Microbe-Derived Butyrate and Its Receptor, Free Fatty Acid Receptor 3, But Not Free Fatty Acid Receptor 2, Mitigate Neointimal Hyperplasia Susceptibility After Arterial Injury

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BACKGROUND: Arterial restenosis after vascular surgery is a common cause of midterm restenosis and treatment failure. Herein, we aim to investigate the role of microbe-derived butyrate, FFAR2 (free fatty acid receptor 2), and FFAR3 (free fatty acid receptor 3) in mitigating neointimal hyperplasia development in remodeling murine arteries after injury.

METHODS AND RESULTS: C57BL/6 mice treated with oral vancomycin before unilateral femoral wire injury to deplete gut microbiota had significantly diminished serum and stool butyrate and more neointimal hyperplasia development after arterial injury, which was reversed by concomitant butyrate supplementation. Deficiency of FFAR3 but not FFAR2, both receptors for butyrate, exacerbated neointimal hyperplasia development after injury. FFAR3 deficiency was also associated with delayed recovery of the endothelial layer in vivo. FFAR3 gene expression was observed in multiple peripheral arteries, and expression was increased after arterial injury. Treatment of endothelial but not vascular smooth muscle cells with the pharmacologic FFAR3 agonist 1-methylcyclopropane carboxylate stimulated cellular migration and proliferation in scratch assays.

CONCLUSIONS: Our results support a protective role for butyrate and FFAR3 in the development of neointimal hyperplasia after arterial injury and delineate activation of the butyrate-FFAR3 pathway as a valuable strategy for the prevention and treatment of neointimal hyperplasia.

Key Words: butyrate ■ neointimal hyperplasia ■ restenosis

Each year in the United States, 7.4 million patients with cardiovascular disease undergo revascularization procedures, such as balloon angioplasty, stenting, and surgical bypass. However, despite decades of advancements in high-intensity statin therapy, antiplatelet therapy, drug-coated balloons, and drug-eluting stents, up to 50% of treated vessels will exhibit recurrent narrowing, or restenosis, within 1 year because of the formation of neointimal hyperplasia, a pathologic response of the blood vessel to the procedure that is distinct from progression of atherosclerosis. Neointimal hyperplasia is a local inflammatory and proliferative process initiated by endothelial denudation. Although both genetic and
environmental factors contribute, the precise nature of gene-environment interactions underlying pathologic intima formation after surgery remains obscure. A growing body of literature supports a direct role for microbiota in cardiovascular disease pathologic features through bioactive microbe-derived chemicals. In humans, patients with symptomatic atherosclerosis have depleted abundance of butyrate-producing microbes *Eubacterium* and *Roseburia* compared with controls. The microbiomes of patients with coronary artery disease have also been found to have depletion of butyrate-producing Lachnospiraceae and Ruminococcaceae compared with patients without coronary artery disease. However, the role of microbe-derived butyrate in the arterial remodeling response after surgery is not well known. We previously demonstrated that antibiotic-treated rats had exacerbated neointimal hyperplasia development after carotid angioplasty, which was associated with diminished butyrate. Butyrate was also found to inhibit vascular smooth muscle cell (VSMC) migration, proliferation, and cell cycle progression in vitro, implicating a beneficial role for butyrate in the arterial remodeling response after surgery. Butyrate is a principal short-chain fatty acid (SCFA) produced in millimolar amounts in the colonic lumen by microbiota. SCFAs exert their physiological effects in part by activating nutrient-sensing free fatty acid GPCRs (G-protein–coupled receptors), such as FFAR2 (free fatty acid receptor 2; also known as GPR43, or FFA2) and FFAR3 (free fatty acid receptor 3; also known as GPR41 or FFA3). Butyrate is passively absorbed from the intestinal lumen and is oxidized by colonic epithelial cells as an energy source. Unused butyrate is taken up into the portal circulation and released into the plasma. The goal of this study is to understand the role of FFAR2 and FFAR3 in mediating the effects of butyrate on neointimal hyperplasia using a mouse model of arterial injury.

