Alteration of Enzyme Expressions in Mevalonate Pathway – Possible Role for Cardiovascular Remodeling in Spontaneously Hypertensive Rats –

Jie Han, PhD; Dong-Mei Jiang, MD; Chang-Qing Du, PhD; Shen-Jiang Hu, MD, PhD

Background: The mevalonate pathway is an important metabolic pathway that plays a key role in multiple cellular processes. The aim of this study was to define whether the enzyme expression in mevalonate pathway changes during cardiovascular remodelling in spontaneously hypertensive rats (SHR).

Methods and Results: Hearts and thoracic aortas were removed for the study of cardiovascular remodeling in SHR and Wistar-Kyoto rats (WKY). The protein expression of the enzymes in hearts, aortas and livers was analyzed by western blot. The histological measurements showed that the mass and the size of cardiomyocytes, the media thickness and the media cross-sectional area (MCSA) of the thoracic aorta were all increased in SHR since 3 weeks of age. In the heart, there was overexpression of some enzymes, including 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), farnesyl diphosphate synthase (FDPS), and geranylgeranyltransferase type I (GGTase-I), and downregulation of squalene synthetase (SQS) in SHR since 3 weeks of age. In the aorta, besides similar expressions of HMGR, SQS, FDPS and GGTase-I as in the heart, there was upregulation of farnesyltransferase α at 16 and 25 weeks of age and of farnesyltransferase β in 25-weeks-old SHR. Western blot demonstrated overexpression of HMGR and downregulation of SQS in SHR livers at all ages tested.

Conclusions: The cardiovascular remodeling of SHR preceded the development of hypertension, and altered expression of several key enzymes in the mevalonate pathway may play a potential pathophysiological role in cardiovascular remodeling. (Circ J 2011; 75: 1409–1417)

Key Words: Cardiovascular remodeling; Gene expression; Mevalonate pathway; Small GTP-binding proteins; Spontaneously hypertensive rats

The mevalonate pathway is an important metabolic pathway that plays a key role in multiple cellular processes by synthesizing sterol isoprenoids, such as cholesterol, and non-sterol isoprenoids, including farnesylphosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). These non-sterol isoprenoid intermediates of the mevalonate biosynthetic pathway play important roles in the post-translational modification of small GTP-binding proteins (GTPases), such as Ras and Rho. Protein prenyltransferases catalyze the addition of isoprenoids, farnesyl or geranyl-geranyl to the C-terminal of GTPases, facilitating their activation.
process of HT in 3-week-old (pre-HT phase), 16-week-old (labile-HT phase), and 25-week-old (established-HT phase) SHR, including 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), phospho-3-hydroxy-3-methylglutaryl-coenzyme A reductase (P-HMGR), farnesyl diphosphate synthase (FDPS), squalene synthase (SQS), farnesyltransferase α (FNTA), farnesyltransferase β (FNTB) and geranylgeranyltransferase type I (GGTase-I) (Figure 1).

**Methods**

**Animal Preparation**
The study was approved by the Institutional Council for Animal Research of Zhejiang University and was performed according to the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**Blood Pressure (BP) and Lipid Analysis**
Male SHR and WKY rats were purchased from Shanghai Laboratory Animal Center (Chinese Academy of Sciences), and housed with free access to water and food. At the ages of 3, 16 and 25 weeks, systolic BP was measured by the tail-cuff method as previously described. After recording BP, the heart, aorta and liver were dissected from the anesthetized animal. A 3-ml blood sample was collected and centrifuged immediately. Serum total cholesterol (STC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) concentrations were determined by commercial enzymatic methods.

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**Table 1.** Left Ventricle and Thoracic Aorta Remodeling Markers and Blood Pressure at 3, 16 and 25 Weeks of Age in WKY and SHR

<table>
<thead>
<tr>
<th>Week</th>
<th>SBP (mmHg)</th>
<th>LVW (g)</th>
<th>LVW/BW (mg/g)</th>
<th>MT (µm)</th>
<th>MCSA (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (n=6)</td>
<td>108±6</td>
<td>0.21±0.01</td>
<td>3.50±0.11</td>
<td>73.35±4.41</td>
<td>0.26±0.02</td>
</tr>
<tr>
<td>16 (n=6)</td>
<td>126±10</td>
<td>0.75±0.03</td>
<td>1.97±0.23</td>
<td>77.98±3.71</td>
<td>0.40±0.03</td>
</tr>
<tr>
<td>25 (n=6)</td>
<td>129±8</td>
<td>0.82±0.05</td>
<td>1.84±0.12</td>
<td>82.33±4.02</td>
<td>0.46±0.02</td>
</tr>
<tr>
<td>SHR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (n=6)</td>
<td>111±5</td>
<td>0.23±0.02</td>
<td>3.96±0.12*</td>
<td>91.05±3.79*</td>
<td>0.36±0.02*</td>
</tr>
<tr>
<td>16 (n=6)</td>
<td>200±9*</td>
<td>1.05±0.05*</td>
<td>3.05±0.17*</td>
<td>115.38±3.67*</td>
<td>0.55±0.03*</td>
</tr>
<tr>
<td>25 (n=6)</td>
<td>215±8*</td>
<td>1.13±0.04*</td>
<td>2.85±0.14*</td>
<td>127.18±4.61*</td>
<td>0.60±0.04*</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM.

