The short-chain fatty acid propionate protects from hypertensive cardiovascular damage


This is a copy of the accepted manuscript, as originally published online ahead of print by the American Heart Association. The original article has been published in:

Circulation
2018 DEC 04 (first published online)
doi: 10.1161/CIRCULATIONAHA.118.036652

Publisher: Wolters Kluwer Health, Inc. on behalf of the American Heart Association, Inc.
The Short-Chain Fatty Acid Propionate Protects from Hypertensive Cardiovascular Damage

Running Title: Bartolomaeus et al.; Short-Chain Fatty Acid Propionate and Hypertension

Hendrik Bartolomaeus, et al.

The full author list is available on page 21.

Address for Correspondence:
Dominik N. Müller, PhD
Experimental and Clinical Research Center & Max Delbruck Center for Molecular Medicine in the Helmholtz Association
Lindenberger Weg 80, 13125 Berlin, Germany
Tel: +49-30-450540286
Fax: +49-30-450540944
Email: dominik.mueller@mdc-berlin.de
Abstract

**Background:** Arterial hypertension and its organ sequelae show characteristics of T cell mediated inflammatory diseases. Experimental anti-inflammatory therapies have been shown to ameliorate hypertensive end-organ damage. Recently, the CANTOS study targeting interleukin-1β demonstrated that anti-inflammatory therapy reduces cardiovascular risk. The gut microbiome plays pivotal role in immune homeostasis and cardiovascular health. Short-chain fatty acids (SCFA) are produced from dietary fiber by gut bacteria and affect host immune homeostasis. Here, we investigated effects of the SCFA propionate in two different mouse models of hypertensive cardiovascular damage.

**Methods:** To investigate the effect of SCFA on hypertensive cardiac damage and atherosclerosis, wild-type NMRI (WT) or ApoE−/− deficient mice received propionate (200mM) or control in the drinking water. To induce hypertension, WT mice were infused with Angiotensin (Ang)II (1.44mg/kg/d s.c.) for 14 days. To accelerate the development of atherosclerosis, ApoE−/− mice were infused with AngII (0.72mg/kg/d s.c.) for 28 days. Cardiac damage and atherosclerosis were assessed using histology, echocardiography, in vivo electrophysiology, immunofluorescence, and flow cytometry. Blood pressure was measured by radiotelemetry. Regulatory T cell (Treg) depletion using PC61 antibody was used to examine the mode of action of propionate.

**Results:** Propionate significantly attenuated cardiac hypertrophy, fibrosis, vascular dysfunction, and hypertension in both models. Susceptibility to cardiac ventricular arrhythmias was significantly reduced in propionate-treated AngII-infused WT mice. Aortic atherosclerotic lesion area was significantly decreased in propionate-treated ApoE−/−. Systemic inflammation was mitigated by propionate treatment, quantified as a reduction in splenic effector memory T cell frequencies and splenic T helper 17 cells in both models, and a decrease in local cardiac immune cell infiltration in WT mice. Cardioprotective effects of propionate were abrogated in Treg-depleted AngII-infused mice, suggesting the effect is Treg-dependent.

**Conclusions:** Our data emphasize an immune-modulatory role of SCFAs and their importance for cardiovascular health. The data suggest that lifestyle modifications leading to augmented SCFA production could be a beneficial non-pharmacological preventive strategy for patients with hypertensive cardiovascular disease.

**Key Words:** Angiotensin II; APOE; Inflammation; Immunology; Metabolite; Microbiome; Short-chain fatty acids; Regulatory T cells; Th17 cells; Bacterial Metabolites
Clinical Perspective

What is new?

- The present study shows that propionate, a short-chain fatty acid produced by intestinal bacteria, has profound beneficial anti-inflammatory properties limiting cardiovascular disease progression in two independent mouse models.
- Oral propionate administration beneficially influenced T helper cell homeostasis, thereby reducing cardiac hypertrophy and fibrosis, susceptibility to cardiac arrhythmias, and atherosclerotic lesion burden.
- Propionate exhibited anti-hypertensive effects in both models.
- Cardioprotective effects of propionate effects are mainly dependent on regulatory T cells.

What are the clinical implications?

