Hydralazine Lowers Serum Triglyceride Levels by Enhancing Farnesoid X Receptor Signaling and Adipose Triglyceride Lipase Expression : In Vivo Evidence from Male Spontaneously Hypertensive Rats

Xuguang Zhang[1)#], Jiali Chen[1)#], Naoki Takeda[1)], Kazuya Kitamori[2)]
Hisao Naito[2)], Tamie Nakajima[3)] and Naoki Tanaka[1)*]

1) *Department of Global Medical Research Promotion, Shinshu University Graduate School of Medicine*
2) *College of Human Life and Environment, Kinjo Gakuin University*
3) *Research Institute of Life and Health Sciences, Chubu University*

Background : Hydralazine (HYD) is a vasodilator widely used for the management of hypertension and related conditions, including heart failure and eclampsia. While previous studies have shown that HYD mitigates hepatic steatofibrosis induced by a high-fat, high-cholesterol diet in spontaneously hypertensive rats (SHRs), its precise effects on lipid metabolism remain unclear. This study investigated the impact of HYD on serum and hepatic lipid profiles and explored potential underlying mechanisms.

Methods : Male SHRs received HYD via drinking water (60 mg/L) for either four or ten weeks, followed by analyses of clinical parameters and hepatic gene expression.

Results : HYD significantly reduced serum triglyceride (TG) levels at both time points. In the liver, HYD markedly upregulated small heterodimer partner, a target gene of the farnesoid X receptor (FXR), as well as adipose triglyceride lipase (ATGL). After ten weeks of HYD treatment, the expression of peroxisome proliferator-activated receptor α was also significantly elevated, coinciding with reduced serum TG levels.

Conclusion : These findings suggest that HYD lowers lipid levels primarily by activating the FXR signaling pathway and enhancing ATGL expression, highlighting a novel pharmacological role of HYD in lipid metabolism.
Shinshu Med J 73 : 165—177, 2025

(Received for publication January 27, 2025 ; accepted in revised form February 18, 2025)

Key words : hydralazine, spontaneously hypertensive rats, triglyceride, farnesoid X receptor, adipose triglyceride lipase

Abbreviations : Abcb11, ATP binding cassette subfamily b member 11 ; Acadl, long-chain acyl-CoA dehydrogenase ; Acox1, peroxisomal acyl-CoA oxidase 1 ; Atgl, adipose triglyceride lipase ; ALT, alanine aminotransferase ; AST, aspartate aminotransferase ; AUC, area under the plasma concentration-time curve ; BA, bile acid ; BP, blood pressure ; BW, body weight ; Cmax, maximum plasma concentration ; Cyp7a1, cytochrome P450 7A1 ; FA, fatty acid ; Fxr, farnesoid X receptor ; Hmgcr, 3-hydroxy-3-methylglutaryl-CoA reductase ; HIP, hexane-isopropanol ; Hmgcs1, 3-hydroxy-3-methylglutaryl-CoA synthase 1 ; HYD, Hydralazine ; LW, liver weight ; NEFA, non-esterified fatty acid ; PL, phospholipid ; Ppar, peroxisome proliferator-activated receptor ; Pxr, pregnane X receptor ; qPCR, quantitative polymerase chain reaction ; SEM, standard error of the mean ; Shp, small heterodimer partner ; SHRs, spontaneously hypertensive rats ; SP, stroke-prone ; T-Chol, total cholesterol ; TG, triglyceride ; WKY, Wistar Kyoto ; VLDL, very-low-density lipoprotein.

* Corresponding author : Naoki Tanaka
Department of Global Medical Research Promotion,
Shinshu University Graduate School of Medicine
3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan
E-mail : naopi@shinshu-u.ac.jp

# Xuguang Zhang and Jiali Chen contributed equally to this work

I Introduction

Hydralazine (HYD) is a direct-acting vasodilator primarily used in the management of severe hypertension and related conditions, including heart failure

and eclampsia[1)2)]. It exhibits high solubility and low permeability[3)] and is primarily metabolized in the liver[4)]. The vasodilatory effect of HYD is predominantly mediated by its inhibition of inositol 1,4,5-trisphosphate-induced calcium ion release from the sarcoplasmic reticulum, thereby preventing myosin phosphorylation in arterial smooth muscle cells[5)-8)]. In recent years, HYD has been found to exert pharmacological effects beyond vasodilation. Notably, it has been shown to scavenge acrolein, a pro-oxidative aldehyde, and to suppress neuroinflammation in a mouse model of experimental autoimmune encephalomyelitis[9)], as well as in APP/PS1 transgenic mice[10)]. Furthermore, HYD has been reported to stabilize genome integrity and extend life expectancy[11)]. These findings suggest that HYD possesses a broader range of beneficial biological activities than previously recognized.

We previously demonstrated that HYD attenuates hepatic steatofibrosis induced by a high-fat, high-cholesterol diet in spontaneously hypertensive SHR/Izm rats (SHRs)[12)]. However, its effects on lipid metabolism remain unclear. To investigate the impact of HYD on serum and hepatic lipid profiles and elucidate potential underlying mechanisms, SHRs were treated with HYD for either 4 or 10 weeks, after which clinical parameters and hepatic mRNA expression levels were analyzed.

Ⅱ Materials and Methods

A Animals and experimental procedures

Male SHRs were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and maintained in accordance with the guidelines established by the Kinjo Gakuin University Animal Center and the Nagoya University Animal Center. All animal experiments were approved by the Experimental Animal Research Committee of Kinjo Gakuin University (Approval #94 and #154) and the Animal Experiments Committee of Nagoya University Graduate School of Medicine (Approval #24247). The rats were housed in a specific pathogen-free environment under controlled conditions (temperature : 23±2℃, humidity : 55±5%, light/dark cycle : 12/12 h) with unrestricted access to a standard control diet and tap water. Body weight (BW) and systolic blood pressure (BP) were monitored biweekly. BP was measured using the tail-cuff method with a SOFTRON BP98A non-invasive BP monitoring system (Softron Co., Ltd., Tokyo, Japan) in a calm state without anesthesia.

Table 1 Nutrient components of SP diet (weight %)

Feed formulation rate	
Carbohydrate	58.2
Crude protein	20.8
Crude lipid	4.8
Crude fiber	3.2
Crude ash	5
Moisture	8

This study comprised two separate experiments. In Experiment 1, male SHRs (8 weeks of age) were maintained on a stroke-prone (SP) diet and randomly assigned to two groups (n=6 per group). The detailed composition of the SP diet is presented in **Table 1**. One group received free access to drinking water containing HYD (60 mg/L), while the control group was provided with water only. Both groups were treated for 4 weeks.