**METHODS**

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

**Animals and Ethics Statement**

All animal studies were conducted in accordance with regulations of the Institutional Animal Care and Use Committee at Northwestern University. All mice were housed in a conventional facility at Northwestern University under a 12-hour light cycle. Standard irradiated chow and autoclaved water were provided ad libitum. Euthanasia was performed by bilateral thoracotomy and sternotomy for terminal sample collection following induction of adequate general anesthesia.

**Antibiotic Treatment**

C57BL/6J mice obtained from Jackson Laboratories (Bar Harbor, ME) underwent bedding mixing for 2 weeks, followed by treatment via the drinking water with vancomycin (0.5 mg/mL), sodium butyrate (100 mmol/L), vancomycin-butyrate, or sodium-matched control drinking water, as previously described. Mice were randomized to treatment groups. Bedding mixing was performed to equilibrate animal housing conditions. After 5 weeks of drinking
water treatment, all animals underwent left femoral artery wire injury. The right side served as the uninjured control. Stool samples were collected before the start of water treatment, before carotid angioplasty, and on the day of euthanasia. Stool pellets were frozen in liquid nitrogen and stored at −80°C until time of use.

FFAR2 and FFAR3 Knockout Mice
Generation of FFAR2 and FFAR3 knockout mice has been previously reported. Heterozygous FFAR2 and FFAR3 mice on a C57BL/6J background were kindly provided by Dr Brian T. Layden (University of Illinois at Chicago) and crossed to produce wild-type (WT) and knockout mice, which were genotyped as previously described.

Unilateral Femoral Artery Wire Injury
This mouse model of neointimal hyperplasia has been previously described. In brief, mice were anesthetized with a single IP injection of a cocktail of ketamine (80 mg/kg) and xylazine (5 mg/kg). A 1.5-cm groin incision was made directly over the left femoral artery. The right femoral artery served as the unoperated control. The common femoral artery was dissected out along its length, including the side branches. Vascular control was obtained proximally and distally with noncrushing vascular clamps. An arteriotomy was made in a medial muscular arterial branch. A 0.014-inch guide wire was inserted through the arteriotomy into the common femoral artery, passed in and out 3 times, and then held in place for 5 minutes. Following injury, the guide wire was removed and the branch was ligated proximal and distal to the arteriotomy. The vascular clamps were removed, and restoration of flow to the common femoral artery was verified. The skin incision was closed with skin clips. At designated time points after surgery, the animals were anesthetized with isoflurane for terminal blood and tissue collection. Whole blood was collected by cardiac puncture. Serum was prepared and stored at -80°C until use. Both femoral arteries are removed after in situ perfusion-fixation with paraformaldehyde.

In Situ Evans Blue Dye Staining
Reendothelialization of the injured artery was assessed by staining with Evans blue dye (Sigma Aldrich, St Louis, MO). Following induction of adequate general anesthesia and creation of a midline laparotomy, 50 µL of 5% Evans blue dye was injected into the abdominal aorta and allowed to circulate before flushing with 1 mL PBS. Both femoral arteries distal to the level of injury were transected. The injured segment of the left femoral artery and the corresponding segment of the right femoral artery were removed, opened longitudinally, and photographed ex vivo adjacent to a ruler. The blue and total area of injury were calculated using ImageJ/FIJI software (National Institutes of Health, Bethesda, MD) after calibration with the ruler. The ratio of blue-stained area/the total area was calculated.

Tissue Processing and Morphometric Analysis
Explanted arteries were fixed, embedded in optimal cutting temperature compound, sectioned, and stained, as previously described. Digital images of hematoxylin-eosin–stained sections were obtained using a Leica DM2000 light microscope with a 20× objective and camera. Morphometric analysis by investigators blinded to the experimental group was performed by using lumen, intima, and media areas and circumference that were measured or calculated using ImageJ/FIJI software after calibration. Sections were sampled at 35-µm intervals along the length of the injured artery (≈2.5 mm in length).