- The data suggest increased attention to propionate in hypertensive patients with a high cardiovascular risk and evidence of ongoing chronic inflammation.
- Oral propionate supplementation is simple, inexpensive and regarded safe in humans.
- Further studies are needed to characterize the effect of propionate supplementation or interventions increasing propionate production by the gut microbiota in humans.
Introduction

Hypertension drives cardiovascular disease by causing an array of pathologic organ sequelae. Hypertensive tissue damage is largely mediated by different immune cells, which are activated in hypertension. Hypertensive stimuli like Angiotensin II (AngII) promote the activation of T cells and macrophages, which subsequently may infiltrate target organs like the heart and the vasculature to cause tissue damage. Increased generation of effector memory T cells (TEM) is indicative of a chronic inflammatory response in hypertension. In particular, T helper cell subtypes Th17 and Th1 may promote hypertension and target organ injury by releasing pro-inflammatory cytokines like interleukin (IL)-17A and interferon-γ (IFN-γ), respectively. In contrast, regulatory T cells (Treg) counterbalance tissue inflammation by secreting anti-inflammatory IL-10. Thus, the extent of hypertensive target organ damage is not solely dependent on the hemodynamic load but additionally depends on the balance of pro- and anti-inflammatory immune cells. In clinical practice, hypertension usually coexists with atherosclerosis and accelerates atherosclerosis progression. Atherosclerosis is similarly a chronic inflammatory disease of the vasculature, although the precise nature of the inflammatory response differs from hypertension. Inflammation and vascular damage are intensified when both hypertension and atherosclerosis are present. Furthermore, experimental and clinical anti-inflammatory approaches, as recently shown in CANTOS trial, ameliorate hypertensive inflammatory tissue damage and atherosclerosis. In particular, enhancement of Treg by adoptive transfer has been shown to reduce effector T cells and ameliorate cardiac damage, hypertension, vascular injury and atherosclerosis.

The gut microbiota is considered important for cardiovascular health and abnormal bacterial communities have been associated with hypertension and atherosclerosis.
Bacterial metabolites mediate interactions with the host. Through resorption and distribution, they can affect intestinal health as well as distant functions of the immune system, the vasculature, and the heart. Short-chain fatty acids (SCFA) are a major class of bacterial metabolites and are mainly produced in the colon by bacterial fermentation of otherwise indigestible polysaccharides (fibers).\(^{20}\) Beyond their importance for intestinal integrity, SCFA have potent anti-inflammatory effects on immune cell functions. The SCFA propionate (C3) has been shown to induce the differentiation and suppressive capacity of CD25\(^+\) Foxp3\(^+\) Treg.\(^{21,22}\) Propionate treatment of murine Treg donors significantly enhanced the protective effect of a Treg adoptive transfer in experimental autoimmune encephalomyelitis.\(^{23}\) In vitro, propionate was shown to enhance the differentiation of different murine T cells dependent on the cytokine milieu.\(^{24}\) Interestingly, propionate promoted a regulatory phenotype among T cells under Th17 polarization conditions.\(^{24}\) Beneficial effects of SCFA have been demonstrated in several other disease models, such as colitis, airway disease, metabolic syndrome or ischemia-reperfusion injury of the kidney.\(^{25}\) Although their mode of action has yet to be elucidated, SCFA may exert their effects via inhibition of histone deacetylases (HDAC) or via G-protein coupled receptors (Gpr) 41, 43 and olfactory receptor (Olfr) 78.\(^{25,26}\) In addition, a direct effect of SCFA on renin release and vasomotor function leading to blood pressure reduction was recently suggested.\(^{26-28}\)

Considering the prominent role of inflammation in both hypertension and atherosclerosis and recent insights about anti-inflammatory and Treg-promoting effect of SCFA, we hypothesized that the SCFA propionate protects from AngII-induced cardiac and vascular damage. To test our hypothesis, we treated AngII-infused wild-type NMRI (WT) and AngII-infused apolipoprotein E knockout (ApoE\(^{-/-}\)) mice with C3 and analyzed inflammatory response, cardiac remodeling, and
atherosclerotic lesion burden. Our data underscore the importance of propionate for cardiovascular health.

Methods

The authors declare that all supporting data and analytical methods are available within the article and its online supplementary files. The data, analytical methods, and study materials that support the findings of this study are available from the corresponding author upon reasonable request.

