Novel Role for Matrix Metalloproteinase 9 in Modulation of Cholesterol Metabolism

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Background—The development of atherosclerosis is strongly linked to disorders of cholesterol metabolism. Matrix metalloproteinases (MMPs) are dysregulated in patients and animal models with atherosclerosis. Whether systemic MMP activity influences cholesterol metabolism is unknown.

Methods and Results—We examined MMP-9–deficient (Mmp9−/−) mice and found them to have abnormal lipid gene transcriptional responses to dietary cholesterol supplementation. As opposed to Mmp9+/+ (wild-type) mice, Mmp9−/− mice failed to decrease the hepatic expression of sterol regulatory element binding protein 2 pathway genes, which control hepatic cholesterol biosynthesis and uptake. Furthermore, Mmp9−/− mice failed to increase the expression of genes encoding the rate-limiting enzymes in biliary cholesterol excretion (eg, Cyp7a and Cyp27a). In contrast, MMP-9 deficiency did not impair intestinal cholesterol absorption, as shown by the 14C-cholesterol and 3H-sitostanol absorption assay. Similar to our earlier study on Mmp2−/− mice, we observed that Mmp9−/− mice had elevated plasma secreted phospholipase A2 activity. Pharmacological inhibition of systemic circulating secreted phospholipase A2 activity (with varespladib) partially normalized the hepatic transcriptional responses to dietary cholesterol in Mmp9−/− mice. Functional studies with mice deficient in other MMPs suggested an important role for the MMP system, as a whole, in modulation of cholesterol metabolism.

Conclusions—Our results show that MMP-9 modulates cholesterol metabolism, at least in part, through a novel MMP-9–plasma secreted phospholipase A2 axis that affects the hepatic transcriptional responses to dietary cholesterol. Furthermore, the data suggest that dysregulation of the MMP system can result in metabolic disorder, which could lead to atherosclerosis and coronary heart disease. (J Am Heart Assoc. 2016;5:e004228 doi: 10.1161/JAHA.116.004228)

Key Words: atherosclerosis • cholesterol • lipid metabolism • liver • matrix metalloproteinase • plasma phospholipase A2

The matrix metalloproteinase (MMP) system comprises 25 different zinc-dependent and multifunctional endoproteases. Although collectively capable of cleaving extracellular matrix components, latent growth factors, cytokines, apolipoproteins, and cell membrane receptors, including the receptors for lipoproteins and metabolic hormones, MMPs are not typically viewed as important metabolic modulators.

We recently reported that the cardiohepatic metabolic phenotype in MMP-2–deficient (Mmp2−/−) mice can be largely explained by a novel heart–liver axis involving myocardial secretion of a unique phospholipase A2 (PLA2) called cardiac secreted PLA2 (sPLA2). Purportedly, the MMP-2–cardiac sPLA2 axis enables the heart to signal to the liver to satisfy its energy needs. In addition, the MMP-2–cardiac sPLA2 system enhances the production of prostaglandin E2 in the heart, brain, and liver. Consequently, a heart-centric mechanism using cardiac sPLA2 as a signal governed by MMP-2 influences the metabolism of other noncardiac organs.
Despite recent advances concerning the MMP-2–cardiac sPLA₂ axis, little is known about the metabolic functions of individual MMPs. In the current investigation, we investigated the potential contribution of MMP-9 to the systemic modulation of metabolism using a murine model of global genetic loss of MMP-9 (Mmp9<sup>−/−</sup>) mice. MMP-9, also known as gelatinase B or 92-kDa collagenase, shares remarkable structural similarities with MMP-2 (and other MMPs) including the presence of a propeptide domain and a catalytic domain with a highly conserved Zn²⁺-binding region; however, the tissue distribution of MMP-9 differs from that of MMP-2. MMP-2, for example, is constitutively expressed in most tissues, whereas MMP-9 expression is induced in response to inflammation.¹⁻⁴ MMP-9 activity contributes to atherosclerotic lesion progression,⁵⁻⁸ pathological cardiovascular remodeling in left ventricular dilatation after myocardial infarction and aortic aneurysm formation, and complications of diabetes mellitus including nephropathy, cardiomyopathy, and retinopathy.⁹,¹⁰ Moreover, obesity increases serum MMP-9 levels in women.¹¹⁻¹² MMP-9–deficient and wild-type (WT) littermate mice are similarly susceptible to obesity induced by a high fat diet (HFD) and exhibit normal adipose tissue development.¹¹⁻¹² Little information, however, is available on the role of MMP-9 in the regulation of lipid metabolism in the liver.

The liver responds to dietary cholesterol by decreasing hepatic cholesterol biosynthesis and increasing biliary cholesterol excretion.¹³⁻¹⁷ Disorders of cholesterol metabolism are strongly linked to the development of atherosclerosis.¹⁸⁻²⁰ MMP-9 activity is dysregulated in patients and experimental animal models with atherosclerosis or coronary artery disease.²¹⁻²³

In this study, we showed that MMP-9 modulates cholesterol metabolism, at least in part, through a novel MMP-9–plasma sPLA₂ axis that affects the hepatic transcriptional responses to dietary cholesterol. Functional studies with mice deficient in other MMPs further indicated that MMP-9 (and the MMP system as a whole) strongly influences cholesterol homeostasis. We propose that dysregulation of the MMP system can result in metabolic disorders that could lead to atherosclerosis and coronary heart disease.

### Materials and Methods

#### Reagents

Sterol regulatory element binding protein 2 (SREBP-2) antibody was purchased from Abcam. Varespladib was from Selleck Chemicals. PNGase F was from Promega. Enhanced chemiluminescence immunoblotting detection reagent was from GE Healthcare. HRP-conjugated antirabbit antibodies and the Bio-Rad Protein Assay kit were from Bio-Rad Laboratories.