*P<0.01 vs. age-matched WKY; *P<0.05 vs. age-matched WKY.

WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; SBP, systolic blood pressure; LVW, left ventricular weight; LVW/BW, ratio of LVW to body weight; MT, media thickness; MCSA, media cross-sectional area.
Mevalonate Pathway in SHR

(test kits from Shanghai Rongsheng Biotech, Inc, Shanghai, China).

**Histological Analysis**

The weight of the left ventricle (LVW) was measured, and the ratio of LVW to body weight (LVW/BW) was calculated as an index of cardiac hypertrophy. Sections of the LV free wall and septum were fixed in 10% neutral formalin, embedded in paraffin, and stained with hematoxylin and eosin. A metal needle was attached to the aortic arch and blood from the arterial tree was washed out with warmed (37°C) saline. The needle was then connected to a perfusion system containing 10% formalin containing phosphate-buffered saline (PBS) solution (mmol/L: NaCl 120; KCl 2.7; PBS 10; pH 7.4 at 25°C). After 30 min perfusion, a 1-cm fragment of the aorta was removed at the level of the diaphragm and stored for 24 h in 10% formalin. The aortic ring was dehydrated in graded ethanol solutions and embedded in paraffin. Three 5-μm-thick sections of each rat’s aorta were cut and stained with hematoxylin and eosin for determination of media thickness (MT) and media cross-sectional area (MCSA), and the mean of these measurements was used as the representative value for each animal. MCSA (mm²) was defined as the area between the internal and external elastic lamina. Morphometric analysis was performed with Image-Pro Plus 6.0.

**Western-Blot Analysis**

Total proteins were isolated from the heart, aorta and liver, and protein concentrations were determined by the Lowry method. Equal amounts of protein were loaded onto polyacrylamide gels, separated, transferred to Immobilon membranes (Millipore), and blocked with 3% bovine serum albumin at room temperature for 1 h. Blots were then probed with antibodies to HMGR (2 μg/ml; 07-457, Millipore, USA), P-HMGR (1 μg/ml; 09-356, Millipore, USA), FDPS (2.5 μg/ml; ab38854, Abcam, UK), SQS (1 μg/ml; sc-49758, Santa Cruz, Japan),

**Table 2. Serum Concentrations of TC, HDL-C and LDL-C in WKY and SHR**

<table>
<thead>
<tr>
<th>Week</th>
<th>STC (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>LDL-C (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (n=6)</td>
<td>2.71±0.06</td>
<td>1.37±0.05</td>
<td>1.10±0.02</td>
</tr>
<tr>
<td>16 (n=6)</td>
<td>2.27±0.14</td>
<td>1.25±0.10</td>
<td>0.85±0.07</td>
</tr>
<tr>
<td>25 (n=6)</td>
<td>2.61±0.21</td>
<td>1.92±0.17</td>
<td>1.16±0.14</td>
</tr>
<tr>
<td>SHR</td>
<td></td>
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</tr>
<tr>
<td>3 (n=6)</td>
<td>2.30±0.17</td>
<td>1.42±0.08</td>
<td>0.65±0.11*</td>
</tr>
<tr>
<td>16 (n=6)</td>
<td>1.34±0.08*</td>
<td>0.80±0.07*</td>
<td>0.34±0.06*</td>
</tr>
<tr>
<td>25 (n=6)</td>
<td>1.51±0.09*</td>
<td>0.92±0.02*</td>
<td>0.60±0.04*</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM.
*P<0.01 vs. age-matched WKY; *P<0.05 vs. age-matched WKY.

TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Other abbreviations see in Table 1.