Animal ethics

All experiments were in accordance with the German/European law for animal protection and were approved by the local ethic committees (G0280/13 and G250/12). Mice were maintained on a 12:12 hour day:night cycle with constant access to food and water.

Angiotensin II-induced hypertension in wild-type mice

Twelve week old male NMRI mice (purchased from Janvier Labs, outbred strain, not genetically altered, referred to as wild-type (WT) in the manuscript), received an angiotensin (Ang)II infusion (1.44 mg/kg/d, Calbiochem) for 14 days via subcutaneous osmotic minipumps (Alzet). Implantation was performed under isoflurane anesthesia. Mice were fed a purified diet low in fiber (Ssniff, S0087-E050). To study the effects of propionate, mice received sodium propionate (200 mM, Sigma Aldrich) or sodium chloride as control (200 mM, Sigma Aldrich) in the drinking water ad libitum. On day 13, echocardiography was performed under isoflurane anesthesia. After 14 days of AngII infusion the mice were sacrificed, blood and organs were collected for further analysis. In a subset of mice, blood pressure was measured using an
implantable subcutaneous radiotelemetry transmitter (DSI), implanted on day -10 prior to AngII infusion.

Treg depletion in WT mice was achieved by intraperitoneal injections of anti-CD25 antibody (clone PC61, kindly provided by T. Hünig, University of Würzburg, Germany; 250 µg on days -1, 2 and 5 of AngII infusion). Control mice received the IgG1 isotype (Bio X Cell). Control and Treg-depleted mice received a low-fiber diet, sodium propionate in drinking water, AngII infusion s.c. and implantable radiotelemetry blood pressure transmitters.

All mice were held in one room of the animal facility. Animals were age-matched, stratified for their body weight and randomized to the different treatment groups (n=18 WT Sham, n=36 WT AngII, n=33 WT AngII+C3, n=4 WT AngII+C3+IgG, n=4 WT AngII+C3+PC61). n=2 mice from the WT AngII+C3 group were excluded because of suture insufficiency at the implantation site of the minipump. Mice assigned to the same treatment group were held in the same cage. Cages were inhabited by n=2-3 mice. All researchers were blinded during the experiment and analysis. Measured values were excluded if a technical failure of the analysis occurred or by statistical testing described below. Animal numbers for the WT experiment were a priori calculated with G*power software (Heinrich-Heine-Universität Düsseldorf, Germany) and an estimated effect size from the literature. For Treg-depletion experiments sample sizes were calculated using G*power and the estimated effect size from the WT experiments. Exact n-numbers are shown in the respective figure.

**Angiotensin II-induced atherosclerosis in ApoE<sup>-/-</sup> mice**

To verify the effects of propionate in another AngII-dependent cardiovascular mouse model, we employed ApoE<sup>-/-</sup> mice. In line with the NC3Rs recommendation, we reduced sample sizes by working without a sham group. ApoE<sup>-/-</sup> mice (Taconic) were backcrossed on a C57BL/6
background at least 10 times. Eight-week old mice received sodium propionate (200 mM, Sigma Aldrich) or sodium chloride as control (200 mM, VWR) in the drinking water ad libitum during the whole experimental period, starting 5 days prior to the implantation of the osmotic minipumps. AngII osmotic minipumps (0.72 mg/kg/d, Sigma) were implanted subcutaneously as described previously. In the fourth week of AngII infusion, systolic blood pressure was measured in conscious mice by tail cuff plethysmography (BP-98A; Softron). During week four, ten measurements per mouse were recorded daily. For habituation during week 3 of AngII minipump implantation, mice were trained daily for 5 consecutive days as follows: Mice were set daily for at least 15 minutes in the mice restrainer; each day at least 20 blood pressure measurements were performed. Systolic blood pressure mean was calculated from all measured days per mouse. After four weeks of AngII infusion mice were sacrificed, blood and organs collected and used for further analyses.

For ApoE−/− experiments, housing, randomization, blinding and exclusion were carried out as described for the WT experiments. n=30 mice were randomized per group. None of the mice were excluded during the experiment. Sample sizes were calculated using G*power and the estimated effect size from the WT experiments. Exact n-numbers are shown in the respective figures.

