Hypertension exacerbates liver injury and hepatic fibrosis induced by a choline-deficient L-amino acid-defined diet in rats

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Abstract. The effect of hypertension on non-alcoholic fatty liver disease (NAFLD) remains unclear at the molecular level. In this study, we investigated the effects of hypertension on the degree of hepatic steatosis, liver injury and hepatic fibrosis induced by a choline-deficient L-amino acid-defined (CDAA) diet in spontaneously hypertensive rats (SHRs). Seven-week-old male SHRs were fed standard chow with high or normal salt concentrations for 7 weeks, followed by a CDAA diet containing high or normal salt for an additional 8 or 24 weeks. Hepatic steatosis was assessed using hepatic triglyceride levels and Oil red O staining. Hepatic fibrosis was evaluated using Sirius red and Azan staining. Systolic blood pressure (SBP) gradually increased with a high-salt diet and was significantly higher after 7 weeks of feeding with high-salt vs. normal-salt chow. After 8 weeks on the CDAA diet, the degree of hepatic steatosis did not differ between the high-salt and normal-salt groups; however, alanine aminotransferase and fasting blood glucose levels were significantly higher and hepatic mRNA levels for interleukin (IL)-10 and heme oxygenase (HO)-1 were significantly lower in the high-salt group compared with the normal-salt group. After 24 weeks on the CDAA diet, the high-salt group had significantly more severe hepatic fibrosis and a higher hepatic mRNA expression of α-smooth muscle actin and lower hepatic IL-10 and HO-1 mRNA levels compared with the normal-salt group. In conclusion, our results indicate that hypertension is a potential risk factor for liver injury and hepatic fibrosis through glucose intolerance and decreased IL-10-mediated or HO-1-induced anti-inflammatory mechanisms.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) includes non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH), and NASH may lead to hepatic cirrhosis or hepatocellular carcinoma (HCC) (1,2). NAFLD/NASH can be viewed as a metabolic syndrome phenotype involving the liver, and the incidence of NAFLD has increased with corresponding increases in risk factors for metabolic syndrome, such as obesity, diabetes and hypertension. Such metabolic risk factors may promote the pathological progression of NAFLD/NASH, and NAFLD advances more rapidly in patients with a greater number of metabolic risk factors (3,4).

The incidence of hypertension is significantly higher in patients with NAFLD compared with the general population (5). Furthermore, a higher incidence of NASH has been observed in patients with NAFLD who also have hypertension and these patients are likely to progress to advanced hepatic fibrosis (6), which suggests that hypertension may promote the onset or progression of hepatitis and hepatic fibrosis. However, the possible association between hypertension and the pathological progression of NAFLD has not been fully established.

Rats fed a choline-deficient L-amino acid-defined (CDAA) diet initially develop fatty liver and hepatitis, and may subsequently progress to hepatic fibrosis and HCC (7). These findings indicate that the rat fed a CDAA diet is an appropriate animal model of the pathology of NASH. The spontaneously hypertensive rat (SHR) is produced by the mating of rats with hypertension. These rats spontaneously develop hypertension at a rate of 100% between the ages of 7 to 15 weeks, even without a high-salt diet, although a high salt load further increases the blood pressure (8).

Carbon tetrachloride (CCl4)-induced hepatic fibrosis in the SHR model is more severe than that in normotensive Wistar-Kyoto (WKY) rats used as controls (9), and SHRs receiving a choline-deficient (CD) diet show severe hepatic steatosis compared with WKY rats receiving the same diet (10). SHRs and WKY rats both originate from Wistar rats, but they diverged

sufficiently long ago to now be essentially separate background strains (11). This is a limitation of the comparison of effects in these 2 types of rats. In addition, several proteins differ in the livers of SHRs and WKY rats, including proteins that induce oxidative stress (11). For this reason, we investigated the effects of hypertension induced by a high-salt diet on the severity of hepatic steatosis, liver injury and hepatic fibrosis induced by a CDAA diet in the SHR model.

Materials and methods

Animals and diets. Six-week-old male SHRs were obtained from Charles River Laboratories (Kanagawa, Japan). The rats were allowed to acclimatize to the laboratory conditions for at least 7 days at a constant temperature of 24˚C with a 12-h light-dark cycle, and fed standard chow (control diet) containing 0.27% NaCl (CE-2; Kyudo, Kumamoto, Japan) and water *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee of Kagoshima University, Kagoshima, Japan. After the acclimatization period, the rats were placed in groups that were fed 3 different diets *ad libitum*: standard chow with high salt (8.0% NaCl) or normal salt (0.27% NaCl) for 7 weeks, followed by a CDAA diet containing a high (8.0% NaCl) or normal salt (0.25% NaCl) level for an additional 8 or 24 weeks (high- and normal-salt groups, respectively); or standard chow with normal salt (0.27% NaCl) for 15 or 31 weeks (control groups). Diets were obtained from Dyets Inc. (Bethlehem, PA, USA) and 30 g per day was administered to equalize the total food intake.