Microbial DNA Preparation
Stool pellets were collected and stored at −80°C before processing. Microbial DNA was extracted with the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol.

16S Ribosomal RNA–Based Analysis of Gut Microbial Composition and Diversity
Sequencing, assembly, taxonomic assignment, and diversity assessments were performed, as previously described.

Stool and Serum Butyrate Quantification
Samples were prepared, as previously described. SCFAs in samples and standards were extracted in 50% acetonitrile, vortexed, and clarified by centrifugation. Supernatant (40 µL) was used for derivatization using 20 µL 200 mmol/L 3-nitrophenylhydrazine in 50% aqueous acetonitrile and 20 µL 120 mmol/L N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide–6% pyridine. The solution then was diluted to 2 mL with 10% aqueous acetonitrile. Derivatized sample (50 µL) was mixed with 50 µL of the internal standards, and 10 µL of the resulting solution was used for liquid chromatography/mass spectrometry. Acetate, propionate, and butyrate, purchased from Sigma-Aldrich, were reacted with 3-nitrophenylhydrazine hydrochloride, which was custom synthesized by IsoSciences Inc (King of Prussia, PA), and N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide at the same condition as the
samples and served as the internal standards. Liquid chromatography/mass spectrometry analysis was performed on a Sciex Qtrap 6500 mass spectrometry system (SCIEX, Framingham, MA) coupled to an Agilent 1290 ultraperformance liquid chromatography system (Agilent Technologies, Santa Clara, CA) using a Phenomenex high-performance liquid chromatography C18 column, 100 Å, 2.6 μm, and operating in multiple reaction mode. A gradient of buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in acetonitrile) was applied as follows: 0 minutes, 15% of buffer B; increase buffer B to 45% in 4 minutes; 45% to 100% in 0.2 minutes; kept B at 100% for 1.8 minutes. The column was equilibrated for 2 minutes at 15% B between the injections. Flow rate was 500 μL/min. The column temperature was 40°C, and the autosampler was kept at 4°C. Precursor (Q1) and product ion (Q3) m/z transitions were 194.1 → 143.1 for acetate, 200.1 → 143.1 for ^13^C_6-acetate, 208.1 → 165.1 for propionate, 214.1 → 143.1 for ^13^C_6-propionate, 222.1 → 137.1 for butyrate, and 228.1 → 143.1 for ^13^C_6-butyrate. The analytes and internal standards eluted at 6 minutes. Acetate and ^13^C_6-acetate peaked at 1.5 minutes, propionate and ^13^C_6-propionate peaked at 1.9 minutes, and butyrate and ^13^C_6-butyrate peaked at 2.6 minutes. All the samples were analyzed in triplicate.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from mouse arteries using the RNeasy kit (Qiagen), and cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen). Gene expression was assessed by quantitative real-time polymerase chain reaction using gene-specific primers of FFAR3 and housekeeping genes obtained from Qiagen: FFAR3 (QuantiTect primer assay QT01299144), β-actin (QT00095242), and GAPDH (QT00199633). Relative gene expression of FFAR3 was compared with the geometric mean of β-actin and GAPDH, as previously described.22

Cell Culture and Scratch Assays

Primary cultures of human umbilical vein endothelial cells (ECs) at low passage were kindly provided by Dr William Muller (Northwestern University) and cultured in EGM-2 Endothelial Cell Growth Medium-2 BulletKit (Lonza, Basel, Switzerland). Human aortic VSMCs were obtained from Lonza and cultured in vascular cell basal medium containing the VSMC growth kit (ATCC, Manassas, VA). Scratch assays were performed, as previously described.13 Cells were washed once, and 1-methylcyclopropane carboxylate (1-MCPC) (Sigma) (50–150 μmol/L) was added to starvation media containing 0% serum. The scratch was photographed at 24 and 48 hours. Treatment groups were performed in triplicate in each experiment. Closure of the scratch was quantified by area (for human umbilical vein ECs) or by cell number (for human aortic VSMCs) using ImageJ/FIJI. All experiments were conducted at least 3 times.