In Experiment 2, male SHRs of the same age and diet regimen as in Experiment 1 were similarly divided into two groups (n=6 per group), with one group receiving drinking water containing HYD (60 mg/L) and the control group receiving water only, both for 10 weeks[12)]. Based on water consumption, the average HYD intake during the treatment period was estimated at approximately 7.47 mg/kg BW/day in Experiment 1 and 6.66 mg/kg BW/day in Experiment 2.

At the end of the study period, animals from both experiments were allowed unrestricted access to food and water until immediately prior to dissection. All rats were anesthetized with pentobarbital (70 mg/kg), after which blood samples were collected and centrifuged at 3,500×*g* for 10 minutes to prepare serum. The rats were then euthanized by an overdose of pentobarbital. The livers were excised, individually

weighed, sectioned into smaller pieces, and snap-frozen. The corresponding serum and liver samples were stored at −80 ℃ until further use and subsequently shipped to Shinshu University School of Medicine for detailed analyses.

B Biochemical analysis

Serum triglyceride (TG), total cholesterol (T-Chol), glucose, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were analyzed by SRL Inc. (Tokyo, Japan). Total liver lipids were extracted using a modified hexane/isopropanol (HIP) method[13]. Approximately 50 mg of liver tissue was homogenized and sonicated in 19 volumes of 50 mM sodium phosphate buffer (NaPi). A 50 μL aliquot of the lysate was transferred to a new tube, followed by the addition of 900 μL of HIP (3 : 2, vol/vol). The mixture was vigorously vortexed for 1 minute and then centrifuged at 2,500 rpm for 5 minutes at 4 ℃. The upper phase was collected into a new tube and subjected to vacuum centrifugation at 40–50 ℃. After evaporation, the lipid extracts were re-dissolved in 100 μL of HIP containing 1 % (wt/vol) Triton X-100 and evaporated again using a vacuum centrifuge. Subsequently, 100 μL of distilled water was added to the dried lipid extract, incubated at 37 ℃ for 30 minutes, and vortexed. Finally, the lipid extracts were solubilized in 1 % Triton X-100/water, and 10 μL of the resulting solution (equivalent to 0.25 mg of liver tissue) was used for the quantification of TG (#632-50991), T-Chol (#294-65801), non-esterified fatty acids (NEFAs) (#279-75401), and phospholipids (PLs) (#433-36201) using commercially available enzymatic assay kits (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan).

C Quantification of mRNA levels

Total RNA was isolated from frozen liver tissue samples (30 mg), which were homogenized and sonicated in 500 μL of TRI reagent (MOR Molecular Research Center, Inc., Cincinnati, OH). The homogenate was incubated at room temperature for 5 minutes, followed by the addition of 100 μL of chloroform. The mixture was vigorously shaken for 15 seconds, incubated at room temperature for 2–3 minutes, and centrifuged at 12,000×*g* for 15 minutes at 4 ℃. The upper aqueous phase was carefully transferred to a new tube, mixed with an equal volume of 70 % ethanol, and vortexed. The resulting solution was then loaded onto a silica membrane column and centrifuged at 12,000×*g* for 1 minute at room temperature. The membrane was subsequently washed, dried, and RNA was eluted using RNase-free water. The extracted RNA was then reverse-transcribed into complementary DNA using the ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan). Quantification of mRNA levels was performed using real-time quantitative polymerase chain reaction (qPCR) with a SYBR qPCR mix (Toyobo Co., Ltd.) on a Thermo Fisher QuantStudio 3 Real-Time PCR Instrument (Thermo Fisher Scientific, Waltham, MA). The mRNA expression levels were normalized to 18S ribosomal RNA (18S rRNA) and expressed as fold changes relative to the control group. Relative quantification of mRNA levels was determined using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in **Table 2**. Primer design and sequence confirmation were conducted using the WebBLAST and PrimerBLAST tools provided by United States National Library of Medicine.

D Statistical analysis

Statistical analyses were performed using the Mann-Whitney U test in SPSS Statistics version 22 (IBM, Armonk, NY). Data are presented as the mean ± standard error of the mean (SEM). A P-value of <0.05 was considered statistically significant.

Ⅲ Results

A HYD reduces serum TG in SP-fed SHRs

BW changes in SP diet-fed SHRs were comparable between the 4-week HYD group and the control group. However, BW was significantly increased in the 10-week HYD group **(Fig. 1A)**. BP was significantly lower in both the 4-week and 10-week HYD groups compared with their respective controls **(Fig. 1B)**. To assess the effects of HYD on liver weight and serum profiles in SHRs, we examined the liver weight-to-body weight (LW/BW) ratio in both experiments. No significant differences in LW/BW ratio were observed between the HYD-treated and control groups **(Fig. 2A)**. However, 4-week HYD treatment resulted in a sig-