#### Animals

All animal protocols and procedures were approved by the University of Alberta animal care committee and conducted in accordance with institutional guidelines issued by the Canada Council on Animal Care. Unless otherwise stated, WT mice aged 10 to 15 weeks were purchased from Charles River Laboratories (Wilmington, MA) or the Jackson Laboratory (Bar Harbor, ME) and compared against age- and sex-matched Mmp7<sup>−/−</sup>– and Mmp9<sup>−/−</sup>– mice purchased from the Jackson Laboratory. Mmp2<sup>−/−</sup>– and Timp2<sup>−/−</sup>– mice were bred at the University of Alberta (Edmonton, Canada). All mice had the C57BL/6 background and were housed in the Health Sciences Laboratory Animal Services of the University of Alberta on a 12-hour light/dark cycle. Mice were fed a chow diet ad libitum (PicoLab Rodent Diet 20; Lab Diet) unless otherwise specified.

At the end of the experiments, mice were euthanized with 65 mg/kg of sodium pentobarbital, the blood was collected with EDTA-coated syringes and tubes, and the organs were excised and snap-frozen in liquid nitrogen. The experiments with the HFD were approved by the local ethics committee (KU Leuven, P06022). Experiments were performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. The genetic background of the MMP-9–deficient mice used in this subset of experiments was 50% CD1 and 50% Sv129 with corresponding WT littermates (Mmp9<sup>−/−</sup>–), as described elsewhere.¹²,²⁴ Male mice (aged 5 weeks) were kept in microisolation cages on a 12-hour light/dark cycle and fed ad libitum with a standard fat diet (KM-04-k12, Muracon, containing 13% kcal as fat, with a caloric value of 10.9 kJ per gram; Carfi) or with an HFD (Harlan Teklad TD.88137; Envigo) containing 42% kcal as fat and a caloric value of 20.1 kJ per gram). All mice had ad libitum access to drinking water. Body weight and food intake were measured at weekly intervals. After 15 weeks, mice were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital (Nembutal; Abbott Laboratories), following overnight fasting. Blood was collected via the retro-orbital sinus with the addition of trisodium citrate (final concentration 0.1 mol/L), and plasma was stored at −80°C. Livers were removed, weighed, divided into portions, and snap-frozen in liquid nitrogen for RNA extraction.

#### In Vivo Responses to Dietary Cholesterol, Fasting, and Fasting–Refeeding

The dietary regimens followed previously described protocols.²⁵ In the cholesterol supplementation studies, the mice (aged 12–14 weeks) were fed chow supplemented with 0%, 0.15%, or 1.5% cholesterol for 2.5 or 6.5 days. In this study, the mice were not fasted before being euthanized. In the fasting and fasting–refeeding studies, mice (aged
10–22 weeks) were fasted for 16 hours or were fasted for 16 hours and then fed a "high carb" mouse diet (TD.88122; Envigo) for 4 hours, and then they were euthanized.

**In Vivo Pharmacological Studies**

To study the contribution of systemic sPLA2 to the lipid metabolic phenotype of MMP-9 deficiency, mice were gavaged with the sPLA2 inhibitor varespladib (10 mg/kg per day). The varespladib stock was prepared in DMSO (Sigma Aldrich), as per the manufacturer’s instructions, and diluted as required. Aqueous DMSO solution of the same concentration as in the varespladib working solution (equivalent to DMSO 2.6 μL/kg per day) was used in control experiments (vehicle). The mice were treated with or without varespladib for 2.5 days prior to cholesterol supplementation, and drug treatment was continued. Mice were then euthanized.

**Metabolic Studies**

Studies were conducted in metabolic cages at the Core Facility of the Cardiovascular Research Center, University of Alberta. Mice were individually housed in Oxymax/CLAMS metabolic chambers (Columbus Instruments) in which O₂ consumption, CO₂ production, food and water consumption, and movement (x and z) were measured over 2 days and 2 nights.

**Quantitative Reverse Transcriptase Polymerase Chain Reaction**

RNA was extracted from tissues by homogenizing 30- to 50-mg pieces of frozen tissue at 4°C in 1 mL of TRIzol reagent (Invitrogen) using the Bullet Blender (Next Advance). RNA was isolated from TRIzol according to the manufacturer’s instructions, and cDNA was generated from RNA using random primers and Superscript II reverse transcriptase. RNA was quantitated in triplicate to obtain a value representative of the relevant tissue for each mouse. Expression analysis of genes was performed by TaqMan quantitative reverse transcriptase polymerase chain reaction using the ABI 7900 HT sequence detection system (Applied Biosystems). For data normalization, both Gapdh and Actb (to confirm interpretation of data relative to Gapdh) were used as internal standards at steady state. The quantitative reverse transcriptase polymerase chain reaction data are shown relative to Gapdh.

**Protein Determinations**

Total protein content was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories) or the Pierce BCA protein assay kit (Thermo Fisher Scientific), according to the manufacturers’ instructions. Hepatic liver SREBP-2 protein levels were assessed by immune blotting. Briefly, 15- to 25-mg liver pieces were homogenized using the Bullet Blender at 4°C in a buffer of 5 mmol/L CaCl₂, 150 mmol/L NaCl, 0.5 mmol/L, and 25 mmol/L Tris (pH 7.4) with a complete protease inhibitor (Roche). The homogenate was incubated for 1 hour at 37°C with 50 U alkaline phosphatase, NP-40 was added to a concentration of 1%, and the samples were sonicated and then incubated for 3 hours at 37°C with PNGase (10 U/μL). The homogenate was diluted at 1:5 (vol/vol) with SDS-PAGE loading buffer (15% SDS, 8 mol/L urea, 10% 2-mercaptoethanol, 25% glycerol, 0.2 mol/L Tris [pH 6.8]), heated at 37°C for 20 minutes, and subjected to 10% SDS-PAGE using the SE260 electrophoresis system (Hoefer). Following electrophoresis, proteins were transferred to a nitrocellulose membrane using the TE22 system (Hoefer). Proteins were visualized with Ponceau S acid stain, blocked in 5% bovine serum albumin in 20 mmol/L Tris and 150 mmol/L NaCl (pH 7.4) containing 0.1% Tween-20, and probed overnight with primary antibodies to SREBP-2. The membrane was then probed for 30 minutes with secondary antibodies and washed in 20 mmol/L Tris and 150 mmol/L NaCl (pH 7.4) containing 0.1% Tween-20 to remove excess antibody. Immunoreactivity was revealed using enhanced chemiluminescence detection reagent.