![Figure 2.](image)

**Figure 2.** Representative images of myocardium and thoracic aorta stained with hematoxylin–eosin. Enlarged myocytes and thickened media of the thoracic aorta are observed in SHR at 3, 16 and 25 weeks (W) of age. Bar = 50 μm in the images of cardiac muscle and thoracic aorta, at ×400 and ×100 magnification, respectively. SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.
FNTA (1 μg/ml; ab77984, Abcam, UK), FNTB (1 μg/ml; ab74206, Abcam, UK) or GGTase-I (1 μg/ml; sc-18996, Santa Cruz, Japan) at 4°C overnight. Blots were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody: goat-anti-rabbit IgG (0.67 μg/ml; ab6721, Abcam, UK) or rabbit-anti-goat IgG (1 μg/ml; A5420, Sigma, USA) for 1 h at room temperature. The immune complexes were visualized by the enhanced chemiluminescence method. Quantification of the bands was carried out using densitometric analysis software (Quantity One, Bio-Rad, CA, USA).

**Figure 3.** HMGR, P-HMGR, SQS, FDPS, GGTase-I, FNTA and FNTB expressions in heart quantified by western blot analysis. (A) Representative blots are shown; GAPDH was used as a loading control. (B–E) The densitometric average was normalized to the values obtained from the analysis of GAPDH as an internal control. Data are expressed as mean±SEM, n=4. *P<0.01 vs. age-matched WKY; #P<0.05 vs. age-matched WKY. FDPS, farnesyl diphosphate synthase; FNTA, farnesyltransferase α; FNTB, farnesyltransferase β; GGTase-I, geranylgeranyltransferase type I; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; SHR, spontaneously hypertensive rats; SQS, squalene synthase; WKY, Wistar-Kyoto rats.
Figure 4. HMGR, P-HMGR, SQS, FDPS, GGTase-I, FNTA and FNTB expressions in aorta quantified by western blot analysis. (A) Representative blots are shown; GAPDH was used as a loading control. (B–G) The densitometric average was normalized to the values obtained from the analysis of GAPDH as an internal control. Data are expressed as mean±SEM, n=4. *P<0.01 vs. age-matched WKY; #P<0.05 vs. age-matched WKY. FDPS, farnesyl diphosphate synthase; FNTA, farnesyltransferase α; FNTB, farnesyltransferase β; GGTase-I, geranylgeranyltransferase type I; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; SHR, spontaneously hypertensive rats; SQS, squalene synthase; WKY, Wistar-Kyoto rats.
To ensure equal protein loading, GAPDH was used as an endogenous control (0.4 μg/ml; ab9485, Abcam, UK).

Statistical Analysis
Statistical analysis results are presented as mean±SEM. All analyses were performed with SPSS (Ver. 13.0; Chicago, IL, USA). One-way ANOVA followed by Bonferroni post hoc test was used to determine significant differences between multiple groups. Statistical significance was set at P<0.05.

Results
BP and Serum Lipid Content
The systolic BP developed gradually over weeks in SHR after 3 weeks of age old, but only slightly in WKY (Table 1). At 3 weeks of age, BP was not significantly different between SHR and WKY, whereas in both the 16- and 25-week-old age groups, BP was significantly higher in SHR than in WKY.

SHR had a lower LDL-C level (P<0.01) but similar values for STC and HDL-C compared with WKY at 3 weeks of age (Table 2). In agreement with previous studies, at the adult stage of 16 and 25 weeks’ old, STC, LDL-C and HDL-C were lower in SHR than in WKY.

Cardiac and Aortic Structural Remodeling
Representative sections of heart and thoracic aorta are shown in Figure 2 and the morphologic data are summarized in Table 1. The results revealed that the heart mass of the SHR was significantly increased as compared with that of WKY at 3 weeks, as evidenced by a LVW/BW ratio of 3.96±0.12 in SHR vs. 3.50±0.11 in WKY (P<0.05). Correspondingly, the cardiomyocyte size was dramatically increased in SHR compared to those in WKY (Figure 2). The heart mass and cardiomyocyte size in SHR remained significantly increased up to 25 weeks of age. MT and MCSA of the thoracic aorta were both increased in SHR when compared with age-matched WKY.
HMGR Expression Levels
HMGR is the rate-limiting enzyme for the mevalonate pathway and is regulated by a negative feedback mechanism mediated by sterols and non-sterol metabolites derived from mevalonate, the product of the reaction catalyzed by reductase (Figure 1). The expression of HMGR in all 3 tissue types was significantly higher in prehypertensive and adult hypertensive SHR (Figures 3–5). In contrast, the expression of P-HMGR, inactivated enzyme reversibly by phosphorylation, was similar in the homogenates from SHR and WKY.