**Statistical analysis**

Outliers identified by Grubbs’ test were excluded and normality was assessed by Kolgomorov-Smirnov test. In groups with n < 5 normality could not be assessed and non-parametric distribution was assumed. To compare two groups, a one-tailed unpaired t-test or one-tailed Mann-Whitney test was used, as appropriate. To compare more than two groups a one-way ANOVA followed by post-hoc Tukey’s test, or a Kruskal-Wallis test with post-hoc Dunn’s test
was used, as appropriate. Survival of the mice was visualized by Kaplan-Meier curves and statistically compared by using a one-tailed Log-rank test. Statistical analyses were performed using GraphPad Prism 6. Blood pressures time courses measured by telemetry were compared using linear mixed model calculations in R (Version 3.1.1 R Foundation). We calculated a model for each time point by including all time points before. Thereby, we received a specific time point for each longitudinal analysis where the p-value fell short of 0.05. To compare the blood pressure area under the curve (AUC) per week of AngII infusion we used a two-way repeated measures ANOVA followed by a post-hoc Sidak’s test (GraphPad Prism 6). A p-value <0.05 was considered statistically significant.

Results

Propionate prevents AngII-induced systemic inflammatory response

To investigate the effect of propionate (C3) on immune homeostasis in an established model for hypertensive cardiac damage, wild-type NMRI mice (WT) were infused with AngII (1.44 mg/kg/d) for two weeks and concomitantly treated with C3 (sodium propionate, 200 mM) or control (sodium chloride, 200 mM) in the drinking water (Figure 1A). Saline-infused WT controls served as non-hypertensive shams. To specifically investigate the effect of exogenous C3, mice were fed a fiber-depleted purified diet to reduce intestinal SCFA production. C3 administration significantly increased C3 serum levels in AngII-infused WT mice as measured by GC-MS (Figure S1A). C3 was well tolerated, as indicated by similar body weights in mice treated with C3 or control (Figure S1B). C3 significantly improved the survival compared to control-treated AngII-infused WT mice (Figure 1B). To investigate the role of C3 on systemic inflammation, spleens were harvested after 14 days and analyzed by flow cytometry. As
compared to sham treated mice, AngII increased splenic CD4+ T_{EM} (CD44+ CD62L-) and conversely decreased CD4+ naïve T cells (T_{N}; CD44- CD62L+), indicating a significant inflammatory response. The increase in T_{EM} and the decrease in T_{N} were prevented by C3 treatment (Figure 1C). CD4+ central memory T cells (T_{CM}; C44+CD62L+) remained unaffected (Figure S1C). Further analysis of splenic Th17 cells revealed an increase in CD4+IL-17A*IL-10- and CD4*RORγt*Foxp3 frequencies after AngII infusion, which was normalized by C3 treatment (Figure 1D-E). Splenic Th1 frequencies, as measured by CD4*Tbet (Figure S1D) and CD4*IFN-γ+ cells (Figure S1E), were not affected. Interestingly, Treg frequencies (CD4*CD25*Foxp3+ and CD4*IL-10*IL-17A-) increased after AngII, signaling a compensatory Treg-response to the hypertensive stimulus, which was not observed upon C3 treatment (Figure 1C-D).

We next tested whether C3 also affects immune homeostasis in hypertensive mice prone to develop atherosclerosis. Therefore, we infused atherosclerosis-prone ApoE-/- mice fed a normal chow with AngII (0.72 mg/kg/d) for 4 weeks and administered C3 or control (200 mM) via the drinking water (Figure 2A). Similar to WT mice, C3 had no nutritive effect (Figure S1F). C3 treatment protected AngII-infused ApoE-/- mice from aortic rupture and thereby reduced mortality during the 4 weeks of AngII infusion (Figure 2B). In congruence with our findings in hypertensive mice without atherosclerosis, C3 reduced splenic T_{EM} and increased splenic T_{N} populations in AngII-infused ApoE-/- (Figure 2C), while splenic T_{CM} were not significantly regulated (Figure S1G). In addition, C3 treatment significantly reduced CD4*Foxp3*RORγt+ Th17 cells, while CD4*CD25*FoxP3+ Treg were not affected (Figure 2D). Concordant with WT, no significant regulation of Th1 cells was observed (Figure S1H). Our data suggest that C3...
treatment ameliorates systemic inflammation in hypertensive mice with and without atherosclerosis.

