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Full-length article

Tissue-specific effects of atorvastatin on 3-hydroxy-3-methylglutaryl-coenzyme A reductase expression and activity in spontaneously hypertensive rats[1]

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Key words

HMG-CoA reductase; atorvastatin; spontaneously hypertensive rat

[1] Project supported by the National Natural Sciences Foundation of China (No 30470715) and the Research Fund for the Doctoral Program of Higher Education of China (No 20040335118), and in part by the E-Institute of Shanghai Municipal Education Commission of China (No E-04010).

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Received 2008-03-18
Accepted 2008-07-08

doi: 10.1111/j.1745-7254.2008.00855.x

Abstract

Aim: Cardiovascular remodeling is closely associated with cholesterol and is attenuated by statins. The spontaneously hypertensive rat (SHR) has a low serum cholesterol level and evident cardiovascular remodeling. The aims of the present study were to characterize the effects of atorvastatin on tissue cholesterol content and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase expression and activity in four tissues from SHR: liver, heart, aorta and kidney. **Methods:** SHR and normotensive Wistar-Kyoto rats (WKY) were treated daily with atorvastatin (50 mg/kg) for 8 weeks. Cholesterol levels of serum and tissues (liver, heart, aorta and kidney) were determined by commercial enzymatic methods. Western blot analysis and high performance liquid chromatogram (HPLC) were used to assay the expression and activity of enzyme respectively. **Results:** Treatment with atorvastatin decreased cholesterol content and HMG-CoA reductase expression and activity in all four tissues of SHR. However, in WKY, atorvastatin only altered HMG-CoA reductase in liver, where the protein expression was upregulated but the enzyme activity was decreased. **Conclusion:** The present study demonstrates that the effects of atorvastatin on tissue cholesterol content and HMG-CoA reductase are strain- and tissue-specific.

Introduction

Early experimental evidence[1] revealed that the spontaneously hypertensive rat (SHR) has lower serum cholesterol level than the normotensive Wistar-Kyoto rat (WKY). Since the liver is the major source of circulating cholesterol[2–4], we can assume that cholesterol synthesis in the SHR liver is rather less than that in WKY. However, the exact levels of cholesterol content and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis[5,6], in the liver of SHR remains unknown.

In addition, some reports[7–9] have revealed that the development of hypertension in SHR is accompanied by cardiovascular remodeling, including cardiovascular hypertrophy and enhanced growth of cardiac fibroblasts and vascular smooth muscle cells. Although cholesterol is an integral component of all eukaryotic cells and is essential for normal cellular functions[10,11], its content or local cholesterol synthesis, especially the HMG-CoA reductase expression and activity, may be changed, and this may be associated with the alterations of cell volume and tissue components in cardiac remodeling in SHR.

Several recent studies support the notion that statins attenuate adverse cardiovascular and kidney remodeling in SHR[12–14]. However, little was known about the effect of statins on HMG-CoA reductase itself in the cardiovascular system of SHR. Therefore, we investigated the effects of atorvastatin on tissue cholesterol content and HMG-CoA reductase expression and activity in these tissues.

The purposes of the present study were to characterize the effects of atorvastatin on tissue cholesterol content and HMG-CoA reductase expression and activity in liver, heart, aorta and kidney in SHR.

Materials and methods

Animals and tissue preparation Eight-week-old male SHR and WKY rats were obtained from the Experimental Animal Center, Chinese Academy of Sciences (grade I, Certificate No SCXK-Hu-20020010, Shanghai, China). The procedures and protocols of the study followed our institutional guidelines and were conducted in accordance with the Animal Care and Use Committee of Zhejiang University. Animals were housed in a room with constant temperature (23±2 °C) and humidity (55%±5%), exposed to a 12:12 h light:dark cycle, and fed a standard diet and water *ad libitum*. Both SHR and WKY rats were randomly divided into two groups with similar body weights (n=6 in each group) to receive atorvastatin or distilled water as a control. Atorvastatin was provided by Pfizer (Dalian, China). The animal received atorvastatin at a dose of 50 mg/kg per day of body weight by gavage. In rats, this dose produces plasma concentrations that are comparable to those achieved after oral administration of common doses of atorvastatin in humans[15,16]. After 10 weeks of treatment, blood samples and tissues of the liver, heart, aorta and kidney were collected.