**In Vitro Assays of PLA₂ Enzymatic Activity and Inhibitor Profiles**

PLA₂ activity was measured by 2 different methods. The Fernandez-Patron laboratory used the commercial assay kit (Cayman) with diheptanoyl thio-phosphatidylcholine as substrate, per the manufacturer’s instructions, to measure PLA₂ activity in tissue homogenates and plasma and to measure the ex vivo tissue release of sPLA2 activity. Confirmatory studies and extended characterization of sPLA2 biochemical properties were performed by the Lambeau laboratory using the highly sensitive [³H]-oleic acid Escherichia coli membrane assay. Because E coli membranes are rich in phosphatidyl ethanolamine and do not contain phosphatidylcholine, the Cayman kit and E coli assay methods display different sensitivities. For comparative biochemical characterization, sPLA₂ activity was assessed in the presence and absence of a panel of inhibitors of various enzyme classes. Briefly, samples were incubated for 15 minutes at room temperature prior to the assay in the presence of different inhibitors: dithiothreitol 10 mmol/L (reducing agent, incubation 30 minutes at 56°C; Euromedex), EDTA 40 mmol/L (a nonspecific inhibitor of Ca²⁺-dependent PLA₂; Euromedex), MJ33 30 μmol/L (PLA₂ inhibitor; Santa Cruz Biotechnology), KH064 10 μmol/L (sPLA₂ inhibitor; Sigma-Aldrich), YM 26734 10 μmol/L (sPLA₂ inhibitor; Santa Cruz Biotechnology), and ME260 10 μmol/L (sPLA₂ inhibitor; Sigma-Aldrich).
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inhibitor; Tocris Bioscience), arachidonyl trifluoromethyl ketone (10 μmol/L (cytosolic PLA₂ and calcium-independent PLA₂ inhibitor; Interchim), N-(p-amylcinnamoyl) anthranilic acid (100 μmol/L; PLA₂ inhibitor, Calbiochem; EMD Millipore), bromoenol lactone (3 μmol/L; calcium-independent PLA₂ inhibitor; Interchim), or heparin 100 μg/mL (sPLA₂ inhibitor; Sigma-Aldrich).

Extraction of Lipids From Tissues

Lipids were extracted from tissues, as described previously.²⁷ Tissue was homogenized in water (8–30 μL/mg) using the Bullet Blender. The homogenate or lysate (500–1000 μL) was mixed at a ratio of 3:4:8 aqueous homogenate/lysate:methanol:chloroform and vortexed for 1 minute, and then centrifuged for 15 minutes to separate phases. The bottom phase containing lipids was transferred into a new tube, dried under argon, and resuspended in 100 μL chloroform. The residue was used for lipid analysis.

Chromatographic Determinations of Lipid Content

Fast-performance liquid chromatography analyses of plasma lipoproteins were performed at the Lipid and Lipid Metabolite Analysis Core Facility, part of the Women and Children’s Health Research Institute and Faculty of Medicine and Dentistry at the University of Alberta. Plasma lipoprotein classes were resolved on a Superose 6 10/300 gel-filtration fast-performance liquid chromatography column run isocratically with 50 mmol/L NaCl buffer on a 1200 series high-performance liquid chromatography system (Agilent Technologies). Cholesterol or triglycerides in lipoproteins were statistically analyzed with the nonparametric t test, as appropriate. ANOVA and repeated-measures analysis was conducted to determine differences among groups in time-course experiments. Differences between groups were analyzed with the nonparametric t test (Mann–Whitney) with statistically significant values of P<0.05.

Results

MMP-9 Modulates Systemic Metabolism and Cholesterol Homeostasis

Chow-fed Mmp9⁻/⁻ mice exhibited several metabolic abnormalities at baseline compared with age-matched WT mice. In the Mmp9⁻/⁻ mice, locomotor activity and respiratory exchange ratio (a measure of energy substrate preference) were lower than in WT mice during the day, suggesting a relatively increased diurnal reliance on fatty acids as fuel; however, the respiratory exchange ratio during the night was higher than in WT mice, suggesting a switch to glucose oxidation. Oxygen consumption, energy expenditure (heat), body weight, and food intake were comparable for Mmp9⁻/⁻ and WT mice (Figure S1).

Analysis of hepatic lipids revealed further metabolic abnormalities in Mmp9⁻/⁻ mice including higher levels of
triglycerides and cholesteryl esters in the liver of Mmp9−/− mice compared with WT mice, particularly when re-fed after overnight fasting (Figure S2A). In Mmp9−/− mice, plasma levels of triglycerides and cholesterol were higher in very low-density lipoproteins and in high-density lipoproteins than in WT mice (Figure 1A). The higher plasma cholesterol levels in Mmp9−/− mice were not associated with abnormal cholesteryl absorption, as demonstrated by the radioactive 14C-cholesterol and 3H-sitostanol absorption assay (Figure 1B). Cholesterol excretion in the stool was unchanged in Mmp9−/− mice (Figure S2B). There were no statistically significant changes in plasma bile acid content (mean levels were increased on average by 7.4% in Mmp9−/− versus WT mice). The bile acid content, however, was higher in the stool of Mmp9−/− mice fed regular chow than in the stool of WT mice, implying a role for MMP-9 in the regulation of cholesterol excretion as bile acids (Figure 1C). When the chow was supplemented with 0.15% cholesterol, bile acid excretion remained high in Mmp9−/− mice and was elevated in WT mice, as expected (Figure 1C).

Quantitative reverse transcriptase polymerase chain reaction analysis indicated no difference between Mmp9−/− and WT mice in the expression of Nr1h3 (encoding liver X receptor α [LXR-α], a major regulator of fatty acid synthesis and cholesterol excretion). Fasn (encodes fatty acid synthase) is an LXR-α target and was also unaltered. Similarly unchanged were Srebfl2 (encodes the transcription factor SREBP-2) and SREBP-2 target genes such as Hmgcr (encodes 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme in the cholesterol and isoprenoid synthesis pathways) and Ldlr (encodes the LDL receptor, which is involved in clearance of low-density lipoprotein from circulation). Paradoxically, LXR-α target genes, the cholesterol transporters ATP-binding cassette G5 and G8 (encoded by Abcg5 and Abcg8) and the SREBP-2 target gene Pcsk9 (encodes proprotein convertase subtilisin/kexin type 9, a protein that binds and negatively regulates hepatic LDLR protein levels)33 were all significantly higher at baseline in Mmp9−/− mice than in WT mice (Figure 2).

