Received: 2 April 2021 | Accepted: 29 June 2021

DOI: 10.1002/prp2.838

ORIGINAL ARTICLE

Alteration of the soluble guanylate cyclase system in coronary arteries of high cholesterol diet-fed rabbits

Masashi Tawa[1] | Katsuya Nakano[1] | Yuka Yamashita[1] | Qiang He[1] | Takayoshi Masuoka[1] | Tomio Okamura[2] | Takaharu Ishibashi[1]

[1]Department of Pharmacology, Kanazawa Medical University, Kahoku, Ishikawa, Japan

[2]Shiga University of Medical Science, Otsu, Shiga, Japan

Correspondence
Masashi Tawa, Department of Pathological and Molecular Pharmacology, Faculty of Pharmacy, Osaka Medical and Pharmaceutical University, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan.
Email: masashi.tawa@ompu.ac.jp

Present address
Masashi Tawa, Department of Pathological and Molecular Pharmacology, Faculty of Pharmacy, Osaka Medical and Pharmaceutical University, Takatsuki, Osaka, Japan

Funding information
This study was partly supported by the President's Discretionary Fund from the Shiga University of Medical Science [No. 1515503BF and No. 1515503CJ to M.T.], the former affiliation of M.T.; the Grants-in-Aid for Scientific Research Program from the Japan Society for the Promotion of Science [No. 17K15579 to M.T.]; and the Grant for Promoted Research from Kanazawa Medical University [No. S2020-1 to M.T.].

Abstract

This study aimed to investigate how atherosclerosis affects the soluble guanylate cyclase (sGC) system in coronary arteries. Rabbits were fed a normal diet for 12 weeks (N group) or a diet containing high cholesterol (1%) for 4 weeks (S-HC group) and 12 weeks (L-HC group). Cholesterol deposition in the intima of coronary arteries was observed in the S-HC group, but the formation of an atherosclerotic plaque was not observed. In contrast, a major plaque developed in the L-HC group. The relaxant response of isolated coronary arteries to sodium nitroprusside (SNP, nitric oxide donor) was not different between the N and S-HC groups, whereas the response in the L-HC group was markedly attenuated. The relaxation induced by BAY 60-2770 (sGC activator) tended to be augmented in the S-HC group, but it was significantly impaired in the L-HC group compared to that in the N group. sGC β1 immunostaining was equally detected in the medial layer of the arteries among the N, S-HC, and L-HC groups. In addition, a strong staining was observed in the plaque region of the L-HC group. cGMP levels in the arteries stimulated with SNP were identical in the N and S-HC groups and slightly lower in the L-HC group than the other groups. BAY 60-2770-stimulated cGMP formation tended to be increased in the S-HC and L-HC groups. These findings suggest that the sGC system was not normal in atherosclerotic coronary arteries. The redox state of sGC and the distribution pattern are likely to change with the progression of atherosclerosis.

KEYWORDS
atherosclerosis, coronary artery, nitric oxide, redox state, soluble guanylate cyclase

Abbreviations: CAD, coronary artery disease; DAB, diaminobenzidine; E_{max}, maximal response; L-HC, long-term high cholesterol diet; MGV, mean gray value; MRP, multidrug resistance-associated protein; N, normal diet; NO, nitric oxide; pEC_{50}, negative logarithm of the concentration that produces one-half E_{max}; PKG, protein kinase G; sGC, soluble guanylate cyclase; S-HC, short-term high cholesterol diet; SNP, sodium nitroprusside.

1 | INTRODUCTION

Soluble guanylate cyclase (sGC) functions as an acceptor of the highly bioactive signaling molecule nitric oxide (NO) to generate cGMP with vasodilating, anti-aggregating, and anti-remodeling activities.[9,20] The first step for NO to activate sGC is to bind to ferrous iron in the heme of sGC. Importantly, NO is less sensitive to the heme of sGC in the ferric state; moreover, it cannot activate the enzyme without a heme moiety. Thus, the conversion of sGC from a reduced form (which contains a heme moiety with ferrous iron) to an oxidized form (which contains a heme moiety with ferric iron) or a heme-free form (which does not contain a heme group) leads to a decrease in NO bioavailability.[26] Accumulating evidence has shown that this sGC redox equilibrium is disrupted in diseased blood vessels.[5,12,21,32,34] This is one of the reasons why drugs targeting the NO/sGC/cGMP pathway are practically used or tested in clinical settings.[26]

Coronary artery disease (CAD) is the most common type of heart disease and is characterized by the narrowing of coronary arteries usually due to the buildup of fatty deposits (atherosclerosis).[18] A decrease in endothelial NO bioavailability in atherosclerotic lesions is an important feature of CAD.[39] In this regard, sGC has attracted attention as a determinant of NO bioavailability in coronary arteries over recent decades. For example, the loss of heme in sGC in coronary arteries has been shown to render the enzyme unable to respond suitably to NO.[25,42] In addition, it has been demonstrated that the balance in the sGC redox state is shifted from a reduced state to an oxidized/heme-free state in in vitro models of CAD where isolated coronary arteries are exposed to hypoxia[33] and oxidative stress conditions.[35] However, there is still no evidence on whether the sGC system is truly impaired in atherosclerotic coronary arteries. This study addressed this issue using a rabbit model of high cholesterol diet-induced coronary atherosclerosis.

2 | MATERIALS AND METHODS

2.1 | Animals

The Animal Care and Use Committee of the Shiga University of Medical Science and the Animal Research Committee of Kanazawa Medical University provided ethical approval for the laboratory animals used in this study (Permit No. 2016-8-4 and 2017-101). A total of 27 male Japanese white rabbits (6-week-old) were obtained from Japan SLC, Inc.. The rabbits were housed in an environmentally controlled room with a 12-h light–dark cycle at the university animal facilities and were allowed free access to food and water. The experimental groups were as follows: (1) normal diet (N) group: fed a standard diet (LRC4, Oriental Yeast Co., Ltd.) for 12 weeks from the age of 6 weeks; (2) short-term high cholesterol diet (S-HC) group: fed a standard diet for 8 weeks from the age of 6 weeks and then a 1% cholesterol diet (LRC4 containing 1% cholesterol, Oriental Yeast Co., Ltd.) for 4 weeks; and (3) long-term high cholesterol diet (L-HC) group: fed a 1% cholesterol diet for 12 weeks from the age of 6 weeks.