Lipid analysis Serum total cholesterol (STC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) concentrations were determined by commercial enzymatic methods (test kits from Shanghai Rongsheng Biotech, Inc., Shanghai, China). The cholesterol contents in the tissues (liver, heart, aorta and kidney) were measured after the lipid was extracted as described by Folch[17]. The amounts of cholesterol were determined using the same kit described above.

Western-blot analysis The total proteins were isolated from liver, heart, aorta and kidney, and protein concentrations were determined by the Lowry method. An aliquot of 10 μg of protein from each sample was separated on 10% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed, and transferred onto nitrocellulose membranes. The membrane was blocked with Tris-buffered saline (TBS, pH 7.6) containing 5% skim milk and 0.05% Tween-20, and then incubated with anti-HMG-CoA reductase rabbit polyclonal antibodies (1:1000 dilution; Upstate, NY, USA) for 12 h at 4 °C. After the membrane was incubated with goat-anti-rabbit IgG conjugated to horseradish peroxidase (1:5000 dilution; MultiSciences, Hangzhou, China) for 1 h at 37 °C, the immune complexes were visualized by the enhanced chemiluminescence (ECL) method. Quantification of the bands was carried out using densitometric analysis software Quantity One (Bio-Rad, CA, USA). To ensure equal protein loading, GAPDH was used as an endogenous control.

HMG-CoA reductase activity determination Tissue (liver, heart, aorta or kidney) endoplasmic reticulum (ER) preparation, HMG-CoA reductase assay, and one-step isolation of mevalonolactone were carried out as previously described[18]. Briefly, the calcium precipitation technique was used to prepare endoplasmic reticulum, and the microsomal protein concentrations were determined by the Lowry method. Incubations for enzyme assay were carried out in a total volume of 150 μL, which contained 0.1–0.8 mg of microsomal protein, 2 mmol/L, NADPH, 30 mmol/L glucose-6-phosphate, 1.75 IU/mL glucose-6-phosphate dehydrogenase, and 0.2 mmol/L HMG-CoA (all from Sigma, St Louis, MO, USA). The reaction time was 45 min, and the samples were incubated for another 30 min at 37 °C to allow complete lactonization of mevalonate. Then mevalonolactone was isolated from the incubation mixture by two successive extractions with 7.5 mL benzene. A 10 mL aliquot of the pooled benzene extracts was transferred to a cryovial and dried at –56 °C in a freeze-drier (Christ Alpha 1-2, PA, USA). Dried mevalonolactone was dissolved in high performance liquid chromatogram (HPLC)-grade water, and the concentration was determined using HPLC[19] (Agilent 1100 HPLC System; Agilent, CA, USA). Chromatographic separations were carried out using an Agilent ZORBA Extend-C18 column (4.6×1.50 cm inner diameter, 3.5 μm; Agilent, CA, USA). Aliquots (100 μL) of each sample were injected into the HPLC system. The mobile phase consisted of HPLC-grade water, and the elution was carried out at 1 mL/min at 37 °C. The detection was carried out at 200 nm. HMG-CoA reductase activity was determined by calculating the concentrations of mevalonolactone and expressed as units per gram of protein (1 unit yields 1 μmol of product per min).

Statistical analysis Results are represented as mean±SD. Statistical analysis was carried out with SPSS 13.0 statistical software. One-way ANOVA followed by *Bonferroni post hoc* test was used to determine significant differences between multiple groups. The significance level was set at P<0.05.

Results

Serum lipid content The SHR control group showed lower STC and LDL-C levels (P<0.05 for each, Table 1) but a similar value of HDL-C compared with the WKY-control group. Long-term treatment with atorvastatin led to a clear reduction of STC and LDL-C in both strains (Table 1).

Table 1. Effects of atorvastatin on serum cholesterol level (mmol/L) in WKY and SHR rats with or without atorvastatin treatment. Data are expressed as mean±SD. $^{c}P<0.01$ *vs* WKY-control; $^{f}P<0.01$ *vs* SHR-control group.

	n	STC	HDL-C	LDL-C
WKY				
Control	6	1.74±0.12	0.91±0.07	0.85±0.05
Atorvastatin	6	1.55±0.11^{c}	0.86±0.12	0.72±0.08^{c}
SHR				
Control	6	1.34±0.15^{c}	0.72±0.09^{c}	0.57±0.07^{c}
Atorvastatin	6	1.08±0.06^{f}	0.66±0.06	0.36±0.06^{f}

Tissue cholesterol profile In liver, cholesterol concentrations were remarkably lower in the SHR-control than in the WKY-control group (Table 2, $P<0.01$). However, in the other three tissues (heart, aorta and kidney), cholesterol levels were comparable between SHR and WKY control groups. Treatment with atorvastatin influenced cholesterol content only in SHR. In the SHR-atorvastatin group, drug intervention significantly reduced the cholesterol level in liver ($P<0.01$), heart ($P<0.05$), aorta ($P<0.05$), and kidney ($P<0.05$) compared with the SHR-control group (Table 2).