Dietary cholesterol inhibits the SREBP-2 pathway to decrease the hepatic synthesis and uptake of cholesterol. At the same time, oxysterols derived from dietary cholesterol activate LXR-α signaling, which, in turn, increases both the synthesis of bile acids from hepatic cholesterol and the excretion of cholesterol into the bile.17,34 As expected, WT mice fed chow supplemented with cholesterol exhibited time-dependent (Figure 2) and concentration-dependent (Figure 3) decreases in the expression of key genes involved in cholesterol synthesis and uptake: Srebfl2, Hmgcr, Ldlr, and Pcsk9. The expression of Fatty acid synthase, a key enzyme in fatty acid biosynthesis, was concentration-dependently downregulated by cholesterol. In addition, dietary cholesterol unexpectedly increased Abcg5 and Abcg8, caused a small decrease in Nr1h3 and Nh1h2 and strongly induced the expression of Cyp7a1 and Cyp27a1, which encode the rate-limiting enzymes in the classical (cholesterol 7α-hydroxylase) and alternate (cholesterol 27α-hydroxylase) bile acid biosynthesis pathways.

In contrast, the gene transcriptional responses to dietary cholesterol were markedly impaired in Mmp9−/− mice (Figures 2 and 3). These findings identify MMP-9 as a novel modulator of the hepatic transcriptional responses to dietary cholesterol.

The MMP-9–Plasma sPLA2 Axis Modulates Hepatic Transcriptional Responses to Dietary Cholesterol

We recently found that in Mmp2−/− mice, the heart secretes an as yet unidentified PLA2 (cardiac sPLA2), which circulates in plasma, acting as a signal that is governed by MMP-2 and that modulates lipid metabolism in the liver.2,3 In Mmp9−/− mice, plasma sPLA2 activity was significantly higher than in WT plasma but otherwise was normal in the heart, as demonstrated by assaying the generation of free thiol from diheptanoyl-thio-phosphatidylcholine (substrate) (Figure 4A). The elevated sPLA2 activity in the Mmp9−/− plasma was confirmed using a highly sensitive (3H)-oleic acid radiolabeled E.coli membranes assay (Figure 4B).

Similar to the sPLA2 that is present in the plasma of Mmp2−/− mice, the sPLA2 activity in Mmp9−/− plasma was calcium dependent and inhibited by the broad-spectrum sPLA2 inhibitor varespladib35 (Figure 4C). Furthermore, the plasma sPLA2 activities from Mmp2−/− and Mmp9−/− mice were inhibited to similar degrees by a panel of PLA2 inhibitors, suggesting that the plasma sPLA2 activities in Mmp2−/− and Mmp9−/− mice result from either the same or structurally homologous enzymes (Figure 4D).

Next, we screened for sPLA2 release from various mouse tissues. Only the spleen—not the heart, thymus, or bone marrow—exhibited an increased ex vivo release of sPLA2 in Mmp9−/− mice relative to WT mice. Consequently, peripheral organs (eg, the spleen) may release sPLA2 into the circulation (Figure S3).

To determine whether the systemic circulating plasma sPLA2 activity mediated the hepatic transcriptional responses to dietary cholesterol in Mmp9−/− mice, we administered the sPLA2 inhibitor varespladib (or vehicle) to Mmp9−/− mice prior to dietary cholesterol supplementation (Figure 5). Administration of varespladib for 5 consecutive days fully normalized the levels of plasma sPLA2 activity in Mmp9−/− mice (Figure 5A). In WT mice, varespladib did not affect the hepatic transcriptional responses to dietary cholesterol (Figure 5B and 5C). In Mmp9−/− mice, varespladib partially normalized the hepatic transcriptional response to cholesterol for genes in the SREBP-2 pathway (Figure 5D and Figure S4); however, varespladib failed to affect mRNA levels of the rate-
Figure 1. MMP-9 deficiency is associated with abnormalities in lipid distribution and excretion. A, Triglyceride and cholesterol levels in lipoprotein fractions of plasma separated by FPLC. Traces correspond to pools of plasma from WT and Mmp9−/− mice (n=4 per genotype). Semiquantitative assessment based on peak heights indicates that Mmp9−/− mice have 2.7-, 4.3-, 1.5-, and 1.2-fold increases in VLDL triglycerides, VLDL cholesterol, LDL cholesterol, and HDL cholesterol, respectively (compared with WT mice). The same volume was injected onto the FPLC. B, Cholesterol absorption measured by radioactive 14C-cholesterol and 3H-sitostanol absorption assay (n=4 per genotype). C, Bile acid content in mouse stool in response to cholesterol (n=5 for WT with normal chow, n=5 for Mmp9−/− with normal chow, n=4 for WT with cholesterol, n=5 for Mmp9−/− with cholesterol). *P<0.05 vs WT, †P<0.05 vs normal chow, t test. FPLC indicates fast-performance liquid chromatography; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MMP, matrix metalloproteinase; VLDL, very low-density lipoprotein; WT, wild type.
limiting enzymes in biliary cholesterol synthesis (Cyp7a1 and Cyp27a) (Figure 5D).

Taken together, the data suggest that MMP-9 deficiency results in a phenotype of relative hepatic insensitivity to dietary cholesterol mediated, at least in part, by systemic sPLA2 activity that is likely contributed by peripheral organs (Figure S5).