At the end of the 12-week feeding period, after 14 h of fasting, the rabbits were deeply anesthetized with sodium pentobarbital (Kyoritsu Seiyaku Co.; 40 mg/kg, i.v.), and blood samples were collected from the inferior vena cava. The rabbits were then injected with heparin (Mitsubishi Tanabe Pharma Co.; 500 U/kg, i.v.), and were sacrificed by bleeding from the abdominal aorta. The heart was excised, and the left coronary artery was isolated.

2.2 | Plasma lipid profiles

Blood samples were centrifuged at 1500*g* for 10 min at 4°C, and the supernatant fraction was used for analysis. Plasma total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides were measured by SRL Inc..

2.3 | Hematoxylin–eosin staining

Redundant portions of coronary arteries were fixed with 10% formaldehyde (Nacalai Tesque) and embedded in paraffin. The samples were cut into 3-μm sections and stained with hematoxylin–eosin according to standard procedures.

2.4 | Vascular reactivity

Coronary arteries were helically cut into strips, with caution, to preserve the endothelium. The strips were then fixed vertically between hooks in a muscle bath (10-ml capacity) containing a modified Ringer-Locke solution with the following composition (in mM): NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25.0, and glucose 5.6. The solution was bubbled with a gas mixture of 95% O_2 and 5% CO_2 (pH 7.4), and the temperature was maintained at 37 ± 0.3°C. The hook anchoring the upper end of the strip was connected to the lever of a force-displacement transducer (Nihon Kohden Kogyo Co.). The resting tension of coronary arteries was adjusted to 0.8 g according to the method of Corr et al.[6] Before starting the experiments, all of the preparations were allowed to equilibrate in the bathing medium for 60–90 min, during which the solution was replaced every 10–15 min.

Isometric contractions and relaxations were displayed on an ink-writing oscillograph. The strips were contracted with endothelin-1 (Peptide Institute; 1–3 nM); there was no significant difference in level of precontraction in all concentration–response experiments (data not shown). The reason why we used endothelin-1 as a preconstrictor is that this peptide produces stable and sustained vasocontraction. After the contraction reached a plateau, concentration–response curves for sodium nitroprusside (SNP; Nacalai Tesque), an NO donor, and BAY 60-2770 (kindly provided by Dr. Johannes-Peter Stasch of the Institute of Cardiovascular Research, Pharma Research Centre, Bayer AG), an sGC activator, were obtained by adding the drug

directly to the bathing media in cumulative concentrations. The former and latter function as a reduced sGC stimulant and an oxidized/heme-free sGC stimulant, respectively;[26] drug characteristics of BAY 60-2770 have been previously presented in detail.[14] At the end of each experiment, papaverine (Dainippon-Sumitomo Pharma Co., 100 μM) was added to induce the maximal relaxation, which was taken as 100% for the relaxation induced by sGC agonists.

2.5 | Immunostaining

The paraffin-embedded sections of coronary arteries were stained with antibodies specific for the sGC β1-subunit (item No. 160897; Cayman Chemical Co.; 1:100) and the protein kinase G (PKG) (item No. ADI-KAP-PK005; Enzo Life Sciences Inc.; 1:500). Briefly, after deparaffinization, the sections were incubated with proteinase K (DAKO) for 15 min at room temperature (for sGC β1) or were autoclaved for 60 min at room temperature (for PKG) to expose the antigenic sites and were washed with Tris-buffered saline. After blocking the endogenous peroxidase activity with 3% hydrogen peroxide for 30 min at room temperature and thorough washes, nonspecific binding on sections was blocked for 10 min with Protein Block Serum-Free (DAKO). The sections were then incubated with primary antibodies for 60 min at room temperature, washed with Tris-buffered saline, and exposed to an EnVision + System-HRP Labeled Polymer anti-rabbit antibody (item No. K4003, DAKO; no dilution) for 30 min at room temperature. After washing with Tris-buffered saline, immunoreactions were visualized using the chromogen diaminobenzidine (DAB; DAKO). Nuclei were counterstained with hematoxylin. As an aside, a negative control was processed in a similar manner in the absence of the primary antibody and showed the absence of nonspecific reactions with the secondary antibody (Figure S1).

To quantify DAB staining intensity, digital images (1600× magnification) were captured from at least four different areas per artery using a NanoZoomer C9600 (Hamamatsu Photonics K.K.) and then deconvolved using ImageJ (NIH) using the color deconvolution plugin. For each monochrome image of the DAB component (brown), the mean gray values (MGVs) in the medial layer and in the plaque region were measured. The MGV can take on any value between 0 (black) and 255 (white). Therefore, the staining intensity was obtained by the difference (255 minus MGV). The values obtained were averaged per artery and then per group.

2.6 | cGMP level measurement

Helically cut strips of coronary arteries were incubated alone or with the addition of SNP (10 nM) or BAY 60-2770 (1 nM) in a modified Ringer-Locke solution (pH 7.4; saturated with a 95%O_2-5%CO_2 gas mixture, 37°C) for approximately 30 min and were then immediately plunged into liquid nitrogen. The tissues were homogenized in 5% trichloroacetic acid at 0°C using a glass homogenizer. After centrifugation at 1500*g* for 10 min, water-saturated ether was added to the collected supernatant, and the residual ether was removed from the aqueous layer by heating the sample to 70°C for 5 min. An aliquot of extract was then used for cGMP determination following the acetylation protocol, using a commercially available enzyme immunoassay kit (item No. 581021; Cayman Chemical Co.). The cGMP level in the tissue was normalized to the protein content measured in the same extract using the Bradford assay.