Table 2 Primer pairs used for qPCR analysis

Gene	Accession #	Primer sequence (5'-3')
18S rRNA	NR_046237	F 5'-GACTCAACACGGGAAACCTC-3'
		R 5'-AGACAAATCGCTCCACCAAC-3'
Abcb11	NM_031760	F 5'-AAGTTCACATCTGTAGGGTCCAA-3'
		R 5'-AGCCCAGTTGAGAACGGTTTA-3'
Acaca	NM_022193	F 5'-CGTTCGCCATAACCAAGTAGA-3'
		R 5'-GCTCTTCGAACATATACCTCCAG-3'
Acadl	NM_012819	F 5'-TGGCGAAATATTGGGCATCT-3'
		R 5'-CGAGCATCCACGTAGGCTTT-3'
Acadm	NM_016986	F 5'-TGCTAGTGGAGCACCAAGG-3'
		R 5'-GCCTTCGCAATAGAGGCAAAG-3'
Acox1	NM_017340	F 5'-ACTATATTTGGCCAATTTTGTGGA-3'
		R 5'-TCGAGGATGAGTTCCGTGGC-3'
Apob	NM_019287	F 5'-ACCATGGAACGAGTGATGCC-3'
		R 5'-GGATCGTTCGGACCTCATCT-3'
Atgl	NM_001108509	F 5'-CTCTCGAAGGCTCTCTTCCC-3'
		R 5'-TTGGTTCAGTAGGCCATTCC-3'
Ccl2	NM_031530	F 5'-TAGCATCCACGTGCTGTCTC-3'
		R 5'-GCTTGGTGACAAATACTACAGC-3'
Cd36	NM_031561	F 5'-TGTACTCTCTCCTCGGATGGC-3'
		R 5'-ATGCTTTCTATGTGGCCTGGT-3'
Cyp27a1	NM_178847	F 5'-GGCACCTTTCCTGAGCTGAT-3'
		R 5'-CTTCCCGAAGGGTACCACAC-3'
Cyp7a1	NM_012942	F 5'-ACCTGCCGGTACTAGACAGC-3'
		R 5'-AACCGTCCTCAAGATGGAGAG-3'
Cyp7b1	NM_019138	F 5'-AGCTTGGCTTGCCTGGAAAG-3'
		R 5'-ATCCATATCCTCTTGCACTTCAC-3'
Cyp8b1	NM_031241	F 5'-GTGTCTCCATATGTCCCGGC-3'
		R 5'-TAGCGAAAGCGTACCTCGTG-3'
Dgat1	NM_053437	F 5'-CATGATGGCTCAGGTCCCAC-3'
		R 5'-AGTAGCTCAGGCCCCTACTG-3'
Dgat2	NM_001012345	F 5'-TACCTACCTCGGATCTCGACC-3'
		R 5'-CGGAGTAGGCAGCGATGAG-3'
Fabp1	NM_012556	F 5'-CCCAGTCATGGTCTCCAGTTC-3'
		R 5'-CCCAGTCATGGTCTCCAGTTC-3'
Fasn	NM_017332	F 5'-TTTCCGTGAGTCCATCCTGC-3'
		R 5'-GGTCGATGAGGGCAATCTGG-3'
Fxr	NM_021745.1	F 5'-TGAGCGTCTACAGCGAAAGTG-3'
		R 5'-GGGATGGTGGTCTTCAAATAAG-3'
Hmgcr	NM_013134	F 5'-TGCAGAGCGATCAGTCTTGG-3'
		R 5'-AATCGTCTCGTGCTGTCGAA-3'
Hmgcs1	NM_017268	F 5'-ATCGCGTTTGGTGCCTGA-3'
		R 5'-AAGGGCAACGATTCCCACAT-3'
Il1b	NM_031512	F 5'-TCCAGTCAGGCTTCCTTGTG-3'
		R 5'-GGGCTTGGAAGCAATCCTTA-3'
Ldlr	NM_175762	F 5'-TTGGCCATCTATGAGGACAAAGT-3'
		R 5'-TGCGTGACGTTGTGAAACAG-3'
Lxra	NM_031627.2	F 5'-AGAGCCTACAGAACTTCGTCC-3'
		R 5'-GGAAGAATCCCTTGCAGCCC-3'
Mttp	NM_001107727	F 5'-GAAGAACTCCTGCAAGCCCT-3'
		R 5'-CTTCAACCACTGCCTTGAGC-3'

Gene	Accession #	Primer sequence (5'-3')
Ntcp	NM_017047	F 5'-GGGACATGAACCTCAGCATCG-3'
		R 5'-CGATCCCTATGGTGCAAGGA-3'
Ppara	NM_013196	F 5'-TCGTGGAGTCCTGGAACTGA-3'
		R 5'-CTTCAGTCTTGGCTCGCCTC-3'
Ppard	NM_013141.2	F 5'-CTCCTGCTCACTGACAGATG-3'
		R 5'-TCTCCTCCTGTGGCTGTTC-3'
Pxr	NM_052980.2	F 5'-GACCTCGGCCCATACGAAAC-3'
		R 5'-TGTCCTGGACTGTTAGGTGG-3'
Shp	NM_057133.1	F 5'-ATCCTCTTCAACCCAGATGTGC-3'
		R 5'-GTGGAAGCCATGAGGAGGATT-3'
Tnf	NM_012675	F 5'-GTGATCGGTCCCAACAAGGA-3'
		R 5'-CGCTTGGTGGTTTGCTACG-3'
Vldlr	NM_013155	F 5'-CCTGCAGACCTGACCAGTTT-3'
		R 5'-CCCAGGCACTGATTGACGTT-3'

F, forward sequence ; R, reverse sequence.
Abcb11, ATP binding cassette subfamily b member 11, Acaca, acetyl-coenzyme a carboxylase alpha
Acadl, acyl-coenzyme a dehydrogenase, long-chain, Acadm, acyl-coenzyme a dehydrogenase, medium-chain
Acox1, acyl-coenzyme a oxidase 1, Apob, apolipoprotein b, Atgl, adipose triglyceride lipase
Ccl2, C-C motif chemokine ligand 2, Cd36, cluster of differentiation 36
Cyp27a1, cytochrome p450 family 27 subfamily a member 1, Cyp7a1, cytochrome p450 family 7 subfamily a member 1
Cyp7b1, cytochrome p450 family 7 subfamily b member 1, Cyp8b1, cytochrome p450 family 8 subfamily b member 1
Dgat1, diacylglycerol o-acyltransferase 1, Dgat2, diacylglycerol o-acyltransferase 2
Fabp1, fatty acid binding protein 1, Fasn, fatty acid synthase, Fxr, farnesoid x receptor
Hmgcr, 3-hydroxy-3-methylglutaryl-coenzyme a reductase, Hmgcs1, 3-hydroxy-3-methylglutaryl-coenzyme a synthase 1
Il1b, interleukin 1 beta, Ldlr, low density lipoprotein receptor, Lxra, liver x receptor alpha
Mttp, microsomal triglyceride transfer protein, Ntcp, sodium taurocholate co-transporting polypeptide
Ppara, peroxisome proliferator-activated receptor alpha, Ppard, peroxisome proliferator-activated receptor delta
Pxr, pregnane x receptor, Shp, small heterodimer partner
Tnf, tumor necrosis factor, Vldlr, very low-density lipoprotein receptor