The resected livers were weighed and used for RNA extraction, or thin slices were immersed in 10% formalin and embedded in paraffin to make 4-μm-thick sections for staining with hematoxylin and eosin (H&E), Oil red O, Azan or Sirius Red. Blood was collected by vena cava puncture following a 12-h fast and then centrifuged. The resulting serum was stored at -80˚C.

Measurement of systolic blood pressure and serum markers. Systolic blood pressure (SBP) was monitored from 7 to 31 weeks using the tail-cuff method (model MK-1030; Muromachi Co., Ltd., Tokyo, Japan). At each time point, SBP was measured for each rat and the mean was calculated. Serum alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels were determined using Spotchem II-liver function 2 (Arkray Inc., Kyoto, Japan). Serum triglyceride and free fatty acid levels were determined by ELISA at SRL, Inc. (Tokyo, Japan). Fasting blood glucose (FBG) and serum immunoreactive insulin (IRI) levels were determined by ELISA (Morinaga Institute of Biological Science, Kanagawa, Japan).

Assessment of hepatic steatosis and hepatic fibrosis. Hepatic steatosis was assessed using hepatic triglyceride levels and Oil red O staining. Hepatic lipids were extracted with chloroform-methanol and measured enzymatically using a commercial kit (L-type Wako TGH; Wako Pure Chemical Industries, Osaka, Japan). Oil red O staining was performed to evaluate the accumulation of fat droplets in hepatocytes in the frozen liver sections. Sirius Red or Azan-stained sections were analyzed to evaluate fibrosis in the liver. Five fields were selected randomly from each right lobe per sample, and samples from 6 to 11 rats in each group were examined. Thus, a total of 30 to 55 fields were analyzed for each group. The ratios of the Oil red O-, Azan- or Sirius Red-stained area to the total area were quantified by image analysis. A spot RT color camera (Visitron Systems Inc., Puchheim, Germany) was used to capture and analyze the fields at x100 magnification. Image analysis was performed using Quick Grain Standard ver. 5.0.3. software (Inotech, Hiroshima, Japan).

Assessment of hepatic mRNA levels for genes associated with liver inflammation and fibrosis. The relative levels of specific mRNAs in the resected livers were assessed by real-time polymerase chain reaction (qPCR) using a One Step SYBR PrimeScript RT-PCR kit II (Takara Bio Inc., Shiga, Japan). Total RNA was extracted from the livers using isogen (Nippon Gene, Tokyo, Japan). The expression levels of target genes were calculated relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an endogenous control gene to normalize the target gene expression levels. All procedures were performed according to manufacturer's instructions. mRNA levels were determined for tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, heme oxygenase (HO)-1, α-smooth muscle actin (SMA), transforming growth factor (TGF)-β1 and tissue inhibitor of metalloproteinase (TIMP)-1. PCR primers were obtained from Takara Bio Inc. The primer sequences used are listed in Table I.

Statistical analysis. A statistical comparison among groups was performed using the Tukey-Kramer test. A Student's t-test was used for a comparison between 2 groups. A value of $P<0.05$ was considered to indicate a statistically significant difference. Data are presented as the means ± standard deviation (SD).

Results

Systolic blood pressure is affected by salt levels, but not by the CDAA diet. SBP gradually increased until 15 weeks in the high- and normal-salt groups. The increase in SBP was significantly greater after 7 weeks on the standard diet in the high-salt group compared with the normal-salt group and this difference continued after the CDAA diet commenced. By contrast, SBP did not differ between the normal-salt groups fed a CDAA or standard diet (Fig. 1).

Metabolic parameters after 8 weeks on the CDAA diet. There were no differences in dietary intake between the high- and normal-salt groups due to dietary restrictions during the study period; however, body weight was significantly lower and the liver/body weight ratio was significantly higher in the high-salt group compared with the normal-salt and control groups after 8 weeks on the CDAA diet (both P-values <0.05). FBG levels were significantly higher in the high-salt group compared with the other 2 groups. Serum insulin levels tended to be higher in the high-salt group compared with the normal-salt group after 8 weeks; however, these levels in the CDAA groups were not significantly higher than those in the control group. Serum triglyceride levels were significantly higher in the CDAA groups compared with the control group; however, there was no difference between the high- and normal-salt groups.

Table I. Oligonucleotide sequences used for qPCR.