2.7 | Statistics

All values are expressed as the mean ± SEM. Univariate comparisons were performed using one-way or two-way ANOVA and the Bonferroni post hoc test. Concentration–response curves were analyzed using nonlinear curve fitting by using GraphPad Prism 7.0 software (GraphPad Software Inc.). The maximal response (E_{max}) and the negative logarithm of the concentration that produces one-half E_{max} (pEC_{50}) were obtained. Comparisons between the concentration–response curves were performed using two-way repeated measures ANOVA and the Bonferroni post hoc test. Differences were considered significant at a $p < .05$.

3 | RESULTS

3.1 | Effects of high cholesterol diet feeding on lipid profiles

Plasma lipid profiles are summarized in Table 1. The total cholesterol levels in the S-HC and L-HC groups were markedly higher than those in the N group; the levels in the L-HC group were significantly higher than those in the S-HC group. Likewise, the LDL cholesterol levels were also higher and statistically significant in the S-HC and L-HC

TABLE 1 Plasma lipid profiles

	N ($n = 8$)	S-HC ($n = 8$)	L-HC ($n = 8$)
Total cholesterol (mg/dl)	32.3 ± 2.6	1858.1 ± 109.5**	2495.6 ± 145.3**,††
LDL cholesterol (mg/dl)	6.9 ± 1.2	931.3 ± 73.0**	1120.0 ± 57.8**
HDL cholesterol (mg/dl)	25.3 ± 1.9	20.6 ± 3.5	14.3 ± 3.0*
Triglycerides (mg/dl)	27.4 ± 1.8	45.1 ± 7.4	138.9 ± 38.4**,†

Data are the mean ± SEM values of eight experiments. *$p < .05$ and **$p < .01$, compared to the N group; †$p < .05$ and ††$p < .01$, compared to the S-HC group. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc test. Abbreviations: L-HC, long-term high cholesterol diet; N, normal diet; S-HC, short-term high cholesterol diet.

TABLE 2 E_{max} and pEC_{50} values

	N ($n = 8$)	S-HC ($n = 8$)	L-HC ($n = 8$)
SNP			
E_{max}	99.2 ± 0.3	98.9 ± 0.6	93.4 ± 2.4*,†
pEC_{50}	7.99 ± 0.16	7.81 ± 0.10	7.20 ± 0.17**,†
BAY 60-2770			
E_{max}	95.9 ± 2.0	96.3 ± 1.3	91.8 ± 2.0
pEC_{50}	8.95 ± 0.14	9.37 ± 0.19	8.25 ± 0.15*,††

Data are the mean ± SEM values of eight experiments. $^{*}p < .05$ and $^{**}p < .01$, compared to the N group; $^{\dagger}p < .05$ and $^{\dagger\dagger}p < .01$, compared to the S-HC group. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc test. Abbreviations: E_{max}, maximal response; L-HC, long-term high cholesterol diet; N, normal diet; pEC_{50}, negative logarithm of the concentration that produces one-half E_{max}; S-HC, short-term high cholesterol diet; SNP, sodium nitroprusside.

groups, while there was no difference between the S-HC and L-HC groups. There was no significant difference in the HDL cholesterol levels between the N and S-HC groups, whereas the levels were significantly lower in the L-HC group than in the N group. In addition, the triglycerides levels in the L-HC group were significantly higher than those in the N and S-HC groups.

3.2 | Effects of high cholesterol diet feeding on the morphology of coronary arteries

Coronary arteries in the N group showed a normal vascular morphology (Figure 1, left images). Cholesterol deposition in the endothelium was seen in coronary arteries in the S-HC group, but the formation of an atherosclerotic plaque was not observed (Figure 1, middle images). On the other hand, a large plaque with luminal stenosis was present in coronary arteries in the L-HC group (Figure 1, right images).

3.3 | Effects of high cholesterol diet feeding on the relaxation of coronary arteries via sGC activation

SNP induced a concentration-dependent relaxation of coronary arteries in the N group. The concentration–response curve for SNP was not different in the S-HC group, but it was shifted to the right in the L-HC

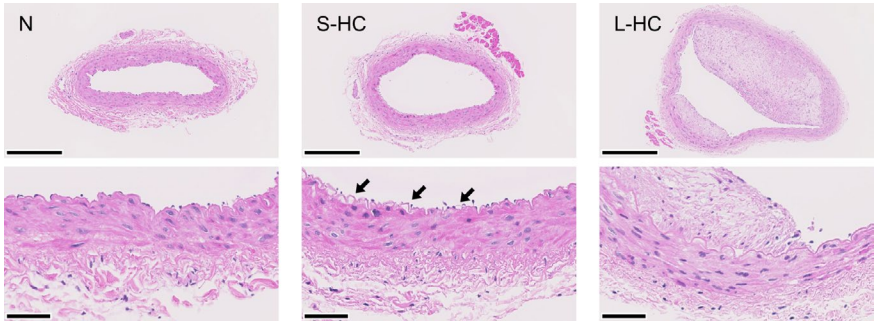

FIGURE 1 Typical images of hematoxylin–eosin-stained rabbit coronary arteries in the N (left images), S-HC (middle images), and L-HC (right images) groups. Scale bars, 250 μm (upper images, 400× magnification) and 50 μm (lower images, 1600× magnification). The arrows indicate cholesterol deposition. Abbreviations: L-HC, long-term high cholesterol diet; N, normal diet; S-HC, short-term high cholesterol diet