**Figure 2.** Hepatic transcriptional responses to dietary cholesterol supplementation. Mice were fed regular chow or chow supplemented with cholesterol (0.15%) for up to 2.5 days. Gene expression analysis was conducted at days 0 and 2.5 (n=4 to 5 mice per time point). *P<0.05 vs WT. †P<0.05 vs 0 days on cholesterol, 1-way repeated-measures ANOVA. Abca1 indicates ATP-binding cassette sub-family A member 1; Abcg5/Abcg8, ATP-binding cassette sub-family G member 5/8; Cyp27a1, sterol 27 hydroxylase; Cyp7a1, cholesterol 7 alpha hydroxylase; Fasn, fatty acid synthase; Hmgcr, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; Ldlr, low density lipoprotein receptor; Mmp, matrix metalloproteinase gene; Nr1h3/Nr1h2, liver X receptor α/β; Pcsk9, proprotein convertase subtilisin/kexin type 9; Srebf1, sterol regulatory element binding protein 1; Srebf2, gene for sterol regulatory element binding protein 2; SREBP, sterol regulatory element binding protein; WT, wild type.

**MMP-9 Does Not Affect Liver Weight or Function in Mice Fed an HFD**

Figures 3 and 6A indicate that either increasing dietary cholesterol from moderate (0.15%) to high (1.5%) over 2.5 days or extending cholesterol administration from 2.5 to 6.5 days resulted in the partial normalization of hepatic gene
transcriptional responses of Mmp9−/− mice, suggesting a finite contribution of MMP-9 to regulation of cholesterol metabolism. We next compared the liver function of Mmp9−/− and WT mice fed an HFD for 15 weeks (from 5 to 20 weeks of age) versus mice fed a standard fat diet. At 5 weeks of age, Mmp9−/− and WT mice had similar body weights. The HFD markedly increased the levels of alkaline phosphatases and alanine and aspartate aminotransferases in both WT and Mmp9−/− mice (versus standard fat diet); however, feeding the HFD did not differently influence plasma lipids, body weight, liver weight, or plasma levels of alkaline phosphatases or alanine and aspartate aminotransferases of Mmp9−/− versus WT mice (Table).

The MMP System Modulates Cholesterol Metabolism

We recently discovered numerous differences in expression of lipid metabolic genes between WT and MMP-2–deficient mice including an upregulation of genes in the SREBP-2 pathway2,32,33 and impaired hepatic transcriptional responses to dietary cholesterol.2

We further compared the hepatic gene transcriptional responses to dietary cholesterol in mice deficient in MMP-2 or MMP-9. Compared with WT mice, mice deficient in either MMP-2 or MMP-9 exhibited abnormal regulation of genes involved in cholesterol metabolism (Figure 6A); however, the
Figure 4. Plasma sPLA2 activity is elevated by MMP-9 deficiency. A, sPLA2 activity in the plasma, liver, and heart of Mmp9−/− mice (n=4 mice per genotype). *P<0.05 vs WT, t test. B, The elevated plasma PLA2 activity in Mmp9−/− mice (compared to WT) was confirmed using 2 unrelated assays: the Cayman sPLA2 assay kit (substrate: di-heptanoyl-thio-PC; n=4 per genotype; *P<0.05 vs WT, t test) and the 3H-oleate E coli membrane assay (data are representative of technical duplicates for a pool of 5 mice per genotype). C, EGTA and varespladib inhibition profiles for the sPLA2 from Mmp9−/− plasma vs heart and plasma from Mmp9−/− mice. The analysis was performed in duplicate using samples from pools of 4 mice per genotype using the 3H-oleate E coli membrane assay. Similar results were obtained using the Cayman assay kit (data not shown). D, Profiling of PLA2 activity inhibition demonstrates that the plasma sPLA2 that is present in Mmp2−/− and Mmp9−/− mice is the same enzyme or very similar enzymes (pools of plasma: n=5 for Mmp2−/− and n=5 for Mmp9−/−). Data are representative of technical duplicates. For comparison, the activity of cardiac sPLA2 from an Mmp2−/− mouse (mouse “E” in figure S3 of Hernandez-Anzaldo et al2) is presented. *P<0.05 vs Mmp2−/− plasma, t test. The x-axis values indicate (0) no inhibitor; (1) EDTA, inhibits Ca2+-dependent PLA2S; (2) dithiothreitol, sulfhydryl redox agent; (3) MJ33, active site-directed PLA2 inhibitor; (4) KH064, sPLA2 inhibitor; (5) YM 26734, sPLA2 (PLA2G2A, PLA2G5) inhibitor; (6) arachidonyl trifluoromethyl ketone, cytosolic PLA2 and iPLA2 inhibitor; (7) N-(p-amylcinnamoyl) anthranilic acid, PLA2 inhibitor; (8) bromoeno lactone, iPLAZ inhibitor; and (9) heparin, inhibits some sPLA2S. AACOCF3 indicates arachidonyl trifluoromethyl ketone; ACA, N-(p-Amylcinnamoyl) anthranilic acid; BEL, bromoeno lactone; cPLA2, cytosolic phospholipase A2; DTT, dithiothreitol; E. coli, Escherichia coli; EDTA, ethylenediaminetetraacetic acid (divalent metal ion chelator); EGTA, ethylene glycol-bis(-aminoethyl ether)-tetraacetic acid (Ca2+ chelator); iPLA2, calcium-independent phospholipase A2; MJ33, 1-hexadecyl-3-trifluoroethylglycero-sn-2-phosphomethanol; MMP, matrix metalloproteinase gene; MMP, matrix metalloproteinase; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; PC, phosphatidylcholine; SH, sulfhydryl; sPLA2, secreted phospholipase A2; WT, wild type; YM 26734, 1,1′-[5-[3,4-dihydro-7-hydroxy-2-(4-hydroxyphenyl)-2H-1-benzopyran-4-yl]-2,4,6-trihydroxy-1,3-phenylene]bis-1-dodecanone.
Figure 5. Circulating systemic sPLA2 modulates hepatic transcriptional responses to dietary cholesterol supplementation. A, Plasma sPLA2 activity of Mmp9−/− mice administered the pan-sPLA2 inhibitor varespladib (10 mg/kg per day) or vehicle for 5 days (n=4 mice per group). *P<0.05 vs WT, t test. †P<0.05 vs untreated, t test. B, Study protocol for varespladib treatment prior to cholesterol supplementation. Mice were fed either regular chow or chow supplemented with 0.15% cholesterol for 2.5 days. Varespladib treatment (10 mg/kg per day for 5 days) started 2.5 days prior to commencement of cholesterol supplementation of the diet. C, Hepatic expression of lipid metabolic genes in WT mice administered varespladib (10 mg/kg per day; n=8 WT without varespladib and n=8 WT with varespladib mice, n=4 per time point). *P≤0.05 vs WT without varespladib at day 2.5. †P<0.05 vs WT without varespladib at day 0. All pairwise multiple comparisons vs control group (Holm–Sidak method), ANOVA. D, Hepatic expression of lipid-metabolic genes in Mmp9−/− mice administered varespladib (10 mg/kg per day; n=8 Mmp9−/− without varespladib and n=8 Mmp9−/− with varespladib, n=4 per time point). *P<0.05 vs Mmp9−/− without varespladib at day 2.5. †P<0.05 vs day 0. All pairwise multiple comparisons vs control group (Holm–Sidak method), ANOVA. Abca1 indicates ATP-binding cassette sub-family A member 1; Abcg5/Abcg8, ATP-binding cassette sub-family G member 5/8; Cyp27a1, sterol 27 hydroxylase; Cyp7a1, cholesterol 7 alpha hydroxylase; Fasn, fatty acid synthase; Hmgcr, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; Ldlr, low density lipoprotein receptor; LXR, liver X receptor; Mmp, matrix metalloproteinase gene; MMP, matrix metalloproteinase; Nr1h3/Nr1h2, liver X receptor a/b; Pcsk9, proprotein convertase subtilisin/kenin type 9; sPLA2, secreted phospholipase A2; Srebf1, sterol regulatory element binding protein 1; Srebf2, gene for sterol regulatory element binding protein 2; SREBP, sterol regulatory element binding protein; WT, wild type.
Figure 6. The MMP system modulates hepatic transcriptional responses to dietary cholesterol. A, Deficiency of MMP-2, MMP-7, or MMP-9 is associated with abnormalities in the hepatic transcriptional responses to dietary cholesterol. Mice were fed either regular chow or chow supplemented with 0.15% cholesterol for 6.5 days. Gene expression analysis was conducted at 0, 2.5, and 6.5 days. Analysis involved 6 WT mice, 8 Mmp2−/− mice, 5 Mmp7−/− mice, and 5 Mmp9−/− mice. For clarity, the symbols indicating statistically significant differences were excluded. An expanded version of these analyses is presented in Figure S6. B, Proposed model for regulation of cholesterol homeostasis by systemic MMP activity. MMP deficiencies (due to genetic deletion or functional blockade) can alter the hepatic cholesterol metabolism. The mechanism by which MMPs act may or may not require the release of sPLA2 activity from peripheral organs and is governed by tissue inhibitors of metalloproteinase. Cyp27a1 indicates sterol 27 hydroxylase; Cyp7a1, cholesterol 7 alpha hydroxylase; Fasn, fatty acid synthase; Hmgcr, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; Ldlr, low density lipoprotein receptor; Mmp, matrix metalloproteinase gene; MMP, matrix metalloproteinase; Nr1h3, liver X receptor α; Pcsk9, proprotein convertase subtilisin/kexin type 9; sPLA2, secreted phospholipase A2; Srebf1, sterol regulatory element binding protein 1; Srebf2, gene for sterol regulatory element binding protein 2; TIMP, tissue inhibitor of metalloproteinase; WT, wild type.