Effect of atorvastatin on HMG-CoA reductase expression and activity The baselines of HMG-CoA reductase expression and activity in all four tissues were strikingly higher in the SHR-control than in the WKY-control group (Figure 1). In WKY, atorvastatin treatment changed HMG-CoA reductase only in the liver, where it remarkably increased the protein expression ($P<0.01$ *vs* WKY-control) but reduced the enzyme activity ($P<0.05$ *vs* WKY-control). These indices were not changed in the other three extrahepatic tissues from the WKY-atorvastatin group. However, both the protein expression and the enzyme activity decreased after 10 weeks of atorvastatin administration in these four tissues of SHR rats.

Discussion

Liver cholesterol is mainly derived from serum LDL, which enters hepatocytes by receptor-mediated endocytosis, as well as from local synthesis within the liver[20] and absorption of dietary cholesterol. Meanwhile, HMG-CoA reductase is the rate-limiting enzyme in local cholesterol biosynthesis[5,6], and the liver is the major source of circulating cholesterol[2–4]. In addition, HMG-CoA reductase expression and activity is modulated through negative feedback regulation by the end product, cholesterol[20].

First, we found that the SHR-control group had lower STC and liver cholesterol contents than the WKY-control group, which was consistent with previous reports that the synthesis of liver cholesterol was abnormally decreased in SHR[1]. Moreover, previous reports[21, 22] revealed that the lower activity of mevalonate pyrophosphate decarboxylase, an important enzyme locating downstream of HMG-CoA reductase, was responsible for the reduced cholesterol synthesis in SHR liver. As a result of lower cholesterol synthesis within the SHR liver, hepatic cholesterol content and serum cholesterol were lower, and correspondingly the HMG-CoA reductase expression and activity were upregulated, probably through negative feedback by the end product, cholesterol[20].

Second, our study revealed that atorvastatin, a competitive HMG-CoA reductase inhibitor, caused a large compensatory induction of hepatic HMG-CoA reductase, but a significant depression of enzyme activity in the WKY liver. This result might be related to the fact that atorvastatin or its active metabolites resides longer in hepatocytes than other statins[23,24]. Therefore, the HMG-CoA reductase activity was competitively inhibited not only *in vivo*, but also *ex vivo* when it was determined in our experiment. Thus, the total enzyme activity obtained by the *ex vivo* test was depressed despite the greater amount of protein. In WKY liver, atorvastatin not only competitively inhibits HMG-CoA reductase activity and

Table 2. Tissue cholesterol concentrations (μmol/g tissue) in liver, heart, aorta and kidney. Data represent the mean±SD. $^{c}P<0.01$ *vs* WKY-control group. $^{e}P<0.05$, $^{f}P<0.01$ *vs* SHR-control group.

	n	Liver	Heart	Aorta	Kidney
WKY					
Control	6	10.32±0.62	5.27±0.30	4.03±0.48	21.17±2.73
Atorvastatin	6	9.51±0.72	5.01±0.56	3.73±0.65	21.88±2.76
SHR					
Control	6	7.55±0.63^{c}	5.29±0.41	4.09±0.56	21.77±2.73
Atorvastatin	6	5.54±1.31^{f}	4.60±0.63^{e}	3.20±0.67^{e}	18.11±2.42^{e}

