can promote weight gain,[11,26-29] no differences in final body weight between strains or diets in our study were observed. We did, however, discover that WKY rats gained the most weight in response to the high-fat diet, which was also evident by increased white adipose tissue weight. This difference may be attributable to a greater level of physical activity. While the current study did not track movement, SHR appeared to move more and were more difficult to restrain than WKY, and other studies have shown evidence of hyperactivity in SHR.[30] This is not surprising since SHR are used as a model for attention deficit hyperactivity disorder, a condition where hyperactivity is a distinct characteristic.[31]

Non-alcoholic fatty liver disease covers a spectrum of disease from steatosis, where there is increased fat in the liver, to NASH where inflammation is evident, through to cirrhosis, which can lead to liver failure.[32,33] Interestingly, we found SHRs to have significantly reduced plasma cholesterol and triglycerides compared to WKY rats regardless of diet. However, in contrast, high-fat diet-fed SHR had increased liver weight and ~10 times more hepatic lipid droplets compared to WKY, indicating significantly increased lipid storage, or steatosis.

Hepatic steatosis results from an increase in hepatic lipid or free fatty acids (FFAs), or the failure to eliminate hepatic lipid. The main sources of FFAs in the liver are non-esterified fatty acids (NEFAs) released from adipose; *de novo* lipogenesis (eg from glucose) involving transcriptional regulation by factors including, proliferator-activated receptor-gamma and SREBP-1c; and FFAs from the diet. Although we did not measure NEFAs and cannot rule out their involvement, it is unlikely that SREBP-1 plays a role since SREBP1c mRNA was reduced in the liver of SHR on a high-fat "Western" diet, compared to WKY. While SHR have a known mutation in SREBP-1, a reduction would likely reduce *de novo* lipogenesis in these animals rather than promote steatosis.[34] In a healthy individual, hepatic lipid is released by the formation

and secretion of very low-density lipoprotein (VLDL) into the bloodstream, which is then converted to low-density lipoprotein (LDL), both contributing to plasma triglycerides and cholesterol.[35] Combining our findings of increased hepatic lipid droplets and reduced plasma cholesterol and triglycerides in high-fat "Western" diet-fed SHR suggests lipid is trapped in the liver of these animals due to an impaired ability to form and secrete VLDL, although this requires further assessment.

In addition to steatosis, we observed hepatocellular ballooning and inflammation in fat-fed SHRs, features essential for diagnosis of NASH.[33,36] The progression of NASH is driven by the hepatic inflammatory response. This can be triggered by a number of factors including oxidative stress, lipotoxicity and metabolic dysfunction as well as the secretion of cytokines and chemokines from adipose tissue or inflammatory cells such as adiponectin, leptin, IL-1β, IL-6, TNFα and MCP-1.[37] SHR have increased oxidative stress[38,39] and are more susceptible to changes in hepatic inflammatory markers,[40] indicating a predisposition to developing NASH. "Western" diet-fed SHR had increased hepatic mRNA expression of TNF-α, IL-1β and IL-6. Although we found no change in MCP-1 after 8-weeks of feeding, this is comparable with others where after 26 weeks, mice with steatosis showed no change in hepatic MCP-1, but increased adipose MCP-1.[41] MCP-1 promotes macrophage recruitment and in response to high-fat feeding, macrophage activation occurs initially, and *transiently*, in the liver, then increasingly in the adipose tissue,[42] suggesting that MCP-1 plays a role in the early stages of NASH, but by 8 weeks, the inflammatory response has progressed, as has disease.

In summary, spontaneous hypertension in rats resulted in reduced weight gain and fat accumulation compared to normotensive WKY rats, when fed a "Western" diet. Hypertensive rats also showed changes in lipid metabolism with reduced plasma cholesterol and triglycerides combined with increased lipid storage in the liver, altered expression of genes regulating lipogenesis and the development of NASH. However, it must be acknowledged this study was relatively short term, and while we did see an effect of diet, longer term feeding would likely exacerbate disease. Further studies are warranted to confirm this.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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