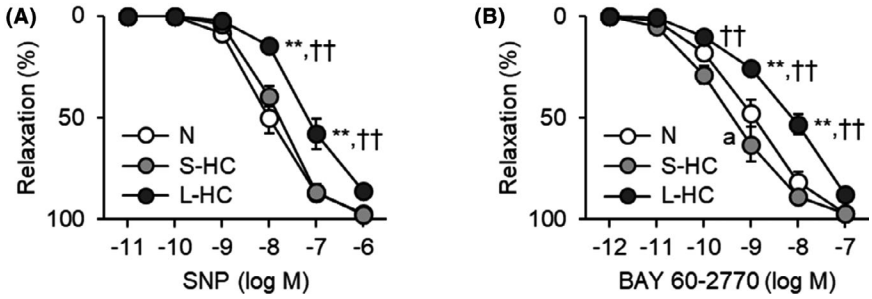

FIGURE 2 (A and B) Vascular responses of rabbit coronary arteries to SNP and BAY 60-2770 in the N, S-HC, and L-HC groups. Each point and bar represent the mean ± SEM values of eight experiments. $^{a}p = .07$, $^{*}p < .05$, and $^{**}p < .01$, compared to the N group; $^{\dagger\dagger}p < .01$, compared to the S-HC group. Statistical analysis was performed using two-way repeated measures ANOVA with Bonferroni post hoc test. Abbreviations: L-HC, long-term high cholesterol diet; N, normal diet; S-HC, short-term high cholesterol diet; SNP, sodium nitroprusside

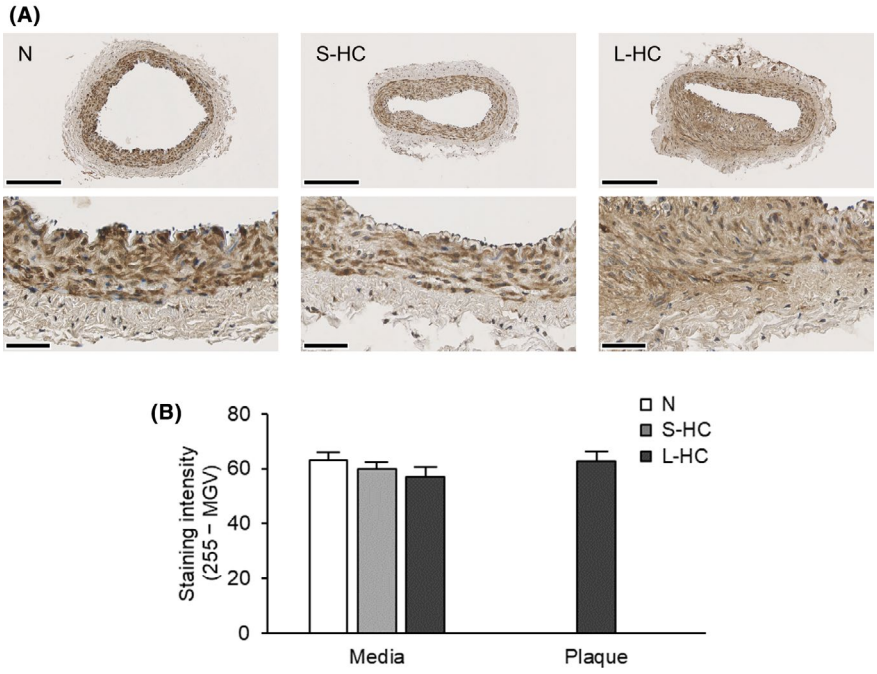

FIGURE 3 (A) Typical images of sGC β1-immunostained rabbit coronary arteries in the N (left images), S-HC (middle images), and L-HC (right images) groups. Scale bars, 250 μm (upper images, 400× magnification) and 50 μm (lower images, 1600× magnification). (B) sGC β1 immunostaining intensity values. Each column and bar represent the mean ± SEM values of eight experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc test. Abbreviations: L-HC, long-term high cholesterol diet; MGV, mean gray value; N, normal diet; S-HC, short-term high cholesterol diet

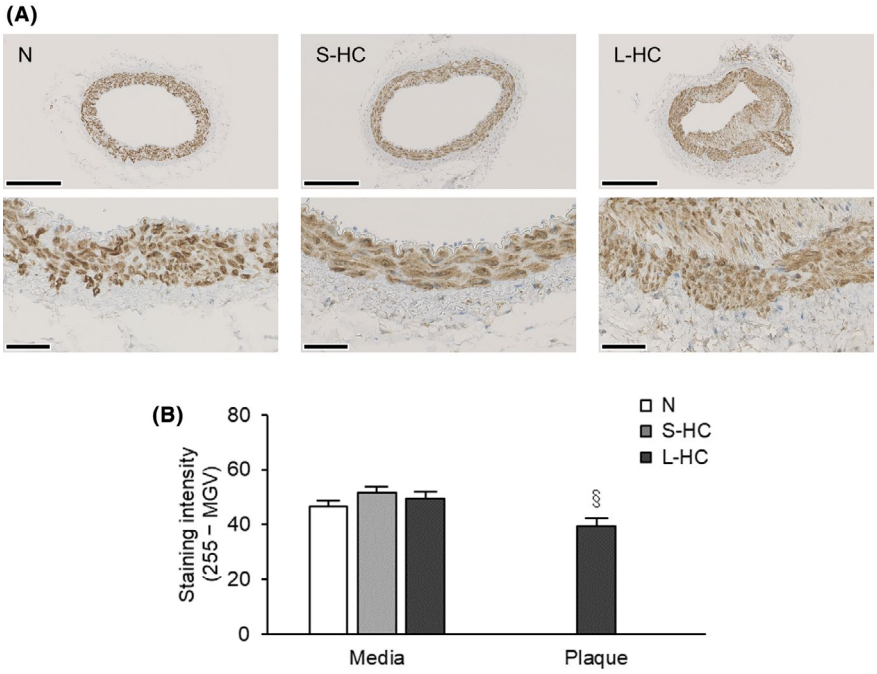

FIGURE 4 (A) Typical images of PKG-immunostained rabbit coronary arteries in the N (left images), S-HC (middle images), and L-HC (right images) groups. Scale bars, 250 μm (upper images, 400× magnification) and 50 μm (lower images, 1600× magnification). (B) PKG immunostaining intensity values. Each column and bar represent the mean ± SEM values of eight experiments. $^{§}p < .05$, compared to the media in the L-HC group. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc test. Abbreviations: L-HC, long-term high cholesterol diet; MGV, mean gray value; N, normal diet; S-HC, short-term high cholesterol diet

group; the relaxant responses at 10 and 100 nM were significantly attenuated in the L-HC group (Figure 2A). In addition, the E_{max} and pEC_{50} values were also significantly lower in the L-HC group than in the N group (Table 2).