Statistical Analysis

Statistical analysis was performed using Prism (GraphPad Software, San Diego, CA). Summary data are expressed for each group as mean±SEM. For comparison between 2 groups, an unpaired Student t test or Mann-Whitney U test was used, depending on the distribution of these data. P<0.05 was considered significant.

RESULTS

Depletion of Microbiota by Oral Vancomycin Exacerabtes Neointimal Hyperplasia After Arterial Injury

Femoral artery wire injury was performed in C57BL/6 males (16–18 weeks old) after treatment with oral vancomycin. Vancomycin-treated mice had significantly more neointimal hyperplasia development 2 weeks after femoral artery wire injury compared with control-treated mice (Figure 1A and 1B), which correlated with significantly lower stool and serum concentrations of major SCFA butyrate, acetate, and propionate (Figure 1C). However, exogenous butyrate replacement in the vancomycin+butyrate–treated group partially mitigated the effect of vancomycin (Figure 1A and 1B). As shown in Figure 1B, neither vancomycin nor vancomycin+butyrate treatment affected the histological features of uninjured (right-sided) femoral arteries. As expected, vancomycin treatment also correlated with significant divergence in β diversity of fecal microbial samples from control samples (Figure 1C). Collectively, these data suggest an inverse correlation between microbe-derived butyrate and neointimal hyperplasia susceptibility after arterial injury.

Effect of FFAR2 and FFAR3 on Neointimal Hyperplasia

FFAR2 and FFAR3 are both GPCRs for butyrate. We observed no gross differences in uninjured FFAR2 and FFAR3 WT and knockout femoral arteries by histological analysis (hematoxylin and eosin, Masson trichrome, or elastin staining) (Figure 2A, 2B, and 2C). Femoral artery wire injury was performed in age- and sex-matched FFAR2 and FFAR3 WT and knockout mice (16–18 weeks old) to clarify the role of these receptors to the arterial remodeling response to injury.

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There was a 1.4-fold increase in neointimal hyperplasia development in FFAR3 knockout mice compared with WT littermates (neointima area, WT versus knockout, 0.015±0.001 versus 0.022±0.002 mm²; \( P=0.01 \)), but no difference between FFAR2 knockout mice and WT littermates (neointima area, WT versus knockout, 0.017±0.001 versus 0.013±0.001 mm²; \( P=\text{not significant} \)). Complete morphometric data, including media area, neointima/(neointima+media) and neointima/media, are shown in the Table. There were no instances of postoperative death or arterial thrombosis. Representative images of arterial cross-sections from injured and uninjured WT and knockout arteries are shown in Figure 2D, and scatterplots of neointima area are shown in Figure 2E. FFAR3 knockout mice also had delayed reendothelialization compared with WT mice, as assessed by in situ Evans blue dye staining of injured arteries 5 days after arterial injury (Figure 3A and 3B). These data suggest a role for FFAR3, but not FFAR2, in modulating neointimal hyperplasia development after arterial injury, potentially by regulation of endothelial recovery.

**Expression of FFAR3 in Murine Peripheral Arteries**

We next assessed the distribution of FFAR3 gene expression in peripheral arteries using quantitative...
Figure 2. FFAR3 (Free fatty acid receptor 3), but not FFAR2 (free fatty acid receptor 2), modulates neointimal hyperplasia development after arterial injury.

Representative arterial sections of femoral arteries from FFAR2 and FFAR3 wild-type (WT) and knockout (KO) mice were analyzed histologically using hematoxylin-eosin (A), Masson trichrome (B), and elastin (C) staining. Bar=50 μm. Lumen orientation of each arterial section is indicated by “L.” Femoral artery wire injury was performed in FFAR2 and FFAR3 KO mice and their respective WT littermates. Mice were euthanized 2 weeks after surgery, and neointimal hyperplasia was assessed in explanted arteries (N=10–12 per group).