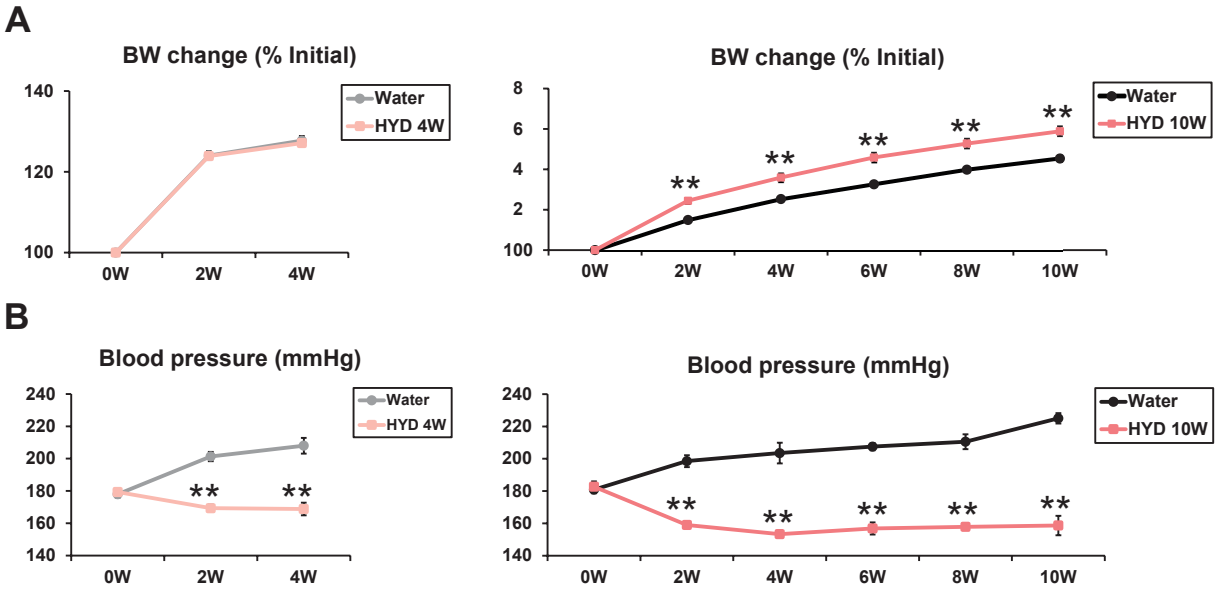

Fig. 1 Body weight changes and blood pressure changes in SHRs treated with HYD for 4 and 10 weeks.
(A) Body weight (BW) changes. (B) Blood pressure changes.
Data are expressed as the mean ± SEM. Statistical analysis was performed using Mann-Whitney U test.
$^{**}P<0.01$ between HYD and control group.

nificant reduction in serum TG levels, a trend that persisted following 10-week HYD treatment **(Fig. 2B)**. Analysis of hepatic lipid content revealed that a 4-week HYD treatment led to an increase in hepatic T-Chol levels, with no significant changes in TG, PL, or NEFA levels. Notably, no significant alterations in hepatic lipid content were observed after 10-week HYD treatment **(Fig. 2C)**.

Serum AST levels were elevated following 10-week HYD treatment ; however, no significant changes were observed in serum ALT levels, a more specific indicator of liver injury than AST, or in hepatic mRNA expression of inflammation-related genes, including tumor necrosis factor-α (*Tnf*), interleukin-1β (*Il1b*), and monocyte chemoattractant protein-1 (*Ccl2*). These findings suggest that HYD treatment did not induce liver injury **(Fig. 2D, E)**.

B HYD enhances TG catabolism in SP-fed SHRs

To investigate the mechanisms underlying HYD-induced reductions in serum TG levels, we analyzed the mRNA expression of genes involved in fatty acid (FA) and TG metabolism. After 4 weeks of HYD treatment, FA uptake-related genes, including CD36 molecule (*Cd36*), low density lipoprotein receptor (*Ldlr*), very-low-density lipoprotein (VLDL) receptor (*Vldlr*) and FA-binding protein 1 (*Fabp1*), remained unchanged. In contrast, genes associated with FA β-oxidation, such as mitochondrial medium-chain and long-chain acyl-CoA dehydrogenase (*Acadm*, *Acadl*) and peroxisomal acyl-CoA oxidase 1 (*Acox1*), showed an upward trend, while those involved in *de novo* lipogenesis, acetyl-CoA carboxylase alpha (*Acaca*) and FA synthase (*Fasn*), were unaffected **(Fig. 3A, B)**.

Similarly, genes related to VLDL secretion, microsomal TG transfer protein (*Mttp*) and apolipoprotein B (*Apob*), showed no significant changes. However, the gene encoding adipose TG lipase (*Atgl*, also known as *Pnpla2*), a key enzyme in TG hydrolysis, was significantly upregulated, whereas TG synthesis-related genes, diacylglycerol O-acyltransferase 1 (*Dgat1*) and 2 (*Dgat2*), remained unaltered **(Fig. 3C)**.

After 10 weeks, these effects were more pronounced, with significant upregulation of *Acadl*, *Acox1*, and *Atgl* **(Fig. 4A, C)**. These findings indicate that HYD promotes hepatic TG catabolism, primarily by enhancing *Atgl* expression and FA degradation.

C HYD enhances bile acid metabolism in SP-fed SHRs

Dysregulation of cholesterol and bile acid (BA) metabolism is implicated in various metabolic disorders in both rodents and humans[14]. To assess the effects of HYD on cholesterol/BA metabolism, we examined the mRNA expression of key enzymes involved in BA synthesis. Cytochrome P450 7A1 (*Cyp7a1*) expression was significantly upregulated following both 4- and 10-week HYD treatments, whereas no significant changes were observed in cytochrome P450 8A1, 27A1 or 7B1 (*Cyp8b1*, *Cyp27a1*, and *Cyp7b1*) **(Fig. 5A)**.

We also analyzed liver BA transporters and cholesterol synthesis enzymes, including sodium taurocholate co-transporting polypeptide (*Ntcp*), ATP-binding cassette subfamily b member 11 (*Abcb11*), 3-hydroxy-3-methylglutaryl-CoA synthase 1 and reductase (*Hmgcs1*, *Hmgcr*). After 4 weeks of HYD treatment, their expression remained unchanged. However, after 10 weeks, *Abcb11*, *Hmgcs1*, and *Hmgcr* were significantly upregulated **(Fig. 5B, C)**.

D Changes in nuclear receptor expression by HYD

Serum TG levels are regulated by various nuclear receptors, including peroxisome proliferator-activated receptors (*Ppars*), pregnane X receptor (*Pxr*), liver X receptor α (*Lxra*), and farnesoid X receptor (*Fxr*), prompting us to examine their expression[15)-20)]. Our analysis showed that *Shp* (small heterodimer partner) expression was significantly increased after 4 weeks of HYD treatment compared to the control group, whereas *Ppara*, *Ppard*, *Fxr*, and *Pxr* expression remained unchanged. Notably, after 10 weeks of HYD treatment, the expression levels of *Ppara*, *Fxr*, *Shp*, and *Pxr* were markedly upregulated **(Fig. 6A, B)**. Given that *Shp*, a well-established FXR target gene, was significantly upregulated in both treatment groups, these findings suggest that FXR activation is another key mechanism underlying HYD's effects.