Gene	GenBank number	Primer sequences
TNF-α	NM_012675	F: 5-TCAGTTCCATGGCCCAGAC-3 R: 5-GTTGTCTTTGAGATCCATGCCATT-3
IL-6	NM_012589	F:5-CCACTTCACAAGTCGGAGGCTTA-3 R: 5-GTGCATCATCGCTGTTCATACAATC-3
IL-10	NM_012854	F: 5-CAGACCCACATGCTCCGAGA-3 R: 5-CAAGGCTTGGCAACCCAAGTA-3
HO-1	NM_012580	F: 5-AGGTGCACATCCGTGCAGAG-3 R: 5-TCCAGGGCCGTATAGATATGGTACA-3
α-SMA	NM_031004.2	F: 5-AGCCAGTCGCCATCAGGAAC-3 R: 5-GGGAGCATCATCACCAGCAA-3
TGF-β1	NM_021578	F: 5-TGCGCCTGCAGAGATTCAAG-3 R: 5-ACGTAACGCCAGGAATTGTTGCTA-3
TIMP-1	NM_053819.1	F: 5-CGAGACCACCTTATACCAGCGTTA-3 R: 5-TGATGTGCAAATTTCGTTCC-3
GAPDH	NM_017008.3	F: 5-GGCACAGTCAAGGCTGAGAATG-3 R: 5-ATGGTGAAGACGCCAGTA-3

TNF, tumor necrosis factor; IL, interleukin; HO, heme oxygenase; TGF, transforming growth factor; SMA, smooth muscle actin; TIMP, tissue inhibitor of metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

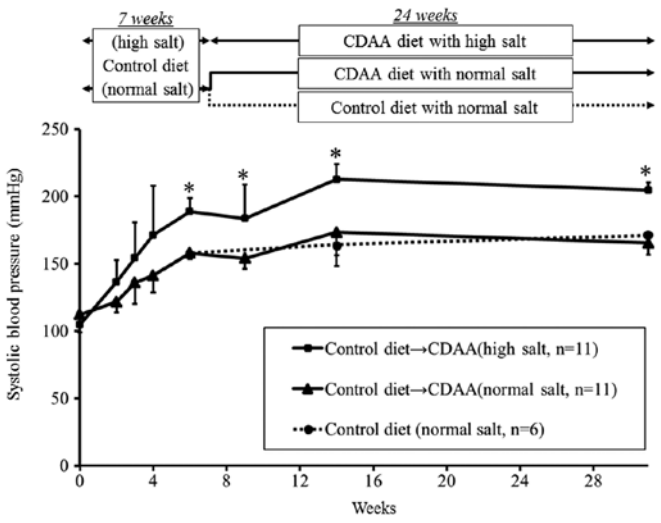

Figure 1. Course of SBP in SHRs fed standard CE-2 chow (control) or a CDAA diet with or without high salt. SBP was measured using the tail-cuff method. SBP gradually increased in the rats fed high-salt chow and was significantly higher after 7 weeks of a high-salt diet compared with a normal-salt diet. *P<0.05 vs. normal-salt groups. SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; CDAA, choline-deficient L-amino acid-defined diet.

Serum free fatty acids were significantly higher in the normal-salt group compared with the high-salt group, and lowest in the control group (Table II).

Severity of hepatic steatosis after 8 weeks on the CDAA diet. Micro- and macrovesicular steatosis was clearly visible in the H&E-stained liver sections from the rats fed a CDAA diet with normal- and high-salt for 8 weeks (Fig. 2A). The hepatic fat area based on Oil red O staining and the hepatic triglyceride content was significantly higher in the CDAA groups compared with the control group (Fig. 2B and C). However, hepatic steatosis did not differ between the 2 salt groups fed a CDAA diet (Fig. 2B and C).

Liver injury after 8 weeks on the CDAA diet. Serum ALT levels were significantly higher in the high-salt group compared with the normal-salt group (P<0.05) (Fig. 3). Serum ALP levels were significantly higher in the high-salt group compared with the control group and showed a tendency to be higher in the high-salt group compared with the normal-salt group (Fig. 3).

Hepatic mRNA levels after 8 weeks on the CDAA diet with high or low salt. The mRNA expression levels for TNF-α and IL-6 did not differ between the 2 salt groups after 8 weeks on the CDAA diet; however, IL-6 levels were relatively higher in the high-salt group compared with the normal-salt and control groups (Fig. 4). By contrast, hepatic mRNA levels for IL-10 and HO-1 were significantly lower in the high-salt group compared with the normal-salt group (Fig. 4). However, the hepatic mRNA level for IL-10 was higher and that for HO-1 was lower in the normal-salt group compared with the control group.

Metabolic parameters after 24 weeks on the CDAA diet. Body weight was significantly lower and the liver/body weight ratio was significantly higher in the high-salt group compared with the normal-salt group after 24 weeks on the CDAA diet. FBG

Table II. Metabolic parameters after 8 weeks on the CDAA diet.