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Our results identify MMP-9 as a new modulator of cholesterol metabolism. Furthermore, the results suggest that dysregulation of MMP-9 activity (and the MMP system as a whole) can alter the hepatic transcriptional responses to dietary cholesterol (Figure 6B), resulting in metabolic disorder that could lead to atherosclerosis and coronary heart disease.

Our study also suggests a novel endocrine mechanism that modulates the sensitivity of the liver to cholesterol: the production and secretion of a unique sPLA2 with plasma levels that could reflect a cumulative release from multiple peripheral organs. Similar to MMP-2,2 MMP-9 acts as a negative regulator of sPLA2 release from the source tissue into the circulation. Our current enzymological data suggest that the plasma sPLA2 activity that is increased in MMP-9– and MMP-2–deficient plasma corresponds to 1 enzyme (or to homologous enzymes). Proteomic studies are under way in our laboratories to identify the relevant variants of sPLA2.

It is noteworthy that in MMP-2–deficient mice, the heart is a major source of plasma sPLA2 activity, whereas in MMP-9–deficient mice, the spleen is a likely source of plasma sPLA2. The reason for this apparent MMP-dependent tissue specificity warrants further investigation, and a possible mechanism has been postulated.42 This MMP-9–plasma sPLA2 axis profoundly influences transcription responses of lipid metabolic genes to dietary cholesterol in the liver, particularly those of enzymes in the SREBP-2 pathway, which is a major regulator of hepatic cholesterol biosynthesis and uptake.43,44

Although intestinal cholesterol absorption is normal in MMP9+/− mice, plasma cholesterol and bile acid excretion are elevated. Most intriguingly, the mRNA levels of Cyp7a1 and Cyp27a1—the rate-limiting enzymes in the classical and alternative bile acid biosynthesis pathways, respectively—are significantly insensitive to dietary cholesterol supplementation. These findings clearly identify MMP-9 as a broad-spectrum MMP inhibitor in a concentration-dependent fashion.38,40 TIMP-2–deficient mice have an increased susceptibility to HFD-induced obesity.41 Figure S6 shows that Timp2−/− mice exhibit abnormalities in both hepatic lipid gene expression and transcriptional responses to dietary cholesterol resembling those of both WT and MMP-deficient mice. Timp2+/− mice, for example, exhibited decreased mRNA levels of Srebf2 and Hmgcr when fed chow, and their response to dietary cholesterol supplementation was different from those of Mmp2+/−, Mmp7+/−, Mmp9+/−, and WT mice (Figure S6).

Additional studies confirmed that MMP expression affects mRNA expression of multiple lipid metabolic genes and their transcriptional responses to dietary cholesterol in the cardiohepatic circuit (Figure S7).