**Propionate attenuates vascular inflammation and atherosclerosis**

Inflammation of the vascular wall is a hallmark of atherosclerosis and is amplified in the presence of elevated AngII levels. To investigate if C3 modulates the atherosclerotic inflammatory response, we analyzed aortic immune cells from AngII-infused ApoE−/− by flow cytometry. Aortic CD4+, CD8+ T cell and F4/80+ macrophage numbers were reduced after C3 treatment (Figure 3A, S2A). Similar to splenic immune cells, the frequencies of aortic CD4+ TEM decreased and CD4+ TN increased after C3 treatment, while CD4+ TCM remained unaltered (Figure 3B, S2B-C). Further verification by immunohistochemical staining was obtained in atherosclerotic plaques of the brachiocephalic artery (BCA). Fewer CD3+ T cells and F4/80+ macrophages were detected in BCA sections from C3 treated AngII-infused ApoE−/− mice (Figure 3C-D). To determine whether these potentially beneficial effects would translate into a reduction in atherosclerotic lesion burden, we performed en face Oil Red O staining of whole aortas. Aortic atherosclerotic lesion burden was significantly reduced in C3 treated mice (Figure 3E). Likewise, considerably less stenosis of the BCA was detected in C3 treated mice (Figure 3F).

Since AngII infusion may induce cardiac remodeling in addition to atherosclerosis, we measured heart weight and analyzed cardiac fibrosis in AngII-infused ApoE−/− by Sirius red staining. Hypertrophy index and interstitial fibrosis were significantly attenuated in C3 treated AngII-infused ApoE−/− (Figure 3G-H). C3 did not affect serum levels of total cholesterol, triglycerides, HDL or LDL cholesterol (Table S1). Taken together, C3 treatment reduced vascular inflammation, atherosclerotic lesion burden and cardiac remodeling in AngII-infused ApoE−/− independent of blood lipid levels.
Propionate ameliorates cardiac immune cell infiltration and remodeling

Alongside vascular injury, hypertension generates an inflammatory response in the heart, which promotes cardiac remodeling.\textsuperscript{30} Flow cytometric analysis of heart-infiltrating lymphocytes on day 14 of AngII infusion in WT mice revealed a significant increase in the number of cardiac CD4\(^+\) T cells, CD8\(^+\) T cells and F4/80\(^+\) macrophages, which was significantly decreased by C3 treatment (Figure 4A-E). These results could be confirmed by analysis of CD4 and CD8 immunofluorescence of cardiac cryosections (Figure S3A-B). We further analyzed the proportion of Th17, Treg and Th1 subsets among infiltrating cardiac T cells. The AngII-induced increase in cardiac CD4\(^+\) ROR\(\gamma\)t\(^+\)Foxp3\(^-\) frequencies was prevented by C3 treatment, while the fraction of CD4\(^+\)FoxP3\(^+\)ROR\(\gamma\)t\(^-\) T cells and CD4\(^+\)T-bet\(^+\) T cells was similar between groups (Figure 4F, S3C). IL-10 mRNA levels in cardiac tissue were analyzed by qPCR. In line with IL-10 expression in CD4\(^+\) splenocytes, C3 prevented the AngII-induced increase in \textit{Il-10} expression (Fig. S3D).

As expected, AngII increased the cardiac hypertrophy index after 14 days of infusion, an effect that was prevented by C3 treatment (Figure 4G). We confirmed this finding using echocardiography, which revealed an increased left ventricular wall thickness after AngII infusion and a significant reduction upon C3 treatment (Figure 4H). Accordingly, the AngII-induced increase in cardiac brain natriuretic peptide (\textit{Nppb}) and \(\beta\)-myosin heavy chain (\textit{Mhy7}) mRNA expression was prevented by C3 treatment as measured by qPCR (Figure 4I-J). C3 treatment also prevented the AngII-induced increase in interstitial and perivascular cardiac fibrosis as measured by fibronectin and collagen I immunofluorescence, respectively (Figure 4K-L). Consistently, the number of cardiac fibroblasts analyzed using fibroblast specific protein (FSP)-1 immunofluorescence was similarly regulated (Figure 4M). Cardiac mRNA expression of
connective tissue growth factor (Ctgf) and neutrophil gelatinase-associated lipocalin (Ngal) also confirmed the anti-fibrotic effect (Figure S3E-F).