The relaxant response of coronary arteries to BAY 60-2770 tended to be potentiated in the S-HC group compared to the N group; $p = .07$ for the response at 1 nM. On the other hand, BAY 60-2770-induced relaxation was significantly attenuated in the L-HC group (Figure 2B). There were no significant differences in the E_{max} and pEC_{50} values between the N and S-HC groups, but these values in the L-HC group were significantly or relatively low (Table 2).

3.4 | Effects of high cholesterol diet feeding on sGC expression in coronary arteries

sGC β1 was detected in the medial layer of coronary arteries in the N group (Figure 3A, left images; Figure 3B). Similar to the N group, a strong sGC β1 staining was observed in the medial layer in the S-HC group (Figure 3A, middle images; Figure 3B). Likewise, the tunica media in the L-HC group showed adequate immunohistochemical signals for sGC β1, as evidenced by the staining intensity values. However, in the L-HC group, an abundant immunoreactivity was also found in the plaque region (Figure 3A, right images; Figure 3B).

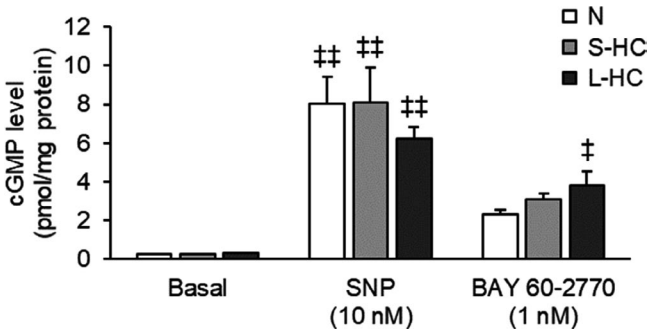

FIGURE 5 cGMP levels in rabbit coronary arteries incubated alone (left side), with SNP (middle), and BAY 60-2770 (right side) in the N, S-HC, and L-HC groups. Each column and bar represent the mean ± SEM values of four experiments with duplicate analyses (total of eight measurements). $^{‡}p < .05$ and $^{‡‡}p < .01$, compared to the respective basal condition. Statistical analysis was performed using two-way ANOVA with Bonferroni post hoc test. Abbreviations: L-HC, long-term high cholesterol diet; N, normal diet; S-HC, short-term high cholesterol diet; SNP, sodium nitroprusside

3.5 | Effects of high cholesterol diet feeding on PKG expression in coronary arteries

The medial layer of coronary arteries in the N group showed a strong positive staining for PKG (Figure 4A, left images; Figure 4B). Similar immunohistochemical expression was observed even in the S-HC and L-HC groups (Figure 4A, middle and right images, respectively; Figure 4B). PKG-immunoreactive signals were also detected in the plaque region in the L-HC groups; however, the staining intensity values were significantly lower than the values in the medial layer (Figure 4A, right images; Figure 4B).

3.6 | Effects of high cholesterol diet feeding on cGMP production via sGC activation in coronary arteries

Basal cGMP levels in coronary arteries were not different among the N, S-HC, and L-HC groups (Figure 5, left side). cGMP formation stimulated with SNP was identical between the N and S-HC groups and was slightly, though not significantly, lower in the L-HC group (Figure 5, middle). There was also no significant difference in the BAY 60-2770-induced cGMP formation among the three groups, although an increasing tendency in the S-HC and L-HC groups was observed (Figure 5, right side).

4 | DISCUSSION

An important objective of this study was to examine the impact of high cholesterol diet feeding for different periods. In short-term feeding, blood cholesterol levels were increased and its deposition in the intima in coronary arteries was observed, suggesting an early phase of coronary atherosclerosis. In these coronary arteries, the vasorelaxant and cGMP-generating actions of SNP were maintained. A similar result was found in a study by Turk et al.,[37] who showed the normal reactivity of coronary arteries to SNP in a porcine model of early stage atherosclerotic vascular disease. Interestingly, both of these actions in response to BAY 60-2770 tended to be augmented. Therefore, it is considered that the level of sGC in the oxidized form or the level of heme-free form was increased; however, since sGC expression in the smooth muscle was unchanged, it remains unclear whether this was due to a shift in the redox state. Supporting our data, Stasch et al. reported that the responsiveness to an sGC activator is potentiated in aortas obtained from high-fat diet-fed ApoE$^{-/-}$ mice and in saphenous arteries obtained from Watanabe heritable hyperlipidemic rabbits, although, unfortunately, no data were obtained on how advanced the atherosclerosis was.[32] Nevertheless, it is suggested that the sGC system in coronary arteries becomes non-normal at an early phase of atherosclerosis.

In long-term feeding, a marked atherosclerotic plaque developed in coronary arteries, wherein the vasorelaxant action of SNP was impaired. In line with this result, several research groups have shown that relaxant responses to NO donors are attenuated in human coronary arteries with atherosclerotic stenosis.[3,11] Although SNP produced some amount of cGMP in coronary arteries with atherosclerotic plaque, it is difficult to determine in which cells/tissues the cGMP was generated because sGC was expressed not only in the medial layer but also in the plaque region. In other words, some of the increased cGMP level may have been generated in the plaque region through the sGC expressed therein. That is, the possibility that the attenuation of SNP-induced vasorelaxation resulted from a decrease in cGMP formation in the smooth muscle cannot be ruled out. In addition, the effect of BAY 60-2770 on vasorelaxation was decreased, whereas the effect of cGMP generation tended to be increased, which can be explained by the above theory. That is, the plaque region may have been rich in oxidized/heme-free sGC, and BAY 60-2770 may have efficiently produced cGMP there. Further studies are required to strengthen this theory, but there is no doubt that the sGC system in coronary arteries changes with the progression of atherosclerosis.