SQS Expression Levels
SQS represents a putative branch point in the isoprenoid biosynthetic pathway, capable of diverting carbon flow specifically to the biosynthesis of sterols, and, hence, is considered to be a potential regulatory point for sterol metabolism14 (Figure 1). Thus, anti-SQS antibody was used to compare differences in cholesterol synthesis signaling pathways from SHR vs. WKY. SQS expression was significantly decreased in heart, aorta, and liver from SHR when compared with that from WKY at all ages (Figures 3–5). These findings could explain the lower serum cholesterol level in SHR.

FDPS, GGTase-I, FNTA and FNTB Expression Levels
To assess differences in non-sterol isoprenoid signaling pathways between SHR and WKY, blots were probed with antibodies to FDPS, GGTase-I, FNTA and FNTB in the western blot analysis. FDPS is a key enzyme in the isoprenoic biosynthetic pathway and directly catalyzes the formation of FPP and geranyl pyrophosphate (GPP; 2 molecules of GPP condense to GGPP),15 which are required for isoprenylation of small GTPases (Figure 1). Farnesyltransferase (FTase: FNTA and FNTB) and GGTase-I are 2 enzymes that carry out the process of prenylation in the cell.16 FTase catalyzes the transfer of a farnesy1 moiety from FPP to a cysteine at the 4th position from the C-terminus of several proteins.17 The β subunit (FNTB) is responsible for peptide-binding. ImmunobLOTS demonstrated greater levels of FDPS and GGTase-I in the heart and aorta from SHR than in those from WKY at all ages tested (Figures 3, 4). A similar pattern of FNTA and FNTB protein expression in different tissues was detected. Aorta exhibited a significant increase in FNTA expression at 16 and 25 weeks, but not in 3-week-old prehypertensive SHR, and FNTB increased only in 25-week-old SHR (Figure 4). FNTA and FNTB protein expressions were not significantly different between SHR and WKY in heart and liver at all ages tested (Figures 3, 5).

Discussion
HT results in a series of pathological changes such as cardiac hypertrophy and aortic remodeling. In the present study, we made some observations related not only to BP, serum lipid content, cardiac hypertrophy and aortic remodeling, but also to the expression of several key enzymes in the mevalonate pathway in SHR. We found a complicated system of enzyme expression between the sterols branch signaling pathway and the non-sterol isoprenoids branch signaling pathway in different tissues (heart, aorta and liver), rat strains and ages.

Our finding that HT did not develop in SHR during the first 3 weeks of life is in agreement with previous reports that the term ‘prehypertensive stage’ should probably be reserved for animals less than 4 weeks’ old.11 From the histological measurements, hypertrophy of the aortic wall and LV were found to precede the development of HT, which is in agreement with most of the previous studies.18–20 These findings suggest that the early changes in cardiovascular structure in neonatal SHR seem not to be in parallel with those in BP, raising the possibility that nonpressor factors may be responsible for this excessive growth.

Targeting small G-proteins and their effectors appears to exert multiple beneficial effects that prevent or cure cardiovascular remodeling under HT conditions.21–23 The Rho family of small GTP-binding proteins, consisting of the Rho, Rac, and Cdc42 subfamilies, regulates many aspects of cytoskeletal function.24 It has been shown recently that activation of the Rho/Rho kinase signal transduction pathway is a principal mechanism of cardiovascular reconstruction in arterial HT.6,7 Rho participates in the angiotensin II or stretch-mediated activation of extracellular receptor kinase (ERK) proteins in the hypertrophy of hypertensive cardiac myocytes and vascular hyperplasia.25,26 Besides, Rac1 and Cdc42 are implicated in hypertrophy effects that are probably mediated through stimulating p21-activated kinases (PAKs), which regulate mitogen-activated protein kinase (MAPK) cascades (c-Jun N-terminal kinase [JNK] and p38-MAPK).27

On the other hand, Ras small G-protein isoforms activate a kinase cascade involving Raf, MAPK and ERK phosphoributes, which in turn induces cell proliferation and growth regulation that lead to both cardiovascular remodeling and chronic contraction of vessels.5,6,8,9 Like all members of the Ras superfamily, the membrane localization and function of Ras and Rho depend on the process of isoprenylation.18 Ras translocation from the cytoplasm to the plasma membrane is dependent on farnesylation by FTase, whereas translocation of Rho is dependent on geranylgeranylation by GGTase-I.29 We found increased expression of HMGR and FDPS and decreased SQS in the heart and aorta from SHR. Other studies have indicated that the enzymes of the non-sterol pathways generally have higher affinities than those of the sterol pathway for mevalonate-derived substrates.30 These findings suggest that when the key enzyme SQS for the biosynthesis of sterols is downregulated in the heart and aorta of SHR, mevalonate-derived substrates may be more preferentially shunted into the high-affinity non-sterol pathways. Therefore, pathologic cardiovascular remodeling and increased vascular contractility in SHR may be caused by toxic accumulation of some intermediates, including FPP and GGPP, which leads to excessive small GTPase activation.