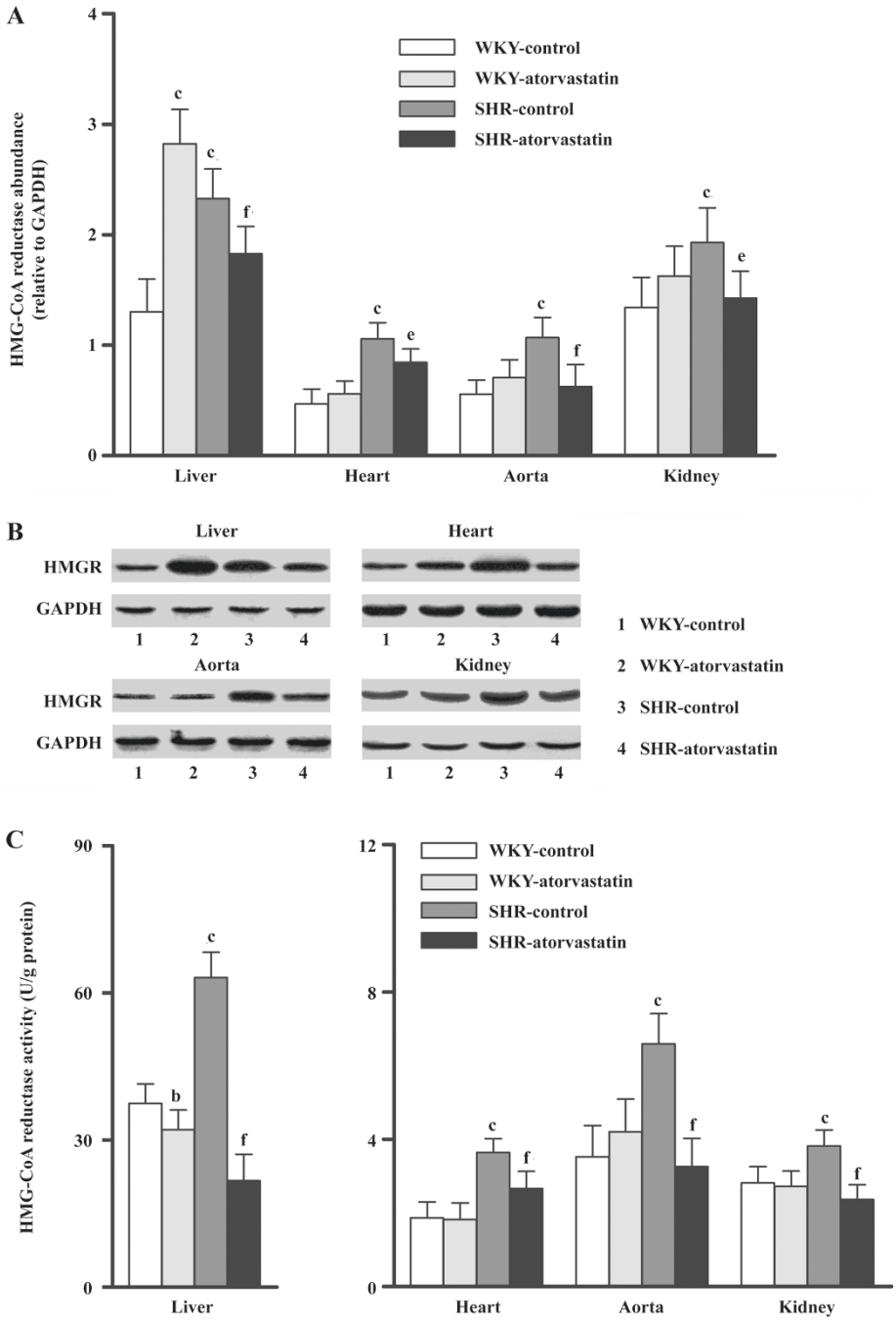

Figure 1. Effects of atorvastatin on HMG-CoA reductase expression and activity in liver, heart, aorta and kidney of WKY and SHR rats. (A) Group data for HMG-CoA reductase levels by Western blot. The densitometric average was normalized to the values obtained from the analysis of GAPDH as an internal control. The results represent the mean±SD of 6 separate experiments. $^{c}P<0.01$ *vs* WKY-control group. $^{e}P<0.05$, $^{f}P<0.01$ *vs* SHR-control group. (B) Representative blots from one experiment showing HMG-CoA reductase expression. (C) Alteration of HMG-CoA reductase activity in WKY and SHR rats with or without atorvastatin treatment. Values are expressed as mean±SD of 6 independent experiments carried out in duplicate. $^{b}P<0.05$, $^{c}P<0.01$ *vs* WKY-control group. $^{f}P<0.01$ *vs* SHR-control group.

cholesterol synthesis[25], but also increases the number of hepatic LDL receptors to enhance the uptake and catabolism of circulating LDL[26–28]. Mainly through these two mechanisms, circulating cholesterol decreased in the WKY-atorvastatin group. Subsequently, HMG-CoA reductase expression was enhanced, also mediated through

end-product feedback[20]. On the other hand, because of the compensatory upregulation of HMG-CoA reductase and the enhanced hepatic uptake of LDL, the content of liver cholesterol in the WKY-atorvastatin group did not drop sharply. In addition, several reports[29,30] revealed that statins increased intestinal cholesterol absorption in humans and animals. This is probably one of the reasons why cholesterol content in the liver of WKY was not affected by atorvastatin.