Parameters	Control (n=6)	CDAA diet	
		Normal-salt (n=11)	High-salt (n=11)
Body weight (g)	349.1 (17.5)	374.6 (17.8)	313.0 (36.4)[b]
Liver weight (g)	9.79 (0.34)	10.69 (1.35)	10.08 (1.39)
Liver weight/body weight (%)	2.81 (0.06)	2.85 (0.32)	3.22 (0.10)[b]
Fasting blood glucose (mg/dl)	96.1 (8.9)	89.5 (17.2)	129.8 (27.4)[b]
Serum insulin (ng/ml)	1.20 (0.60) (n=4)	0.69 (0.36)	1.24 (1.13) (n=9)
Triglyceride (mg/dl)	15.8 (2.4)	29.0 (4.8)[a]	29.2 (8.6)[a]
Free fatty acids (mEq/l)	520.8 (46.3)	772.0 (95.2)[a]	551.0 (108.0)[c]

Data are shown are the means ± standard deviation (SD). [a]P<0.05 vs. control, [b]P<0.05 vs. control and normal-salt group, [c]P<0.05 vs. normal-salt group. CDAA diet, choline-deficient L-amino acid-defined diet.

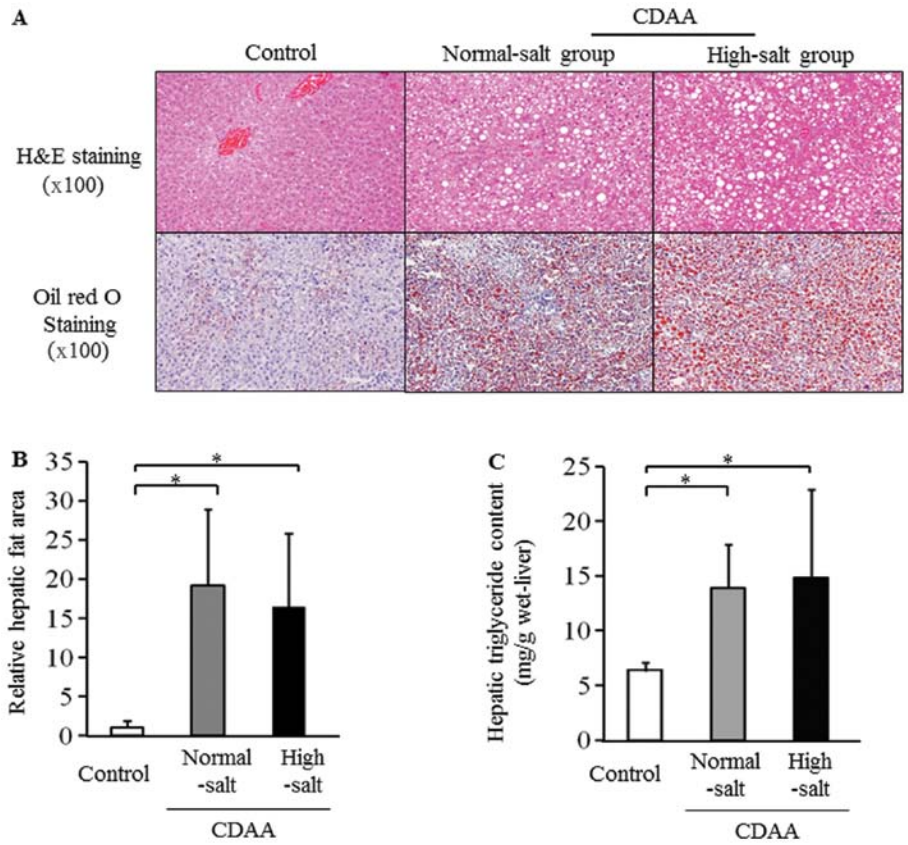

Figure 2. Histological evaluation of hepatic steatosis and hepatic triglyceride content in rats fed standard chow (control), a CDAA diet with normal salt (normal-salt group), and a CDAA diet with high salt (high-salt group) at week 15 (8 weeks of a CDAA diet). Hepatic steatosis was evaluated by (A) hematoxylin and eosin (H&E) and Oil red O staining, and (B) imaging analysis using Oil red O staining, and (C) hepatic triglyceride levels. Hepatic steatosis in the normal- and high-salt groups was more severe compared with the control group, based on imaging analysis and hepatic triglyceride levels. The degree of hepatic steatosis did not differ between the normal- and high-salt groups. Values are the means ± standard deviation of 6 or 11 rats. *P<0.05. CDAA, choline-deficient L-amino acid-defined diet.

levels were relatively higher in the high-salt group compared with the normal-salt and control groups. Serum insulin levels tended to be higher in the high-salt group compared with the normal-salt group; however, these levels were lower than those in the control group. Serum triglyceride levels were significantly higher in the CDAA groups compared with the control group, but did not differ between the high- and normal-salt groups. The levels of serum free fatty acids were significantly higher in the normal-salt group compared with those in the control group (Table III).