Ⅳ Discussion

This study demonstrates that the antihypertensive drug HYD significantly reduces serum TG levels after both 4- and 10-week treatments. The common changes

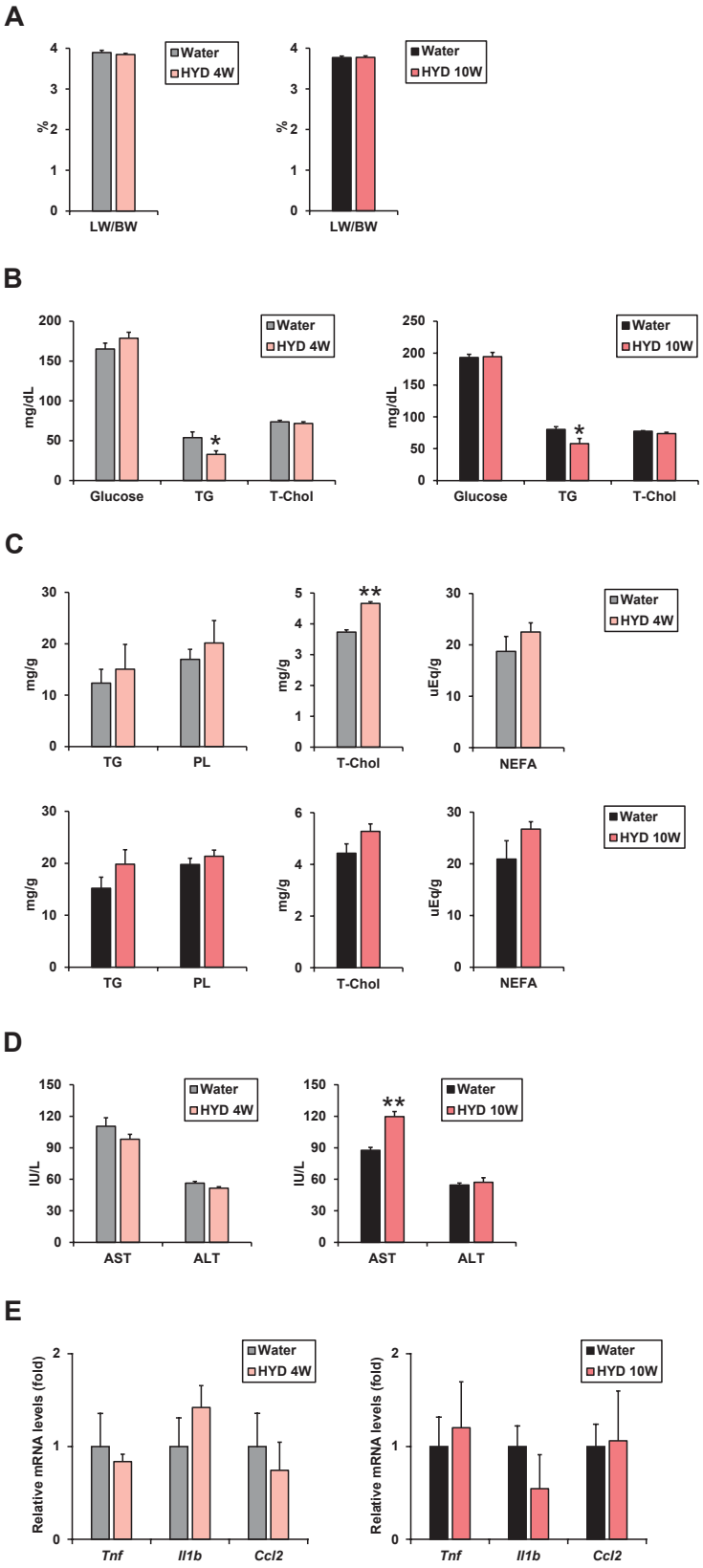

Fig. 2 Anthropometric and biochemical parameters in SHRs treated with HYD for 4 and 10 weeks.
(A) Liver/body weight (LW/BW) changes. (B) Serum glucose, TG and T-Chol levels. (C) Hepatic lipid content levels. (D) Serum AST and ALT levels. (E) Hepatic mRNA levels of genes associated with inflammation.

The mRNA levels were normalized to those of 18S rRNA. Data are expressed as the mean ± SEM. Statistical analysis was performed using Mann-Whitney U test. $^{*}P<0.05$, and $^{**}P<0.01$ between HYD and control group.