**D.** Representative photomicrographs of arterial sections from injured FFAR2 WT and KO arteries are shown (left panel). There was no significant difference in neointimal hyperplasia (P=0.91) between FFAR2 WT and KO mice (right panel) (N=10-12 per group).

**E.** Representative photomicrographs of arterial sections from injured FFAR3 WT and KO arteries are shown (left panel). There was a significant increase in neointimal hyperplasia in FFAR3 KO compared with WT mice (right panel) (N=10–12 per group). Magnification and lumen orientation of arterial sections in D and E are the same as in A through C. Lines on each scatterplot represent mean±SEM, and statistical analyses were performed with the Mann-Whitney U test (D and E).
real-time polymerase chain reaction. FFAR3 mRNA was detected at varying levels in uninjured WT carotid artery, aorta, and femoral artery (Figure 4A). In the femoral artery, FFAR3 mRNA increased 3-fold by 3 days after injury, as shown in Figure 4B.

Table. Morphometric Parameters of FFAR2 and FFAR3 WT and Knockout Femoral Arteries 2 Weeks After Wire Injury

| Parameter                        | FFAR2                  | P Value | FFAR3                  | P Value |
|----------------------------------|------------------------|---------|------------------------|---------|
|                                  | WT                     | Knockout|                       | WT                  | Knockout| |
| Neointima, mm²                   | 0.017±0.001            | 0.013±0.001| 0.91                  | 0.015±0.001| 0.022±0.002| 0.01*   |
| Media, mm²                       | 0.0134±0.0006          | 0.0147±0.002| 0.53                  | 0.0155±0.0004| 0.0136±0.0004| 0.007*  |
| Neointima/(neointima+media)      | 0.375±0.016            | 0.439±0.034| 0.07                  | 0.418±0.016| 0.526±0.019| 0.001*  |
| Neointima/media                  | 0.756±0.062            | 0.775±0.263| 0.13                  | 0.709±0.036| 1.518±0.177| 0.007*  |

Values presented are mean±SEM. FFAR2 indicates free fatty acid receptor 2; FFAR3, free fatty acid receptor 3; and WT, wild type.

*P<0.05.

Figure 3. FFAR3 (Free fatty acid receptor 3) modulates endothelial recovery after arterial injury. FFAR3 wild-type (WT) and knockout (KO) mice underwent femoral artery wire injury. At 3 or 5 days after injury, in situ Evans blue staining was performed. Arteries were explanted, opened longitudinally, and photographed adjacent to a ruler. A, Representative arteries explanted from an uninjured and injured femoral artery at day 0 (left panel) and at 5 days (right panel), showing increased recovery of the endothelium (ie, lack of blue staining) in the WT compared with the KO artery. Dotted lines indicate area of blue staining. B, Blue staining was quantified in each artery at 3 and 5 days. There was no significant difference in staining between WT and KO mice at 3 days (P=0.18). Lines on each scatterplot represent mean±SEM. N=5 to 6 per group per time point. Statistical analysis was performed with the Mann-Whitney U test (B). L indicates left; and R, right.

Figure 4. Ffar3 gene expression in murine peripheral arteries. A, Total RNA was isolated from the indicated peripheral arteries of adult C57BL/6 mice, and cDNA was reverse transcribed, as described in the Methods section. Ffar3 mRNA was quantified by quantitative polymerase chain reaction and gene expression was normalized to the geometric mean of Actb and Gapdh housekeeping genes and expressed as Ffar3 relative expression. B, Relative expression of Ffar3 in uninjured and injured femoral arteries. Arteries were harvested 3 days after injury. Four arteries were pooled together in each RNA sample and hence each bar represents 12 to 16 pooled arteries. Statistical analysis was performed with the Mann-Whitney U test (B).
Effect of Pharmacologic FFAR3 Agonists of EC and VSMC Migration and Proliferation