SCFA have been attributed histone deacetylase (HDAC) inhibitory properties\textsuperscript{31} and HDAC inhibition is known to inhibit AngII-induced cardiac hypertrophy and fibrosis.\textsuperscript{32} To address this potential mechanism of action, we cultured rat neonatal cardiomyocytes \textit{in vitro} in the presence or absence of AngII and tested the effect of C3 on atrial natriuretic peptide (Nppa) mRNA expression as a sensitive hypertrophy marker in comparison to the known class I and II HDAC inhibitor Trichostatin A (TSA). In contrast to TSA, C3 did not reduce \textit{Nppa} mRNA expression (Figure S4), suggesting that the effect of C3 on AngII-induced cardiac hypertrophy is HDAC-independent.

**Effect of propionate depends on Treg**

C3 has been shown to promote Treg generation and function.\textsuperscript{22} We hypothesized that the observed beneficial effects of C3 in AngII-infused hypertensive WT mice are Treg-dependent. To test this hypothesis, we depleted Tregs in C3-treated AngII-infused WT mice by injecting anti-CD25 antibody (i.p. injections of antibody clone PC61 on days -1, 2 and 5 of AngII infusion). We assessed inflammation and cardiac fibrosis in comparison to non-depleted C3-treated AngII-infused WT mice receiving IgG control antibodies i.p. Treg depletion was well-tolerated and had no effect on body weight (Figure S5A). On day 14 of AngII infusion, splenic Treg were still significantly depleted compared to the IgG control group (Figure 5A). Treg-depleted AngII-infused WT mice treated with C3 displayed a significant increase in splenic CD4\textsuperscript{+}IL-17A\textsuperscript{+} cell frequencies compared to non-depleted mice (Figure 5B), reinforced by a similar trend in splenic CD4\textsuperscript{+}RORyt\textsuperscript{+} Foxp3\textsuperscript{-} frequencies (Figure S5B). Splenic Th1 cells, as measured by IFN-\gamma and T-bet expression in CD4\textsuperscript{+} cells, were not regulated (Figure S5C-D). The
inhibitory effect of C3 on splenic CD4+ T_{EM} frequencies was abrogated in Treg-depleted mice (Figure 5C), without altering T_{CM} or T_{N} populations (Figure S5E-F). Significantly more CD4+ and CD8+ lymphocytes could be detected in heart sections of Treg-depleted AngII-infused mice treated with C3 (Figure 5D-E). Cardiac hypertrophy measured by echocardiography and hypertrophy index was only non-significantly increased in Treg-depleted C3 treated AngII-infused mice compared to mice injected with IgG (Figure 5F Figure S5G). However, hearts from Treg-depleted mice displayed a significantly increased interstitial and perivascular fibrosis, as well as increased numbers of FSP-1+ cells (Figure 5G-I). These findings suggest that Treg may partially mediate the cardioprotective effects of C3.

**Moderate blood pressure lowering effect of propionate**

Recent studies have shown C3 may directly influence vasomotor function. To achieve C3-induced vasorelaxation in isolated perfused kidneys, very high, supra-physiological concentrations (3-100 mM) were needed (data not shown). Additionally, atomic force microscopy-based nanoindentation measurements in ApoE^{−/−} mice revealed that C3 treatment softens endothelial cells compared to control treatment (data not shown). Next, we examined whether chronic oral C3 treatment influences blood pressure in AngII-infused WT mice and performed continuous radiotelemetric blood pressure measurements. Systolic and diastolic blood pressures in C3 treated WT were not affected in the initial phase but were lowered towards the second week of AngII infusion, reaching statistical significance from day 11 and day 12 on (Figure 6A, C). Calculating the area under the curve (AUC) for both weeks of AngII infusion separately, a significant difference in systolic and diastolic blood pressure was seen only for the second week (Figure 6B, D). This pattern was confirmed in the ApoE^{−/−} model, as tail cuff measurements of the systolic blood pressure were significantly lower in C3 treated mice during
the last week of AngII infusion (Figure S6A). In parallel with the reduced blood pressure, endothelial dysfunction in both mouse models was significantly ameliorated by C3 treatment as shown by ex vivo analysis of endothelium-dependent relaxation (Figure S6B, C). To analyze whether C3 affects endothelial dysfunction in an immune cell-free setting, we incubated isolated mesenteric rings from untreated healthy mice with IL-17A and AngII for 24 hours in vitro (Figure S6D). Co-incubation with C3 under cell culture conditions did not prevent endothelial dysfunction, making a direct endothelium-mediated effect of C3 less likely (Figure S6D). To elucidate if the blood pressure lowering effect of C3 is influenced directly by Treg, we measured blood pressure by radiotelemetry in C3 treated AngII-infused WT mice receiving the Treg-depleting anti-CD25 antibody or IgG control. However, systolic and diastolic blood pressures were similar in the initial and late phase of AngII-infusion. (Figure S6E, F). Therefore, blood pressure lowering effect of chronic C3 treatment cannot be ascribed to a single mechanism.