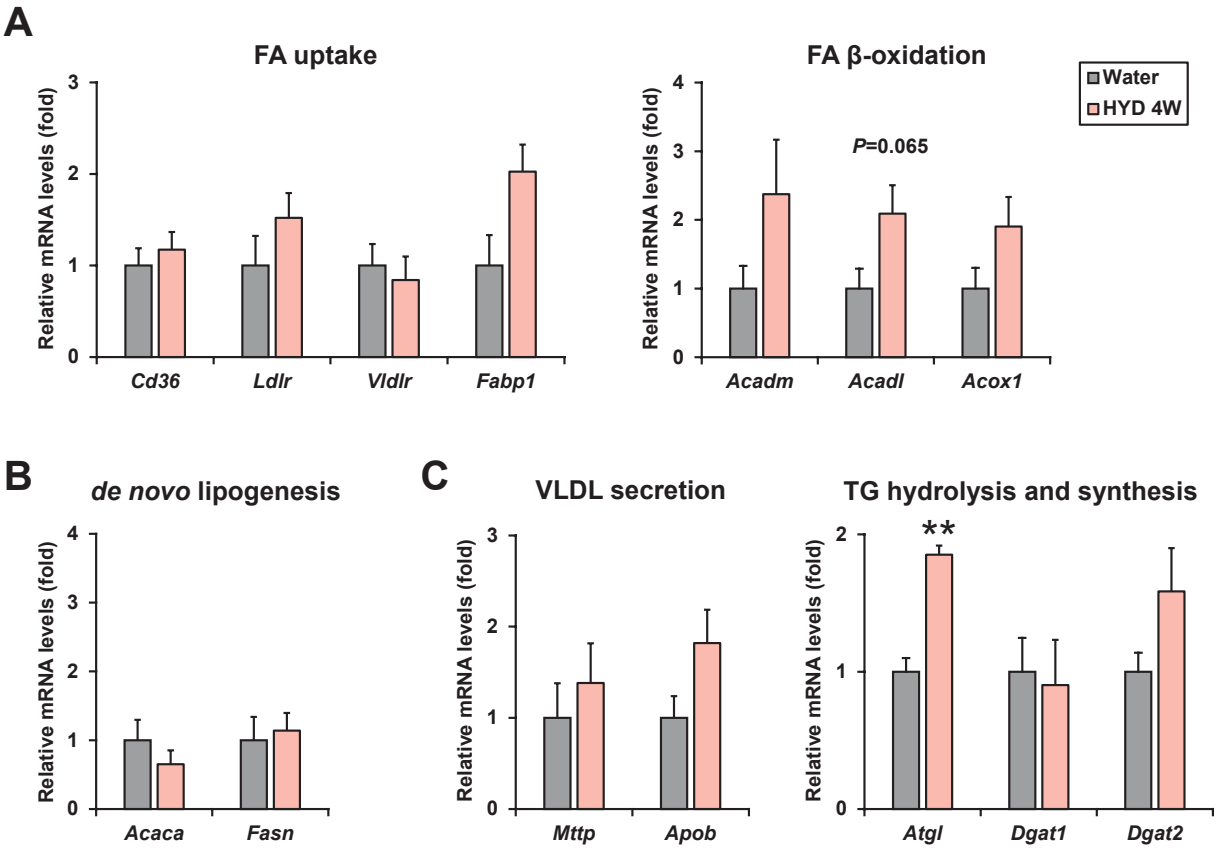

Fig. 3 The mRNA expression levels of genes associated with FA/TG metabolism in SHRs treated with HYD for 4 weeks.

The hepatic mRNA levels of genes associated with FA uptake and FA β-oxidation (A), *de novo* lipogenesis (B), VLDL secretion and TG hydrolysis and synthesis (C) were measured using qPCR method. The mRNA levels were normalized to those of 18S rRNA. Data are expressed as the mean ± SEM. Statistical analysis was performed using Mann-Whitney U test. $^{**}P<0.01$ between HYD and control group.

in gene expression observed in both treatment groups, including upregulation of *Atgl* and *Shp*, a typical target gene of FXR, suggest that increased ATGL expression and FXR activation are key mechanisms through which HYD modulates lipid metabolism.

Since the 4-week and 10-week HYD treatment experiments were conducted at different times, individual differences among the rats were inevitable. Although we maintained identical housing conditions, batch variation and aging effects cannot be completely ruled out. Some genes exhibited differential expression between the two treatment groups, while others showed a gradual increase from 4 to 10 weeks. These findings suggest a progressive effect of HYD, likely mediated by FXR activation and ATGL upregulation.

To better approximate clinical conditions, we used SHRs, which have been reported to exhibit higher serum TG levels and BP than Wistar Kyoto (WKY) rats under the same housing conditions[21]. Given our focus on lipid metabolism, SHRs were considered a suitable model, as their elevated TG levels may enhance the detection of HYD-induced changes. Future studies using additional models, such as high-fat diet-induced obesity and metabolic syndrome models, will be necessary to generalize our findings.

To ensure the clinical relevance of our experimental dose, we evaluated pharmacokinetic parameters. In this study, HYD was administered at approximately 7 mg/kg/day in drinking water. Previous pharmacokinetic studies have reported that oral administration of 7.5 mg/kg of HYD to Wistar rats resulted in a maximum plasma concentration (Cmax) of 213 ng/mL and

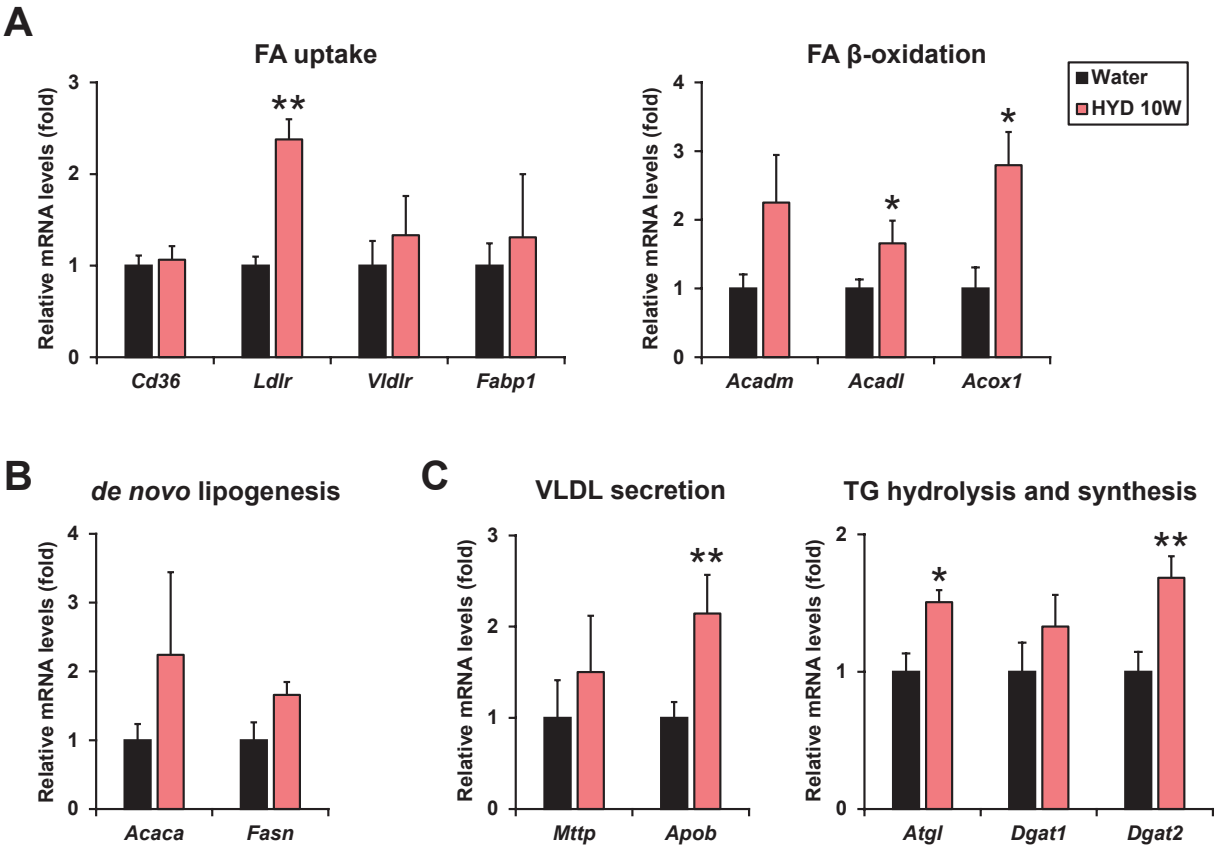

Fig. 4 The mRNA expression levels of genes associated with FA/TG metabolism in SHRs treated with HYD for 10 weeks.