Table III. Metabolic parameters after 24 weeks on the CDAA diet.

Parameters	Control (n=6)	CDAA diet	
		Normal-salt (n=11)	High-salt (n=11)
Body weight (g)	375.7 (17.9)	399.6 (17.5)	303.9 (16.5)[b]
Liver weight (g)	12.48 (0.91)	10.97 (0.46)[a]	9.47 (0.95)[b]
Liver/body weight ratio (%)	3.33 (0.29)	2.75 (0.09)[a]	3.11 (0.21)[c]
Fasting blood glucose (mg/dl)	86.7 (16.4)	103.7 (16.6)	119.2 (41.2)
Serum insulin (ng/ml)	2.29 (0.63)	0.20 (0.16)	0.56 (0.27)
Triglyceride (mg/dl)	20.5 (3.6)	45.0 (1.3)[a]	38.2 (14.3)[a]
Free fatty acids (mEq/l)	537.7 (167.5)	804.2 (90.3)[a]	668.0 (210.2)

Data are shown are the means ± standard deviation (SD). [a]P<0.05 vs. control, [b]P<0.05 vs. control and normal-salt group, [c]P<0.05 vs. normal-salt group. CDAA diet, choline-deficient L-amino acid-defined diet.

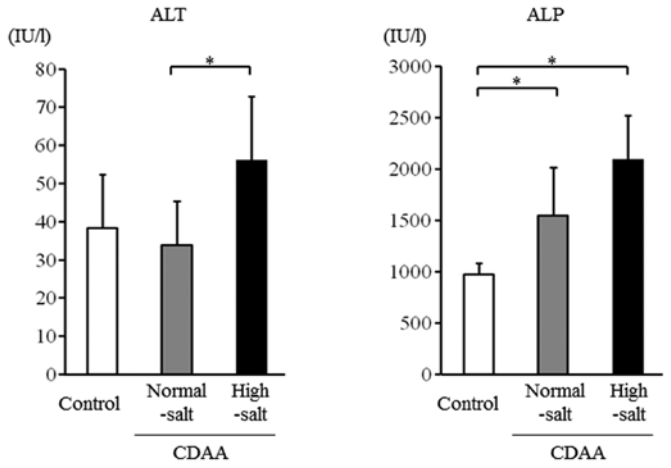

Figure 3. Serum levels of ALT and ALP at week 15 (8 weeks of a CDAA diet). Serum ALT levels were significantly higher in the high-salt group compared with the normal-salt group. Serum ALP levels were significantly higher in the high-salt group compared with the control group. Values are the means ± standard deviation of 6 or 11 rats. *P<0.05. ALT, alanine aminotransferase; ALP, alkaline phosphatase; CDAA, choline-deficient L-amino acid-defined diet.

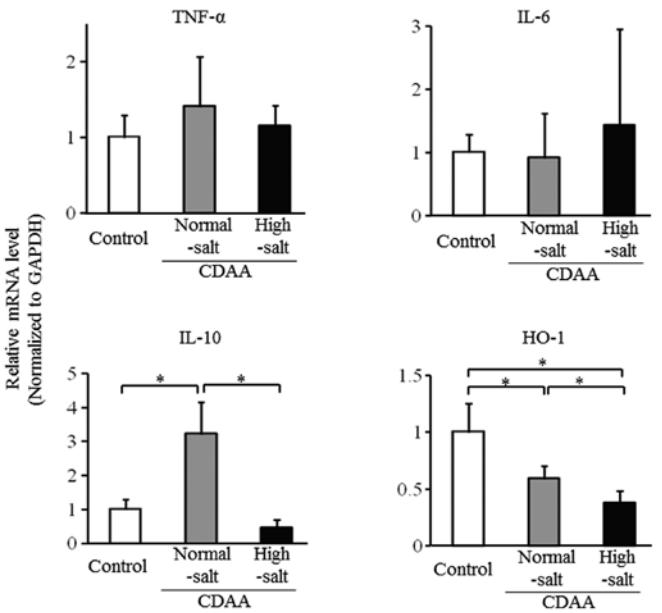

Figure 4. Hepatic mRNA levels of genes involved in inflammation assessed by qPCR at week 15. The hepatic IL-10 mRNA level was significantly higher in the normal-salt group compared with the control and high-salt groups. The hepatic HO-1 mRNA level differed significantly among the 3 groups and was lowest in the high-salt group followed by the normal-salt group. Hepatic TNF-α and IL-6 mRNA levels did not differ among the 3 groups. Values are the means ± standard deviation of 6 or 11 rats. *P<0.05. qPCR, real-time polymerase chain reaction; IL, interleukin; HO, heme oxygenase; TNF, tumor necrosis factor. CDAA, choline-deficient L-amino acid-defined diet.