Interestingly, we also found greater levels of GGTase-I in the aortas and hearts from SHR than from WKY at all ages tested. Thus, the relative increased GGPP level may be catalyzed for more post-translational modification of signaling proteins such as Rho. As for the key enzyme FTase for farnesylation, FNTA and FNTB expressions exhibited a significant increase only in the aorta of adult SHR, but the relative increase in the level of intracellular mevalonate-derived substrates could be much higher than in WKY due to higher expression of HMGR or FDPS also in the heart, which could lead to a larger pathway flux and production of isoprenoids for modification of Ras. These findings are supported by the finding that Rho and Ras expressions and activities are enhanced in the heart and vessels of SHR.31–33 Hence, we suggest that the reverse effect of statins (HMGR inhibitors),34–36 and bisphosphonates (FDPS inhibitors) for cardiovascular remodeling31 are mediated by their ability to block the synthesis of important isoprenoid intermediates. Although statins primarily act to block HMGR, which is the rate-limiting enzyme in the de novo synthesis of cholesterol.
in the liver, many of the cholesterol-independent effects of statins may be mediated by inhibition of isoprenoid synthesis, which leads to inhibition of the intracellular signaling molecules Rho, Rac, Cdc42 and Ras. In particular, inhibition of Rho and one of its downstream targets, Rho kinase, may be the predominant mechanism contributing to the pleiotropic effects of statins.

It has been proposed that the development of the cardiovascular structure before adulthood has a major influence on BP in later life. In particular, structural changes in the vasculature form the core of the hypothesis that arterial hypertrophy and subsequent narrowing of the vascular lumen may be responsible for the development of HT in SHR. Moreover, in the HT phase, the agonists reported to activate small GTPases in SHR and several other hypertensive rat models include thrombin, platelet-derived growth factor, and angiotensin II. Activity is also regulated by cell–cell adhesion molecules, mechanical stretch and shear stress.

Our data showed that the key enzymes of the isoprenoid biosynthetic pathway remained overexpressed in the heart and aorta of adult SHR, which implies that the agonists activating small GTPases may simultaneously induce changes in the expression of key enzymes to produce more isoprenoids for modification of small GTPases. Hence, excessive activation of small GTPases, cardiovascular remodeling and HT may create a vicious cycle.

The liver is the major source of circulating cholesterol, and we found that SHR showed a lower STC level at all age stages tested, which was consistent with previous reports that the synthesis of liver cholesterol is abnormally decreased in SHR. We also demonstrated for the first time that the lower SQS in the SHR liver, which catalyzes the first committed step of the specific hepatic cholesterol biosynthesis at the final branch point of the cholesterol biosynthetic pathway, was probably responsible for the reduced cholesterol synthesis. As a result of lower cholesterol synthesis within the SHR liver, the STC level was lower and consequently HMGR expression was upregulated in the liver, at least in part, probably through negative feedback by the endproduct of cholesterol.

In conclusion, the results of the present study suggest that alteration of the expression of key enzymes in the mevalonate pathway in the heart and aorta is implicated in the process of cardiovascular remodeling in SHR, probably through activation of small GTPases. In addition, increased small GTPase activity also occurs in the brain and kidneys of SHR and other hypertensive rat models, including stroke-prone SHR and deoxycorticosterone acetate-salt rats, and this may also play a role in regulating the pathophysiology of HT. Increases in both membrane-bound RhoA and Rho kinase activity in the brainstem of these hypertensive animals have also been detected, suggesting a possible interplay between central RhoA/Rho kinase and the sympathetic nervous system in the development of HT. Additionally, enhanced activity of Rho A has been detected in the pregglomerular microvascular smooth muscle cells of SHR kidneys, which may contribute to the elevated vascular resistance via alterations in vascular reactivity observed in the established stages of HT. Therefore, the changes in the mevalonate pathway in the brain and kidney of SHR and other hypertensive models should be explored in further studies.

Although there are many essential differences between experimental and clinical studies, our study indicates that interfering with the upregulation of key enzymes and biosynthesis of isoprenoids in the mevalonate pathway may open the field for novel therapeutic indications and give many possible therapeutic targets beyond arterial HT in the future.

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References