Oxidative stress is a risk factor for a shift of the sGC redox state in coronary arteries.[35] Of note, many studies have shown that blood and tissue levels of oxidative stress biomarkers are upregulated within 1–2 weeks after starting high cholesterol diet feeding in rabbits.[16,17,24] Therefore, our model irrespective of short-term or long-term feeding may have been accompanied with oxidative stress conditions. If that is the case, our view that the level of sGC in the oxidized/heme-free form was increased in coronary arteries of high cholesterol diet-fed rabbits becomes more mature. In this regard, whether the treatment with oxidative stress modulators affects vascular responses of atherosclerotic coronary arteries to SNP and BAY 60-2770 is also of great interest.

sGC expression was evident in the plaque region in this study. This result is in line with previous reports showing a strong

expression of sGC in intimal plaques of cholesterol-fed rabbit aortas,[15] in atherosclerotic lesions of ApoE$^{-/-}$ mouse aortas,[36] and in atherosclerotic plaques of human carotid arteries.[30] In addition, it has also been reported that atherosclerotic plaques in aortas obtained from chronically hypercholesterolemic rabbits have a weak but NO-sensitive sGC activity, suggesting the presence of sGC.[23] Since the cGMP generated in atherosclerotic lesions is capable of inhibiting macrophage foaming,[36] the stimulation of cGMP production in the plaque region might be desirable from a therapeutic perspective. That is, the fact that BAY 60-2770 produced cGMP normally, or more strongly, in coronary arteries with atherosclerotic plaque, although the vasorelaxant responsiveness was decreased, is of great importance.

The vascular effects of cGMP are mediated through the activation of PKG; therefore, normal protein expression of PKG is required for cGMP to induce vasorelaxation. PKG expression in the medial layer was normal even in coronary arteries with plaque formation, suggesting that the cause of impairment in relaxant responses to SNP and BAY 60-2770 was not due to a decrease in PKG. In contrast, PKG was also present in the plaque region, although the amount was small. These expression patterns are consistent with the results obtained in hypercholesterolemic rabbit aortas[23] and in neointima-formed swine and human coronary arteries.[2]

Coronary atherosclerosis has features different from those of other arterial atherosclerosis.[7,22,29] Therefore, we performed some analysis in iliac arteries. The results showed that there was neither apparent atherosclerotic plaque formation, nor an altered responsiveness to SNP and BAY 60-2770, even in rabbits fed a high cholesterol diet for 12 weeks (data not shown). The reason for this discrepancy might be that coronary arteries are more prone to cholesterol-induced atherosclerosis than lower extremity arteries.[13] Regarding iliac arteries, long-term feeding is probably required for the changes seen in coronary arteries to be observed.

Several lines of evidence indicate that stimulation of the sGC system is beneficial for preventing the progression of coronary atherosclerosis.[10,19,40,41] As described above, this study showed that sGC is more abundantly expressed in the oxidized/heme-free form in atherosclerotic coronary arteries. In this regard, there is an interesting report by Ahrens et al.,[1] who reported that a higher level of oxidized/heme-free sGC is present in the platelets obtained from CAD patients. In addition, the stimulation of oxidized/heme-free sGC in monocytes under inflammatory conditions can potently inhibit tissue factor procoagulant activity.[31] Taken together, the oxidized/heme-free sGC expression is likely to be upregulated in various types of cells/tissues involved in the development and/or progression of atherosclerosis. Therefore, sGC activators may have great potential as therapeutic drugs for coronary atherosclerosis. A previous study using a high fat, high cholesterol diet-fed ApoE$^{-/-}$ mouse model demonstrated that chronic treatment with an sGC activator suppresses atherosclerotic plaque formation in aortas and aortic roots.[38] Whether sGC activators are effective for preventing the progression of atherosclerosis, even in coronary arteries, is of great interest and, therefore, a future study subject.

This study focused on sGC and did not address the influence of high cholesterol diet feeding on the cGMP removal system. Intracellular cGMP is reduced through the degradation by phosphodiesterases or through extracellular export via multidrug resistance-associated proteins (MRPs).[28] Phosphodiesterase 1 expression has been found to be increased in neointimal lesions in human coronary arteries.[4] In addition, an upregulated MRP4 and MRP5 expression has also been shown in human atherosclerotic coronary arteries.[8,27] cGMP, which was measured in this study, may have been detected even after being affected by enhanced removal system(s). However, the lack of examination on how the cGMP removal system was changed is a limitation of this study.

The most notable limitation of this study is that the mechanism by which the relaxant response of coronary arteries in the L-HC group to BAY 60-2770 was impaired, although cGMP production was not decreased remains unclear. Although this study did not examine the cell types in which sGC was present (e.g., smooth muscle cells, fibroblasts, or macrophages), this information may be a clue to solving the puzzle. In addition, since sGC expression is regulated not only post-transcriptionally, but also transcriptionally, it is also of great interest whether gene expression of sGC in coronary arteries is altered by atherosclerosis.

In conclusion, the sGC system in coronary arteries was demonstrated to be in the non-normal state at an early phase of atherosclerosis. In addition, the redox state and the distribution pattern are likely to change with the progression of atherosclerosis. Given these findings, sGC activators are expected to become promising drugs for the treatment of coronary atherosclerosis.

ACKNOWLEDGMENTS

The authors thank the animal care staff of the Shiga University of Medical Science and the Kanazawa Medical University for their kind and tireless assistance. We also thank the staff of the Bioscience Business Division, KAC Co., Ltd. (Shiga, Japan) for their support during the immunohistochemical experiments.