Severity of hepatic fibrosis after 24 weeks on the CDAA diet with high or low salt. The CDAA diet was administered from week 7 to week 31 (a total of 24 weeks) with or without high salt for the induction of liver fibrosis (Fig. 1). Liver fibrosis assessed by Sirius Red and Azan staining was significantly more severe in the high-salt group compared with the normal-salt and control groups (Fig. 5). The hepatic mRNA levels for α-SMA were significantly higher in the high-salt group compared with the normal-salt and control groups (Fig. 6), and hepatic TGF-β1 and TIMP-1 mRNA levels tended to be higher in the high-salt group compared with the normal-salt group. By contrast, hepatic mRNA levels for IL-10 and HO-1 were significantly lower in the high-salt group compared with those in the normal-salt group, whereas the hepatic mRNA level of IL-10 was significantly higher and that of HO-1 tended to be lower in the normal-salt group compared with the control group (Fig. 6).

Discussion

The prevalence of NAFLD including NASH has increased with an increase in the incidence of metabolic syndrome, which includes obesity, diabetes, dyslipidemia and hypertension (12-16). Hypertension, high levels of ALT and insulin resistance have all been associated with NASH and liver fibrosis (6); however, the effect of hypertension on NAFLD or NASH at the molecular level is uncertain. In this study,

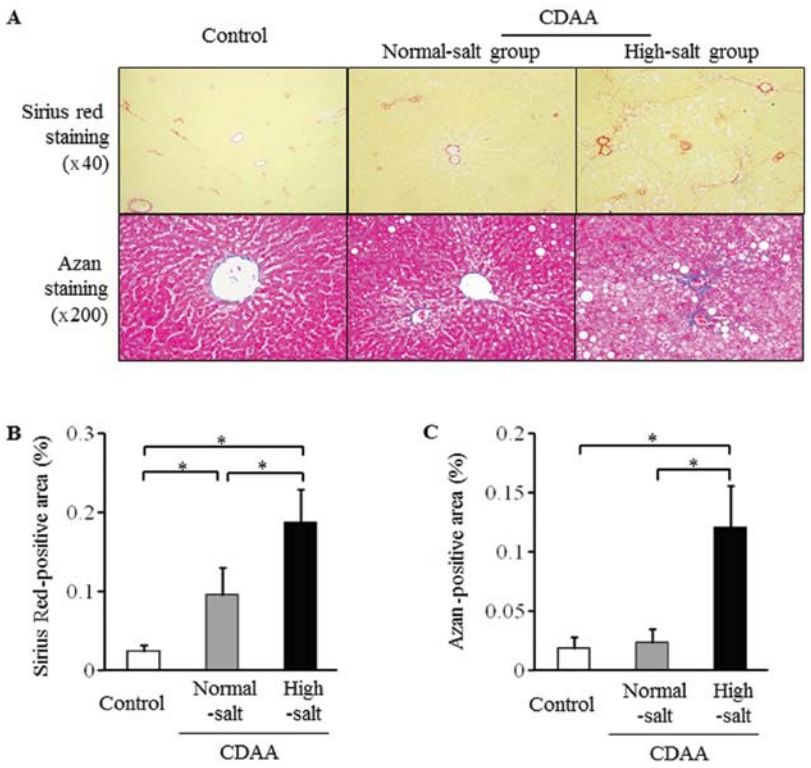

Figure 5. Histological evaluation of hepatic fibrosis in rats fed a CDAA diet for 24 weeks. Hepatic fibrosis was evaluated by (A) Sirius Red and Azan staining. The hepatic fibrosis area was determined by imaging analysis using (B) Sirius Red staining and (C) Azan staining. Hepatic fibrosis in the high-salt group was significantly more severe compared with the normal-salt and control groups. Values are the means ± standard deviation of 6 rats. *P<0.05. CDAA, choline-deficient L-amino acid-defined diet.