### Discussion

Table. Effect of MMP-9 Deficiency on Body and Liver Weight and Plasma Hepatic Enzyme Activities

| Variable       | SFD   | HFD   |
|----------------|-------|-------|
|                | Mmp9+/+ | Mmp9−/− | Mmp9+/+ | Mmp9−/− |
| Experiments, no. | 7     | 7     | 7       | 11     |
| Body weight start, g | 21±1.2 | 19±1.0 | 20±1.0  | 17±1.4 |
| Body weight end, g  | 34±1.8 | 33±1.6 | 39±1.3  | 38±2.2 |
| Liver weight, mg    | 1153±135 | 963±55 | 1343±60 | 1533±215* |
| AP, U/L            | 39±0.9 | 37±1.8 | 246±51* | 221±31* |
| AST, U/L           | 24±2.8 | 28±4.5 | 219±54* | 302±45* |
| ALT, U/L           | 14.0±1.8 | 13±1.8 | 156±46* | 272±56* |

Male mice were fed an SFD or an HFD for 15 weeks. Data are shown as mean±SEM of the number of experiments in each group. AP indicates alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HFD, high fat diet; MMP, matrix metalloproteinase; SFD, standard fat diet.*P<0.05 vs SFD. Diet composition is described in Materials and Methods.

Transcriptional responses to dietary cholesterol of MMP-2–deficient mice were, generally, not the same as those of MMP-9–deficient mice. Chow-fed MMP-2–deficient mice, for example, exhibited higher Srebf2 mRNA expression than MMP-9–deficient and WT mice (Figure 6A and reference2). When the chow was supplemented with cholesterol for 2.5 days, the Srebf2 mRNA levels of MMP-2–deficient mice dropped but remained above those of WT mice. Chow-fed MMP-9–deficient mice exhibited similar Srebf2 mRNA expression as WT mice but had a delayed response to dietary cholesterol supplementation. The response to cholesterol of Fasn was also different in MMP-2–deficient and MMP-9–deficient mice. Deficiency of either MMP-2 or MMP-9 markedly impaired the response of Cyp27a1 and Cyp7a1 to dietary cholesterol supplementation, whereas WT mice had a dramatic response.

Taken together, our results suggest that individual MMPs can modulate the hepatic transcriptional responses to dietary cholesterol in an MMP-specific fashion. This notion was supported by analysis of mice deficient in MMP-7, a “minimal” MMP produced in Kupffer cells and hepatocytes.36 MMP-7 contains a conserved catalytic domain but lacks the fibronectin repeats and hemopexin domain present in MMP-2 and MMP-9.4 Deficiency of MMP-7 induced hepatic insensitivity to dietary cholesterol that resembled the phenotype of MMP-9–deficient mice rather than that of MMP-2 deficient mice (Figure 6A).

Systemic MMP activity is regulated by tissue inhibitors of metalloproteinase (TIMPs) that form MMP/TIMP complexes37 that can affect MMP activation, activity, and binding to interaction partners and substrates.38 TIMP-2 is both an activator of MMP-2 and a broad-spectrum MMP inhibitor in a concentration-dependent fashion.38,40 TIMP-2–deficient mice have an increased susceptibility to HFD-induced obesity.41
as an important modulator of cholesterol homeostasis and suggest the existence of MMP-regulated pathways of bile acid synthesis and cholesterol excretion. This latter notion is supported by earlier research linking PLA2 activity to gallstone formation, although these earlier studies do not invoke regulation of either PLA2 activity or biliary cholesterol synthesis by MMPs. Similarly, PLA2 activity has been linked previously to remodeling of lipoproteins and cardiovascular disease, although these earlier studies focused on group II PLA2 (PLA2G2A; which is not made by C57BL mice) and lipoprotein lipase, PLA2s that do not fit the activity or inhibition profile of the plasma sPLA2 activity that is elevated in mice deficient in either MMP-9 (as shown in this paper) or MMP-2. Previous research has attributed roles in cholesterol metabolism to several sPLA2 family members including PLA2G1B, PLA2G2E, PLA2G5, PLA2G10, and PLA2G12B (the latter is not catalytically active). These earlier findings are consistent with our current results.

It appears that MMP-9 affects cholesterol metabolism in ways that we could not predict. We did not expect, for example, that the mRNA levels of ABCG5/8 would be higher in the liver of Mmp9−/− compared with WT mice fed chow or that CYP7 and CYP27 gene expression would remain almost unchanged when Mmp9−/− mice were fed cholesterol. These findings are very interesting. Notably, it has been reported previously that PLA2G10 influences gene expression of ABCG5/8 and LXR-α. In the case of the former gene, PLA2G10 activity works in a manner opposite to that of MMP-9 deficiency, whereas in the case of the LXR-α, PLA2G10 works in a manner similar to MMP-2 deficiency. Consequently, it appears that the convergence of MMP- and PLA2-dependent pathways can strongly affect lipid-metabolic gene transcription in somewhat unintuitive ways.

Our operating hypothesis is that sPLA2 release into plasma is globally regulated by proinflammatory cytokines and MMPs in an MMP- and tissue-specific fashion. Downstream hydrolysis of target phospholipids by the sPLA2 might influence liver metabolism and affect cholesterol homeostasis. Importantly, not all lipid-metabolic abnormalities associated with MMP-9 deficiency were normalized by systemic sPLA2 inhibition using varespladib; therefore, other MMP-9–dependent but sPLA2 activity–independent mechanisms are likely at play. A putative mechanism is the MMP-9 proteolytic processing of cell membrane lipoprotein receptors and lipid transporters such as CD36. Furthermore, MMPs bind to lipoprotein and cargo receptors. The LDLR-related protein 1 (LRP1) is a member of the LDLR superfamily, also known as CD91. LRP1 is an endocytic receptor for multiple extracellular ligands including apolipoprotein E–containing lipoproteins such as chylomicron remnants (which carry dietary cholesterol and other lipids to the liver) and plasma MMPs such as MMP-2, MMP-9, and MMP-13, including complexes of these MMPs with TIMPs.