1-MCPC is a known pharmacologic agonist of FFAR3. Because FFAR3 appears to both modulate in vivo recovery of the endothelial layer after femoral artery wire injury and be expressed in the arterial wall, we next examined the effect of FFAR3 agonism in vitro. Human umbilical vein ECs and human aortic VSMCs were subjected to in vitro scratch assays with increasing doses of 1-MCPC or vehicle. Human umbilical vein ECs demonstrated augmentation in closure of the scratch area after treatment with 1-MCPC (Figure 5A). In contrast, 1-MCPC had no effect on human aortic VSMCs in the scratch assay (Figure 5B). These data suggest that activation of FFAR3 by the pharmacologic agonist 1-MCPC stimulates EC but not VSMC migration and proliferation, providing phenomenological evidence of cell-specific activity of FFAR3.

DISCUSSION

The microbe-derived metabolite butyrate has been shown to have myriad physiological effects, including in the cardiovascular system, where it has a regulatory role in hypertension,23–26 hypertrophic cardiomyopathy,27 and atherogenesis.28 Prior work by others has also demonstrated that butyrate has cell-specific effects on gene transcription because of its activity as a histone deacetylase inhibitor.29 Butyrate and other major SCFAs, propionate and acetate, are also ligands of FFAR2 and FFAR3, which are GPCRs that couple the Gq (FFAR2) and/or Gi/o (FFAR2 and FFAR3) pathways.15,30 In this study, we demonstrate that antibiotic treatment alters gut microbial communities, depletes systemic SCFA concentration, and exacerbates neointimal hyperplasia development in a murine model of arterial injury, which is attenuated by exogenous butyrate dietary supplementation; that murine FFAR3 is present in multiple peripheral arteries; that FFAR3, but
not FFAR2, knockout mice have worsened neointimal hyperplasia development and delayed endothelial recovery after arterial injury compared with WT controls; and that pharmacologic FFAR3 agonists promote EC but not VSMC migration and proliferation. This study adds to our previous data suggesting a role for gut microbes in arterial remodeling after vascular surgery, and specifically provides a mechanistic link between microbe-derived butyrate, FFAR3, the endothelial response to injury, and neointimal hyperplasia.

This study corroborates our earlier study in rats and work by many others that antibiotics significantly alter the gut microbial community and deplete butyrate. We specifically found that serum levels of butyrate were significantly depleted but still detectable after vancomycin treatment despite the steep concentration gradient of SCFAs between the intestinal lumen and periphery. In humans, although there is an association between free fatty acids and atherosclerotic disease, the association between free fatty acids and arterial restenosis after cardiovascular procedures is unknown. We used oral vancomycin as a tool to reduce gut biomass and thus the primary source of butyrate production. Although vancomycin has been shown to be toxic to ECs in vitro, this was demonstrated only with doses expected after intravenous infusions lasting 24 to 72 hours. Because oral vancomycin is not absorbed systemically, we would not anticipate any vancomycin-related change in FFAR3 expression or endothelial function. Moreover, we did not observe any histologic changes in uninjured mouse femoral arteries after oral vancomycin treatment (Figure 1B).

Existing data on the gene expression pattern of FFAR3 in the vasculature are also conflicting. A survey of tissues from adult C57BL/6 mice assessed transcript levels for 353 GPCRs and identified undetectable relative expression of FFAR3 in the aorta and low levels in the vena cava. However, Pluznick et al. identified FFAR3 gene expression by real-time polymerase chain reaction in WT murine aorta, renal artery, and iliac artery, with relative expression highest in the renal artery>iliac artery>aorta. Interestingly, Natarajan et al. further localized aortic FFAR3 gene expression to the endothelial layer, because expression was not detected after endothelial denudation. EC expression of FFAR3 was also reported by Li et al. Like Pluznick et al., we also found differential FFAR3 gene expression in peripheral arteries (carotid>aorta>iliofemoral). Because our injury model is in the femoral artery, we also assessed for change in gene expression 3 days after injury and noted a marked increase in gene expression corresponding to a time point when the endothelial layer is recovering. Like others, we found that commercially available antibodies to FFAR3 did not provide specific staining and hence were not used to assess protein expression.