The hepatic mRNA levels of genes associated with FA uptake and FA β-oxidation (A), *de novo* lipogenesis (B), VLDL secretion and TG hydrolysis and synthesis (C) were measured using qPCR method. The mRNA levels were normalized to those of 18S rRNA. Data are expressed as the mean ± SEM. Statistical analysis was performed using Mann-Whitney U test. $^{*}P<0.05$, and $^{**}P<0.01$ between HYD and control group.

an area under the plasma concentration-time curve (AUC) of 553 ng·h/mL[22]. In contrast, in healthy adults receiving an oral dose of 1.6 mg/kg (100 mg in 62.5 kg of BW), the Cmax reached 362 ng/mL and the AUC was 794 ng·h/mL[23]. Given these parameters, the HYD dose used in our study falls within a clinically relevant range and is not extremely high.

HYD increased ATGL mRNA expression in the liver, supporting its role in TG hydrolysis. ATGL catalyzes the breakdown of TG into FAs and glycerol and is regulated via multiple pathways, including the β-adrenergic receptor-cAMP/PKA pathway[24]. While the precise molecular mechanism underlying HYD-induced ATGL upregulation remains unclear, it may represent a compensatory response to enhanced sympathetic nerve tone following lowered BP. Hepatic ATGL plays a crucial role in TG metabolism by channeling hydrolyzed FAs toward β-oxidation and inducing PPARα activation in hepatocytes and mice[25)26)]. Notably, PPARα activation is a well-established mechanism for lowering serum TG levels[15)19)20)]. In this study, HYD treatment increased the mRNA expression of *Ppara* and β-oxidation-related genes, such as *Acadl* and *Acox1*, after 10 weeks of HYD treatment. These findings suggest that ATGL upregulation is a key contributor to the TG-lowering effect of HYD.

Another key finding is that HYD upregulated *Shp*, which is a downstream of FXR. Since SHP is a crucial transcriptional repressor in BA/cholesterol metabolism[13], the upregulation of *Shp* suggested FXR activation by HYD. The finding that *Abcb11*, another FXR target gene, was upregulated after 10 weeks of

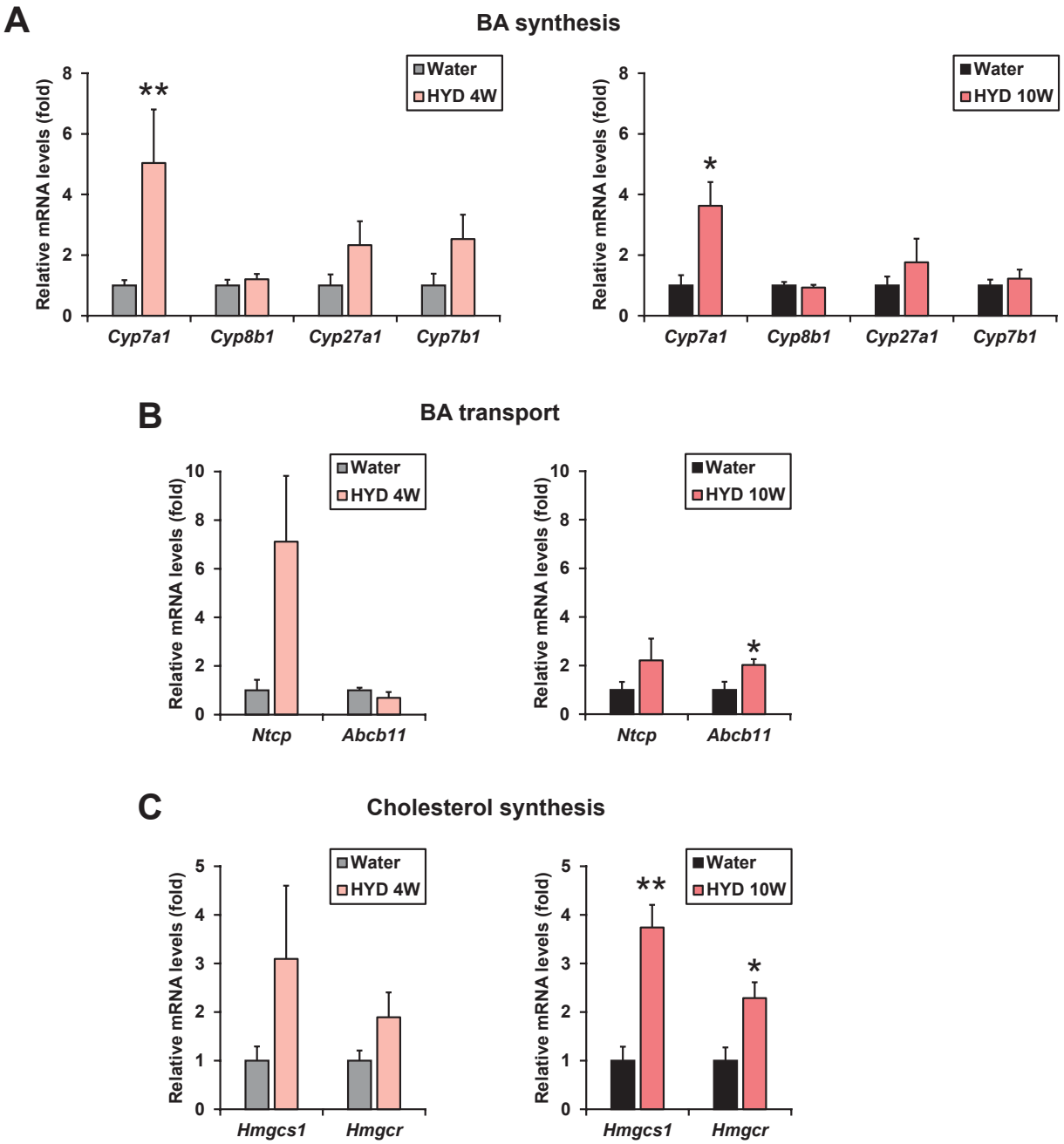

Fig. 5 The mRNA expression levels of genes associated with bile acid and cholesterol metabolism in SHRs treated with HYD for 4 and 10 weeks.

The hepatic mRNA levels of genes associated with BA synthesis (A), BA transport (B) and cholesterol synthesis (C) were measured using qPCR method. The mRNA levels were normalized to those of 18S rRNA. Data are expressed as the mean ± SEM. Statistical analysis was performed using Mann-Whitney U test. $^{*}P<0.05$ and $^{**}P<0.01$ between HYD and control group.

HYD treatment supported this notion.