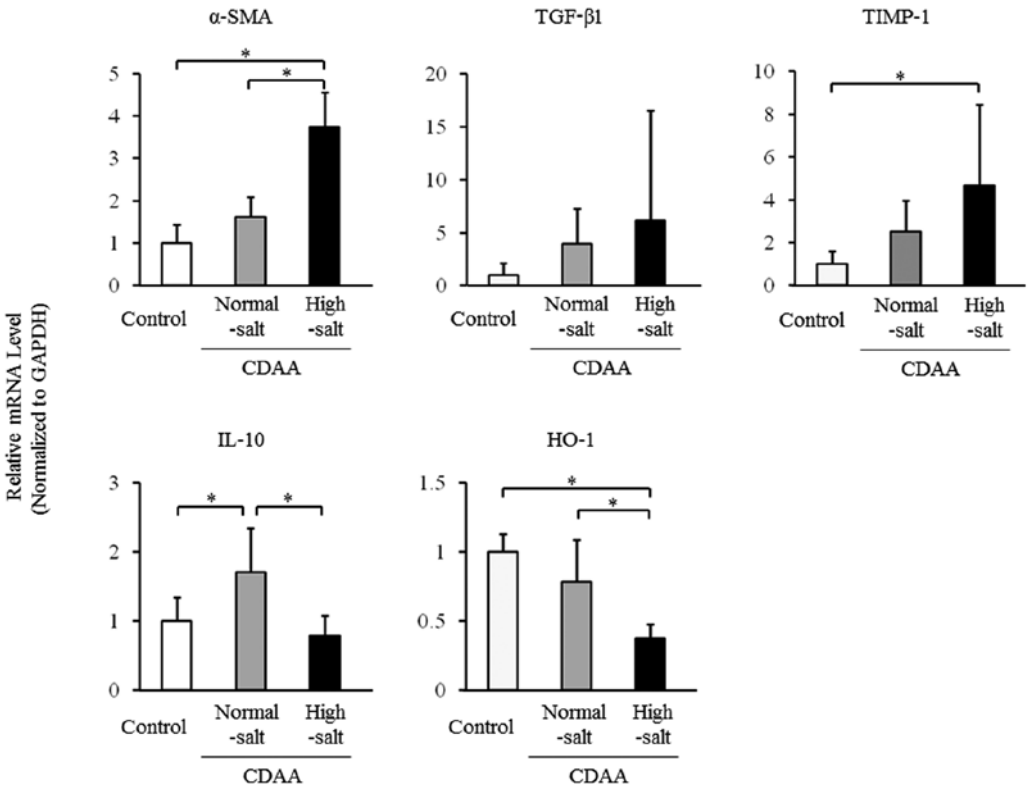

Figure 6. Hepatic mRNA expression of genes involved in hepatic fibrosis. Expression levels were assessed by qPCR. The hepatic α-SMA mRNA level was significantly higher in the high-salt group compared with the normal-salt and control groups. Hepatic TIMP-1 mRNA expression in the high-salt group was significantly higher than that in the control group. The hepatic IL-10 mRNA level was significantly higher in the normal-salt group compared with the high-salt and control groups. The hepatic HO-1 mRNA level was significantly lower in the high-salt group compared with the normal-salt and control groups. Values are the means ± standard deviation of 6 rats. *P<0.05. qPCR, real-time polymerase chain reaction; SMA, smooth muscle actin; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; IL, interleukin; HO, heme oxygenase; CDAA, choline-deficient L-amino acid-defined diet.

we investigated the mechanisms through which hypertension affects the degree of hepatic steatosis, liver injury and hepatic fibrosis induced by a CDAA diet in a hypertensive rat model. The results revealed that hypertension induced by a high-salt diet had a negligible effect on the progression of hepatic steatosis in rats, but may be a potential risk factor for liver injury and hepatic fibrosis due to glucose intolerance and decreased IL-10-mediated and HO-1-induced anti-inflammatory effects.

Ikuta *et al* concluded that hypertension may have an effect on the progression of NASH (10) and Hsu also suggested that hypertension may induce severe hepatic fibrosis (9). However, these reports are limited due to their dependence on comparing SHRs and normotensive WKY rats, which are clearly different despite having a similar origin (11). By contrast, we adjusted blood pressure using 2 salt levels in the SHR model. In our study, SBP gradually increased over the study period and the increase in SBP was significantly more severe in the high-salt group compared with the normal-salt group. By contrast, SBP did not differ between the normal-salt group fed a CDAA diet and the controls fed a standard diet with a normal-salt level. SBP was also suppressed by an anti-hypertensive agent (data not shown). Thus, these results demonstrate that the increase in SBP in the SHR model was independent of diet, apart from the salt level, and therefore our results reveal a direct mechanistic effect of hypertension on NAFLD/NASH.