Third, both the activity and the expression of HMG-CoA reductase were reduced by atorvastatin treatment in SHR liver. The level of HMG-CoA reductase is controlled by many interactions, including synthesis and degradation of the protein, which can result in changes of over 200-fold in intracellular levels of the enzyme[20]. Therefore, we suggest following mechanisms: (1) SHR might have defects in end product feedback mechanism of hepatic HMG-CoA reductase; and (2) SHR might have an enzyme conformation more vulnerable to atorvastatin, with a faster degradation rate, since several studies[25,31–32] have shown that statins modify the conformation of HMG-CoA reductase and hence its degradation. Therefore, we can infer that in the livers of the SHR-atorvastatin group, degradation of HMG-CoA reductase may exceed the enzyme compensatory synthesis induced by atorvastatin, and the amount of HMG-CoA reductase decreased. Due to the decrease of HMG-CoA reductase and the prolonged residence of atorvastatin in the hepatocytes[23,24], enzyme activity declined even more, and the liver cholesterol content dropped markedly.

Fourth, data from our study revealed that the SHR-control group had lower serum LDL levels and comparable cholesterol content of extrahepatic tissues (heart, aorta and kidney). We also found that the HMG-CoA reductase expression and activity of these tissues were upregulated. Extrahepatic tissues also have local cholesterol synthesis[33], but under physiological conditions, serum LDL probably supplies their cholesterol needs[34,35]. These results suggest that the supply of LDL to extrahepatic tissues may be insufficient due to low circulating LDL, and subsequently, local cholesterol synthesis increased, probably *via* upregulation of HMG-CoA reductase expression and activity.

Finally, HMG-CoA reductase expression and activity did not change in extrahepatic WKY tissues after atorvastatin treatment. These tissues may take up enough LDL from the circulation, despite the decreased serum cholesterol level after atorvastatin treatment. Since the HMG-CoA reductase in these tissues was relatively inactive, atorvastatin had little effect on the enzyme. However, we found that HMG-CoA reductase expression and activity of extrahepatic SHR tissues decreased after atorvastatin treatment. This was consistent with the results from the livers of rats in the SHR-atorvastatin group, indicating the inhibition of local cholesterol synthesis after atorvastatin treatment. Because the local cholesterol synthesis was depressed by atorvastatin, and the circulating LDL supply was insufficient, the cholesterol content in extrahepatic tissues of the SHR-atorvastatin group declined.

In conclusion, this study demonstrated that: (1) both the liver and the extrahepatic tissues of SHR showed abnormal cholesterol biosynthesis where the key enzyme, HMG-CoA reductase, was in an abnormally upregulated state; and (2) the effects of atorvastatin on tissue cholesterol content and HMG-CoA reductase are strain- and tissue-specific.

Limitations Although this study proposed several possible reasons why the effects of statin on HMG-CoA reductase were too different between SHR and WKY, the exact mechanisms should be explored in further study. Also, to understand more precisely the effects of atorvastatin on tissue cholesterol content, morphological examination of these tissues are needed to provide more solutions. In addition, the intestinal source of cholesterol was not investigated in our study and further study will be necessary to further clarify the relevance of the findings of the study.

Acknowledgements

We express our sincere gratitude to Prof Iain C BRUCE (University of Hong Kong) for checking the English. We also thank Jian-zhong SHENTU from the Department of Clinical Pharmacology, The First Affiliated Hospital, College of Medicine, Zhejiang University for his excellent technical support on HPLC.

Author contribution

Shen-jiang HU, Guo-ping CHEN, and Lei YAO designed research; Guo-ping CHEN, Lei YAO, and Xian LU performed research; Liang LI contributed new analytical tools and reagents; Guo-ping CHEN, and Lei YAO analyzed data; Guo-ping CHEN wrote the paper.

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