DISCLOSURE

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Tawa, Okamura, and Ishibashi participated in research design, and performed data analysis and interpretation of the manuscript. Tawa, Nakano, Yamashita, He, and Masuoka conducted experiments. Tawa wrote or contributed to the writing of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Masashi Tawa https://orcid.org/0000-0002-9282-0828

REFERENCES

1. Ahrens I, Habersberger J, Baumlin N, et al. Measuring oxidative burden and predicting pharmacological response in coronary artery disease patients with a novel direct activator of haem-free/oxidised sGC. *Atherosclerosis*. 2011;218:431-434. https://doi.org/10.1016/j.atherosclerosis.2011.06.042
2. Anderson PG, Boerth NJ, Liu M, McNamara DB, Cornwell TL, Lincoln TM. Cyclic GMP-dependent protein kinase expression in coronary arterial smooth muscle in response to balloon catheter injury. *Arterioscler Thromb Vasc Biol*. 2000;20:2192-2197. https://doi.org/10.1161/01.atv.20.10.2192
3. Berkenboom G, Unger P, Fontaine J. Atherosclerosis and responses of human isolated coronary arteries to endothelium-dependent and -independent vasodilators. *J Cardiovasc Pharmacol*. 1989;14(Suppl 11):S35-S39.
4. Cai Y, Nagel DJ, Zhou Q, et al. Role of cAMP-phosphodiesterase 1C signaling in regulating growth factor receptor stability, vascular smooth muscle cell growth, migration, and neointimal hyperplasia. *Circ Res*. 2015;116:1120-1132. https://doi.org/10.1161/CIRCRESAHA.116.304408
5. Cheng D, Talib J, Stanley CP, et al. Inhibition of MPO (Myeloperoxidase) attenuates endothelial dysfunction in mouse models of vascular inflammation and atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2019;39:1448-1457. https://doi.org/10.1161/ATVBAHA.119.312725
6. Corr L, Burnstock G, Poole-Wilson P. Effects of age and hyperlipidemia on rabbit coronary responses to neuropeptide Y and the interaction with norepinephrine. *Peptides*. 1993;14:359-364. https://doi.org/10.1016/0196-9781(93)90053-j
7. Dalager S, Paaske WP, Kristensen IB, Laurberg JM, Falk E. Artery-related differences in atherosclerosis expression: implications for atherogenesis and dynamics in intima-media thickness. *Stroke*. 2007;38:2698-2705. https://doi.org/10.1161/STROKEAHA.107.486480
8. Dazert P, Meissner K, Vogelgesang S, et al. Expression and localization of the multidrug resistance protein 5 (MRP5/ABCC5), a cellular export pump for cyclic nucleotides, in human heart. *Am J Pathol*. 2003;163:1567-1577. https://doi.org/10.1016/S0002-9440(10)63513-4
9. Derbyshire ER, Marletta MA. Structure and regulation of soluble guanylate cyclase. *Annu Rev Biochem*. 2012;81:533-559. https://doi.org/10.1146/annurev-biochem-050410-100030
10. Dhawan V, Handu SS, Nain CK, Ganguly NK. Chronic L-arginine supplementation improves endothelial cell vasoactive functions in hypercholesterolemic and atherosclerotic monkeys. *Mol Cell Biochem*. 2005;269:1-11. https://doi.org/10.1007/s11010-005-1810-4
11. Förstermann U, Mügge A, Alheid U, Haverich A, Frölich JC. Selective attenuation of endothelium-mediated vasodilation in atherosclerotic human coronary arteries. *Circ Res*. 1988;62:185-190. https://doi.org/10.1161/01.RES.62.2.185
12. Geenen IL, Kolk FF, Molin DG, et al. Nitric oxide resistance reduces arteriovenous fistula maturation in chronic kidney disease in rats. *PLoS One*. 2016;11:e0146212. https://doi.org/10.1371/journal.pone.0146212
13. Kamimura R, Suzuki S, Sakamoto H, Miura N, Misumi K, Miyahara K. Development of atherosclerotic lesions in cholesterol-loaded rabbits. *Exp Anim*. 1999;48:1-7. https://doi.org/10.1538/expanim.48.1
14. Knorr A, Hirth-Dietrich C, Alonso-Alija C, et al. Nitric oxide-independent activation of soluble guanylate cyclase by BAY 60–2770 in experimental liver fibrosis. *Arzneimittelforschung*. 2008;58:71-80. https://doi.org/10.1055/s-0031-1296471
15. Laber U, Kober T, Schmitz V, et al. Effect of hypercholesterolemia on expression and function of vascular soluble guanylyl cyclase. *Circulation*. 2002;105:855-860. https://doi.org/10.1161/hc0702.103975
16. Lee LS, Cho CW, Hong HD, Lee YC, Choi UK, Kim YC. Hypolipidemic and antioxidant properties of phenolic compound-rich extracts from white ginseng (Panax ginseng) in cholesterol-fed rabbits. *Molecules*. 2013;18:12548-12560. https://doi.org/10.3390/molecules181012548
17. Lefer AM, Ma XL. Decreased basal nitric oxide release in hypercholesterolemia increases neutrophil adherence to rabbit coronary artery endothelium. *Arterioscler Thromb*. 1993;13:771-776. https://doi.org/10.1161/01.atv.13.6.771
18. Libby P. Mechanisms of acute coronary syndromes and their implications for therapy. *N Engl J Med*. 2013;368:2004-2013. https://doi.org/10.1056/NEJMra1216063
19. Loaldi A, Polese A, Montorsi P, et al. Comparison of nifedipine, propranolol and isosorbide dinitrate on angiographic progression and regression of coronary arterial narrowings in angina pectoris. *Am J Cardiol*. 1989;64:433-439. https://doi.org/10.1016/0002-9149(89)90417-7
20. Lucas KA, Pitari GM, Kazerounian S, et al. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev*. 2000;52:375-414.
21. Martin E, Golunski E, Laing ST, Estrera AL, Sharina IG. Alternative splicing impairs soluble guanylyl cyclase function in aortic aneurysm. *Am J Physiol Heart Circ Physiol*. 2014;307:H1565-H1575. https://doi.org/10.1152/ajpheart.00222.2014
22. Matsuo Y, Takumi T, Mathew V, et al. Plaque characteristics and arterial remodeling in coronary and peripheral arterial systems. *Atherosclerosis*. 2012;223:365-371. https://doi.org/10.1016/j.atherosclerosis.2012.05.023
23. Melichar VO, Behr-Roussel D, Zabel U, et al. Reduced cGMP signaling associated with neointimal proliferation and vascular dysfunction in late-stage atherosclerosis. *Proc Natl Acad Sci USA*. 2004;101:16671-16676. https://doi.org/10.1073/pnas.0405509101
24. Moriel P, Okawabata FS, Abdalla DS. Oxidized lipoproteins in blood plasma: possible marker of atherosclerosis progression. *IUBMB Life*. 1999;48:413-417. https://doi.org/10.1080/713803534
25. Patel D, Alhawaj R, Kelly MR, et al. Potential role of mitochondrial superoxide decreasing ferrochelatase and heme in coronary artery soluble guanylate cyclase depletion by angiotensin II. *Am J Physiol Heart Circ Physiol*. 2016;310:H1439-H1447. https://doi.org/10.1152/ajpheart.00859.2015
26. Sandner P, Zimmer DP, Milne GT, Follmann M, Hobbs A, Stasch JP. Soluble guanylate cyclase stimulators and activators. *Handb Exp Pharmacol*. 2021;264:355-394. https://doi.org/10.1007/164_2018_197
27. Sassi Y, Lipskaia L, Vandecasteele G, et al. Mrp4 is a transmembrane export pump acting as an endogenous regulator of cyclic- nucleotides dependent pathways. *Biophys J*. 2009;96(Suppl 1):273A. https://doi.org/10.1016/j.bpj.2008.12.1351
28. Schneider EH, Seifert R. Inactivation of non-canonical cyclic nucleotides: hydrolysis and transport. *Handb Exp Pharmacol*. 2017;238:169-205. https://doi.org/10.1007/164_2016_5004
29. Shiomi M, Ito T, Tsukada T, Yata T, Ueda M. Cell compositions of coronary and aortic atherosclerotic lesions in WHHL rabbits differ. An immunohistochemical study. *Arterioscler Thromb*. 1994;14:931-937. https://doi.org/10.1161/01.atv.14.6.931
30. Sigala F, Efentakis P, Karageorgiadi D, et al. Reciprocal regulation of eNOS, H2S and CO-synthesizing enzymes in human atheroma: correlation with plaque stability and effects of simvastatin. *Redox Biol*. 2017;12:70-81. https://doi.org/10.1016/j.redox.2017.02.006
31. Sovershaev MA, Egorina EM, Hansen JB, et al. Soluble guanylate cyclase agonists inhibit expression and procoagulant activity of tissue factor. *Arterioscler Thromb Vasc Biol*. 2009;29:1578-1586. https://doi.org/10.1161/ATVBAHA.109.192690