Although butyrate has been shown to have multiple effects in ECs, including regulation of vascular adhesion molecule expression, decreased inflammasome activation, reduced inflammatory cytokine release to inflammatory stimuli, reduction of the oxidative stress response, and modulation of cell proliferation and angiogenesis in a dose-specific manner, and enhancement of the efficacy of induced pluripotent stem cell derivation from human adult or fetal fibroblasts, the distinction between FFAR3-mediated effects and effects mediated by histone deacetylase inhibition is unclear. Thus, we used a pharmacologic agonist of FFAR3, 1-MCPC, to specifically investigate the effect of cell-specific effects of FFAR3. Furthermore, because re-endothelialization strategies halt VSMC proliferation and decrease neointima formation, we examined the role of 1-MCPC on both ECs and VSMCs in culture. Our data that 1-MCPC stimulates EC but not VSMC migration and proliferation, in combination with our observation that FFAR3 knockout mice had delayed endothelial recovery in vivo, implicate a protective effect of FFAR3 activation in the arterial remodeling response after injury.

Previous studies of FFAR3 knockout mice have suggested a role for FFAR3 in inflammation, asthma, metabolic disorders, diabetes mellitus, immune function, and sympathetic tone. This is the first report linking FFAR3 to the arterial injury response, and opens the door for future exploration of FFAR3 agonists as therapeutics or adjuncts for the prevention of neointimal hyperplasia after cardiovascular procedures.

There are several limitations to this study. Because concomitant vancomycin+butyrate treatment ameliorated the effect of vancomycin alone on neointimal hyperplasia, it is possible that other microbe-derived SCFAs, including propionate and acetate, which are also ligands for FFAR2 and FFAR3, have similar or related effects. However, the mitigation of the vancomycin phenotype with concomitant butyrate supplementation justifies our current focus on butyrate rather than other SCFAs. Strategies to boost in vivo butyrate in FFAR3 WT and knockout mice are under active investigation and will help strengthen the link between butyrate, FFAR3, and restenosis without concern for a competing effect of vancomycin. Furthermore, although we used human instead of mouse ECs and VSMCs for in vitro studies because of the availability of the human cells, other investigators have observed significant differences in the responses of human and mouse monocytes to stimulation by FFAR2 and FFAR3 agonists. This could be addressed by repeating the in vitro experiments using mouse-derived WT and knockout ECs and VSMCs or using species-specific FFAR3 knockout strategies (eg, small interfering RNA) to test cell-specific effects of FFAR3 activation. These gene knockdown strategies and the use of other pharmacologic receptor agonists...
and antagonists of FFAR3 will also strengthen the link and provide important insights into the signaling events mediating FFAR3 activation and EC migration and proliferation. Furthermore, our study does not address the molecular mechanism by which FFAR3 modulates EC migration and proliferation or the possibility that modulation of systemic inflammation by FFAR3, and not direct FFAR3-dependent effects on ECs at the local site of arterial injury, contributes to neointimal hyperplasia; both possibilities are areas currently under active investigation. Finally, although the larger study includes mice from both sexes, females will be analyzed and reported separately as there is known sexual dimorphism in both neointimal hyperplasia susceptibility and the microbiome.71

In summary, the present study demonstrates that microbe-derived butyrate has a protective role in neointimal hyperplasia development after arterial injury, which may be mediated by promotion of EC migration and proliferation in an FFAR3-dependent manner. The results highlight the potentially important role of the microbe-host interaction in the arterial remodeling response after cardiovascular surgery.

ARTICLE INFORMATION
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Disclosures
None.

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