FXR is a master regulator of not only BA metabolism but also glucose and lipid homeostasis. FXR activation has been shown to induce PPARα expression via SHP[27], thereby promoting hepatic FA uptake and β-oxidation, ultimately leading to reduced serum TG levels[15]. Thus, FXR activation followed by PPARα upregulation represents another potential mechanism underlying the TG-lowering effect of HYD. To our knowledge, this is the first study suggesting that

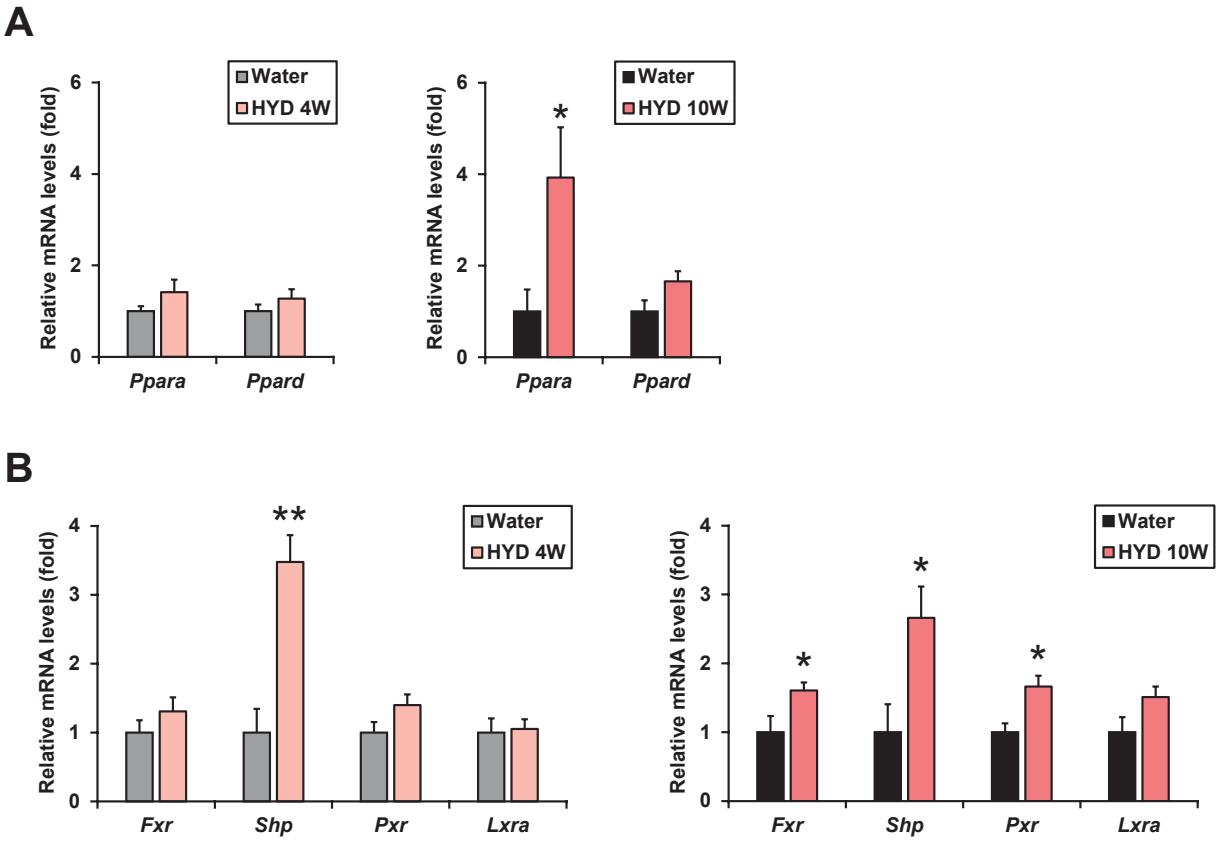

Fig. 6 The mRNA expression levels of genes encoding transcriptional factors in SHRs treated with HYD for 4 or 10 weeks.

The hepatic mRNA levels of PPARs (A) and other metabolic regulators (B) were measured using qPCR method. The mRNA levels were normalized to those of 18S rRNA. Data are expressed as the mean ± SEM. Statistical analysis was performed using Mann-Whitney U test. $^{*}P<0.05$, and $^{**}P<0.01$ between HYD and control group.

HYD possesses FXR-activating properties. Interestingly, other 1,4-dihydropyridine calcium channel blockers, such as cilnidipine and nicardipine, have also been reported to bind and activate FXR, exerting hepatoprotective effects[28].

In the 10-week treatment group, the increased expression of *Hmgcs1* and *Hmgcr* suggests enhanced cholesterol-to-BA conversion via *Cyp7a1* induction. Given that *Cyp7a1* expression is regulated by activation of PXR[29)-31)], its upregulation may further contribute to enhanced BA metabolism in HYD-treated rats.

We propose that the TG-lowering effect of HYD is primarily attributable to FXR activation and ATGL upregulation. We acknowledge that co-administration of HYD with FXR and/or ATGL inhibitors would provide more definitive mechanistic evidence. However, potential pharmacokinetic interactions and off-target effects may complicate interpretation. While these approaches are beyond the scope of this study, they represent important directions for future research. In particular, knockout models would be invaluable in minimizing confounding effects from pharmacokinetic interactions. Further studies incorporating these methodologies will be essential for a more comprehensive understanding of this mechanism.

To the best of our knowledge, this is the first study to demonstrate the TG-lowering effects of HYD beyond its antihypertensive action, along with potential underlying mechanisms. However, several limitations should be acknowledged. First, validation in other animal models of dyslipidemia and metabolic dysfunction is required. Second, this study was conducted exclusively in male SHRs because estrogen and progesterone may influence hepatic lipid metabolism[32].

Future investigations using female SHRs and/or male/female WKY rats will be necessary to strengthen our conclusions. Lastly, it remains unclear whether the TG-lowering effect of HYD is directly mediated by FXR and/or ATGL. Studies employing whole-body or hepatocyte-specific *Fxr*- and *Atgl*-deficient mice are needed to address this critical question[33)34)].

V Conclusions

This study uncovered an unexpected pharmacological effect of HYD in lowering serum TG levels, likely mediated by enhanced ATGL expression and FXR signaling. These findings provide novel insights into the broader metabolic impact of antihypertensive agents on nuclear receptors and lipid metabolism.

Author Contributions

N. Tanaka, and XZ contributed to conception and design of the study. KK, HN and TN conducted animal experiments. XZ, JC, and N. Takeda collected the data and performed statistical analysis. XZ, JC, and N. Tanaka wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

The work was supported by Grants-in-Aid for Scientific Research (B15H04788, C16K00877, C18K10033, and C19K10583).

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(2025. 1. 27 received ; 2025. 2. 18 accepted)