32. Stasch JP, Schmidt PM, Nedvetsky PI, et al. Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. *J Clin Invest*. 2006;116:2552-2561. https://doi.org/10.1172/JCI28371
33. Tawa M, Geddawy A, Shimosato T, Iwasaki H, Imamura T, Okamura T. Soluble guanylate cyclase redox state under hypoxia or hypoxia/reoxygenation in isolated monkey coronary arteries. *J Pharmacol Sci*. 2014;125:169-175. https://doi.org/10.1254/jphs.14046FP
34. Tawa M, Shimosato T, Sakonjo H, et al. Chronological change of vascular reactivity to cGMP generators in the balloon-injured rat carotid artery. *J Vasc Res*. 2019;56:109-116. https://doi.org/10.1159/000498896
35. Tawa M, Okamura T. Soluble guanylate cyclase redox state under oxidative stress conditions in isolated monkey coronary arteries. *Pharmacol Res Perspect*. 2016;4:e00261. https://doi.org/10.1002/prp2.261
36. Tsou CY, Chen CY, Zhao JF, et al. Activation of soluble guanylyl cyclase prevents foam cell formation and atherosclerosis. *Acta Physiol*. 2014;210:799-810. https://doi.org/10.1111/apha.12210
37. Turk JR, Henderson KK, Vanvickle GD, Watkins J, Laughlin MH. Arterial endothelial function in a porcine model of early stage atherosclerotic vascular disease. *Int J Exp Pathol*. 2005;86:335-345. https://doi.org/10.1111/j.0959-9673.2005.00446.x
38. van Eickels M, Wassmann S, Schäfer A, Bauersachs J, Strobel H, Rütten H. Role of the sGC activator ataciguat sodium (HMR1766) in cardiovascular disease. *BMC Pharmacol*. 2007;7(Suppl 1):S4. https://doi.org/10.1186/1471-2210-7-S1-S4
39. Vanhoutte PM. Endothelial dysfunction: the first step toward coronary arteriosclerosis. *Circ J*. 2009;73:595-601. https://doi.org/10.1253/circj.CJ-08-1169
40. Wang BY, Singer AH, Tsao PS, Drexler H, Kosek J, Cooke JP. Dietary arginine prevents atherogenesis in the coronary artery of the hypercholesterolemic rabbit. *J Am Coll Cardiol*. 1994;23:452-458. https://doi.org/10.1016/0735-1097(94)90433-2
41. Wei CY, Wang YM, Han L, et al. Nitrate esters alleviated coronary atherosclerosis through inhibition of NF-κB-regulated macrophage polarization shift in epicardial adipose tissue. *J Cardiovasc Pharmacol*. 2020;75:475-482. https://doi.org/10.1097/FJC.0000000000000818
42. Zhang B, Alruwaili N, Kandhi S, et al. Inhibition of ferrochelatase impairs vascular eNOS/NO and sGC/cGMP signaling. *PLoS One*. 2018;13:e0200307. https://doi.org/10.1371/journal.pone.0200307

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Tawa M, Nakano K, Yamashita Y, et al. Alteration of the soluble guanylate cyclase system in coronary arteries of high cholesterol diet-fed rabbits. *Pharmacol Res Perspect*. 2021;9:e00838. https://doi.org/10.1002/prp2.838