Hypertension has been shown to reduce the plasma levels of IL-10 (17) and we also found that hypertension inhibited the expression of IL-10 in the livers of CDAA-fed rats. Treatment with IL-10 *in vivo* has been shown to significantly reduce SPB in hypertensive mice (18). IL-10 is a potent anti-inflammatory cytokine that plays a pivotal role in the regulation of immune and inflammatory responses. The inhibition of IL-10 has been shown to promote the expression of inflammatory cytokines, reduce insulin signaling and activate gluconeogenic and lipidogenic pathways in an animal model of diet-induced fatty liver disease (19). In addition, the upregulation of HO-1 has been shown to induce a reduction in the levels of cytokines, adhesion molecules, chemokines and neutrophil accumulation, and to ameliorate organ injury in a state of shock (20-22). As previously demonstrated, the induction of HO-1 reduces hepatic ischemia reperfusion injury (23), hepatic injury after trauma hemorrhage (24) and renal oxidative stress in diabetic SHRs (25). The inhibition of HO-1 may also be associated with acetaminophen-induced liver injury (26). Thus, HO-1 is thought to play a protective role in several organs under various deleterious conditions. IL-10 also inhibits hepatitis (27,28) and this inhibition may occur through a mechanism involving increased HO-1 expression induced by an increase in IL-10 levels (29). Furthermore, in our study, in the CDAA-fed rats, the normal-salt group showed higher levels of IL-10 and lower levels of HO-1 compared with the control group. This higher level of IL-10 may be due to a feedback mechanism against hepatitis induced by CDAA, although the CDAA diet may reduce HO-1 expression. By contrast, hypertension inhibited IL-10 and HO-1 expression, with a resultant increase in ALT to a level higher than that caused by the CDAA diet alone. In addition, indirect evidence indicates that IL-10 exerts a protective effect on endothelial function in diabetes and hypertension (17,30). IL-10 has also been shown to inhibit the activation of hepatic stellate cells and decrease hepatic fibrosis and apoptosis in a CCl4-induced rat liver fibrosis model (31,28). These findings suggest that CDAA-induced hepatic fibrosis progresses more in SHRs fed a high-salt diet compared with those fed a normal-salt diet due to the inhibition of IL-10 and reduced HO-1 expression. Thus, we speculate that the normal-salt group had increased IL-10 expression to compensate for liver injury and hepatic fibrogenesis induced by the CDAA diet, but the high-salt group could not compensate in the same manner due to the inhibition of IL-10 and HO-1 by hypertension.

Hypertension is associated with NAFLD/NASH, increased diastolic blood pressure has been linked to hepatic fat content (32), and the incidence of NAFLD in patients with systolic hypertension is twice that in the general population (33). Donati *et al* also found that the prevalence of fatty liver in patients with hypertension without diabetes and obesity was 3-fold higher than that in patients with normal blood pressure (34). These patients have higher homeostasis model assessment-insulin resistance (HOMA-IR) scores, and hypertension may trigger insulin resistance, which may induce fatty liver. However, our study on rats showed that hypertension caused by a higher salt load did not influence the degree of fatty deposition in the liver. The CD diet model does not present with insulin resistance (10) and hyperinsulinemia was not observed in CDAA loading in the SHR model with or without high salt, compared with the control group fed a standard diet in the current study. By contrast, FBG and serum insulin levels were higher in the high-salt group compared with the normal-salt group. HOMA-IR calculated from FBG and serum insulin levels in the high-salt group was higher than that in the normal-salt group (data not shown). Therefore, hypertension may induce glucose intolerance or insulin resistance in the SHR model. Hepatic steatosis induced by a CDAA diet may generally be severe, but insulin resistance should not be critical in hepatic steatosis induced by a CDAA diet, which may mask the effect of hypertension on hepatic steatosis. Therefore, the effect of hypertension on the severity of hepatic steatosis requires further investigation using other fatty liver models.

There are well known associations between hypertension and insulin resistance (35-37). Higher insulin resistance may lead to hypertension due to a decrease in nitric oxide (NO), the facilitation of a sympathetic nerve response and an increase in vascular smooth muscle contraction, while hypertension induced by a high-salt diet can increase insulin resistance (38,39). In our study, there was a possible association between hypertension induced by a higher salt load and glucose intolerance or insulin resistance in the CDAA-fed SHR model. Insulin resistance is linked to hepatic fibrosis (40), and peroxisome proliferator-activated receptor (PPAR)-γ agonists that improve insulin resistance also improve the pathology of NAFLD/NASH (41). The finding that hypertension induced by a higher salt load contributed to the progression of hepatic fibrosis suggests that glucose intolerance or insulin resistance induced by hypertension may be factors in the acceleration of hepatic fibrosis.

The pathological progression of NAFLD/NASH is not yet fully understood (42), but the 'two hit theory' is widely supported as the pathogenic mechanism of NASH (43). In this theory, it is proposed that the accumulation of triglycerides in

hepatic cells (first hit) and then oxidative stress, lipid peroxidation and endotoxins increase cytokine levels and insulin resistance, which then leads to liver injury and progression to hepatic fibrosis (second hit). Our results suggest that the reduced expression of IL-10 and/or HO-1 with a consequent reduction in the inhibition of anti-inflammatory effects, and increased glucose intolerance or insulin resistance, may be involved in the hypertension-mediated exacerbation of liver injury and the acceleration of hepatic fibrosis. Thus, hypertension may be a contributing factor to the second hit rather than the first hit in NASH. Furthermore, our results suggest that aggressive anti-hypertensive therapy is required in patients with NAFLD/NASH accompanied by hypertension for the prevention of cardiovascular diseases and the inhibition of the progression of hepatic diseases.

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