LRP1-mediated endocytosis removes MMPs from the circulation. The hemopexin domain of MMP-9, which is conserved in MMP-2 but absent in MMP-7, contains the high-affinity binding site required for LRP-mediated endocytosis of MMP-9. The endocytosis of MMP-9 results in catabolism of the extracellular matrix by regulating extracellular proteinase activity. Conversely, LRP1–mediated endocytosis of MMPs reduces cell surface levels of LRP1. Consequently, MMPs compete with apolipoprotein E–containing lipoproteins such as chylomicron remnants and intermediate-density LDL for LRP-mediated endocytosis, providing mechanisms through which the MMP system can influence systemic lipid metabolism. Further research is warranted to establish the precise mechanisms by which MMPs regulate cholesterol metabolism.

Some of the effects of MMP-9 deficiency were obliterated when the cholesterol level of the diet was sufficiently high (eg, by supplementation of the chow diet with 1.5% cholesterol or by prolonged administration of an HFD). These data suggest that the intracellular cholesterol-sensing mechanisms remain functional in MMP-9 deficiency. Furthermore, the individual contribution of MMP-9 to cholesterol homeostasis, although significant, may be relatively small. Most interestingly, similar to MMP-9–deficient mice, the lack of any of MMP-2, MMP-7, or TIMP-2 resulted in a significant perturbation of hepatic transcriptional responses to dietary cholesterol. These observations demonstrate a potentially important role of MMP-9 (and the MMP/TIMP system as a whole) in the modulation of cholesterol metabolism. As such, the current research substantially expands previous reports by our groups and other investigators and points to important metabolic actions of MMPs.

In most in vitro and in vivo studies of cholesterol metabolism, alterations in MMPs and TIMPs are not normally monitored; therefore, detection of the modulatory effects of individual MMPs and TIMPs on cholesterol metabolism has likely been precluded. These effects could be profoundly important if ≥1 MMP or TIMP was altered, genetically or pharmacologically, and should not be neglected.

Insufficiencies in hepatic cholesterol uptake tend to elevate plasma cholesterol favoring extrahepatic cholesterol deposition and the development of atherosclerotic plaque at sites of vascular injury, a process in which the role of MMPs (including MMP-9) is well documented but poorly understood. Restoring the sensitivity of the liver to cholesterol is crucial for managing hypercholesterolemia and atherosclerosis. Our data suggest that cholesterol homeostasis could be targeted through broad-spectrum pharmacological manipulation of the proteolytic activity of MMPs. The efficacy of therapeutic strategies directed at decreasing plasma cholesterol would be enhanced if MMP-2, MMP-7, and MMP-9 levels were kept normal. Whether
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Disclosures

None.

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Figure S1. Systemic metabolic profile of MMP-9 deficient mice.
(A) Oxygen consumption. (B) Carbon dioxide production. (C) Heat / Energy expenditure (normalized to body weight). (D-F) Locomotor activity. (G) Body weight. (H) Total food consumption. The studies were conducted in metabolic cages. n=7 WT mice, n=5 Mmp9−/− mice. *: P≤0.05 vs. WT.
Figure S2. Supplemental quantitative analysis of lipids.

(A) Esterified lipids were elevated in livers from Mmp9\(^{-/-}\) mice compared to livers from WT mice, particularly when the mice were fasted-and-refed a high carbohydrate diet. In contrast to Mmp9\(^{-/-}\) mice, mice lacking MMP-7 (Mmp7\(^{-/-}\)) had a different hepatic lipid profile. \(n=4\) mice per group, except \(n=3\) for Mmp7\(^{-/-}\) fasted-and-refed. \(\dagger P\leq0.05\) vs. fasted for each genotype.

\(\ast P\leq0.05\) vs. WT fasted-and-refed.

(B) Cholesterol excretion was unchanged in Mmp9\(^{-/-}\) mice. \(n=4\) mice per genotype.
Figure S3. Spleen is a likely source of plasma sPLA₂ in MMP-9-deficient mice. sPLA₂ activity was analyzed in duplicate using pools of the indicated tissues. n=3 WT and n=4 Mmp9⁻/⁻ mice. *: P<0.05 vs. WT.
Figure S4. SREBP-2 expression in response to dietary cholesterol and varespladib. **Left:** Western blot showing amount of hepatic SREBP-2 protein in *Mmp9* mice in response to varespladib and dietary cholesterol supplementation. The experiment involved *n*=4 to 5 mice per group (or treatment). For analysis, livers were pooled, homogenized and the fraction containing nuclei was subjected to western blot analysis with SREBP-2 antibodies. **Right:** Quantitative analysis for two independent preparations and western blots. *: *P*≤0.05 vs. untreated.
**Figure S5. Proposed model.** MMP-9 regulates cholesterol metabolism through PLA₂-dependent and PLA₂-independent mechanisms. Important elements are: peripheral organs (e.g., the spleen) acting as source of plasma sPLA₂ activity and MMP-9 (inhibitor of sPLA₂ release from peripheral organs). Once in the circulation, sPLA₂ acts on plasma lipoproteins or target organs (e.g., the liver) to release lipid mediators from phospholipids that ultimately influence cholesterol metabolism. Furthermore, the direct action of MMP-9 in the liver may influence hepatic cholesterol through as yet unclear PLA₂-independent pathways.
Figure S6. Supplement to Figure 7A containing the quantitative analysis of hepatic transcriptional responses to dietary cholesterol for the indicated genes and genotypes. 

\( n = 6 \) WT mice, \( n = 8 \) Mmp2\(^{-/-}\) mice, \( n = 5 \) Mmp7\(^{-/-}\) mice, \( n = 5 \) Mmp9\(^{-/-}\) mice and \( n = 5 \) Timp2\(^{-/-}\) mice. *: \( P \leq 0.05 \) vs. WT. †: \( P \leq 0.05 \) vs. 0 days on cholesterol.
Figure S7. Extended quantitative analysis of the relative mRNA expression of cardiac and hepatic lipid metabolic and metalloproteinase genes and their response to dietary supplementation with 0.15% cholesterol for 2.5 days in mice deficient in one of several MMPs. 

n = 6 WT mice, n = 8 Mmp2⁻/⁻ mice, n = 5 Mmp7⁻/⁻ mice, n = 5 Mmp9⁻/⁻ mice. Slots without bars: Gene expression was not determined.