**Propionate reduces susceptibility to ventricular arrhythmias**

To further explore if the beneficial effects of C3 treatment on AngII-induced cardiac remodeling lead to an improved functional outcome, we assessed the susceptibility of AngII-infused WT mice treated with C3 or control to ventricular arrhythmias by in vivo cardiac electrophysiological studies. Ventricular tachyarrhythmias are prognostically relevant in hypertensive heart disease.\(^{33}\)

Susceptibility to ventricular tachyarrhythmias was significantly lower in C3 treated animals (five of seven C3-treated mice could not be triggered at all), whereas sustained tachyarrhythmias could be triggered in 85% of control-treated mice (Figure 7A, B). Connexin (Cx)43, a major gap junction protein required for electrical integrity, was relocated from the intercalated disk to the lateral border of the cardiomyocytes upon AngII infusion as shown by immunofluorescence
Consequently, the degree of Cx43 colocalization with N-cadherin (localized at the intercalated disc) was reduced upon AngII infusion in comparison to sham-infused mice and maintained by concomitant C3 treatment (Figure 7C). These data show that C3 improves cardiac electrical remodeling.

Discussion

Metabolites released by the gut microbiota exert an important influence on cardiovascular health of their host. SCFA, end products of bacterial metabolism in the intestine, which are primarily derived from dietary fibers, have been attributed health-promoting properties in several diseases, particularly due to their potent action on immune cells. Epidemiological studies suggest that sufficient fiber intake may be beneficial in hypertension, but we lack a comprehensive understanding of the underlying mechanisms. The current study demonstrates that the SCFA propionate prevents target organ damage in hypertensive mice with and without atherosclerosis by maintaining immune homeostasis.

Hypertension stands out among health risk factors as it promotes several cardiovascular diseases including hypertensive heart disease and atherosclerosis. Beyond blood pressure control, the need to address the inflammatory response to hypertensive stimuli has been recognized. Particularly, effector T cells and macrophages are activated in hypertension, and mediate damage to the heart and the vasculature. Experimental evidence suggests that immunosuppression or adoptive transfer of Treg limits hypertensive target organ damage, though potential side effects prevent further translation of these interventions.

Propionate improved the survival in both WT and ApoE−/− mice and attenuated the systemic T cell response to AngII as indicated by reduced splenic T_{EM} frequencies together with
less pro-inflammatory Th17 cells. More importantly, this anti-inflammatory effect was also
detectable in the respective target organs, since less T cells and macrophages infiltrated the heart
and the aorta, respectively. AngII-induced cardiac hypertrophy and fibrosis were attenuated by
propionate in both mouse models. In ApoE+/− mice propionate treatment led to a reduced
atherosclerotic lesion burden despite unaltered blood lipid levels.

Our data expand on recent observations demonstrating cardio- and renoprotective effects
of a high-fiber diet in uninephrectomized DOCA-treated hypertensive mice.28 The authors
ascribe the observed cardio-renal protection to acetate, another SCFA, and demonstrate a
beneficial gene regulation in hearts and kidneys from uninephrectomized DOCA mice fed a
high-fiber diet or supplemented with acetate. The authors also describe decreased blood
pressures at the end of the treatment28. Our current study expands on the cardiovascular
protective effects of SCFA, pointing to an important contribution of immune homeostasis in the
SCFA-mediated effect.

Activation of T cells can be observed in response to hypertensive stimuli, indicated by
increased TEM.36 The balance of Treg and effector T cells is critically important in hypertension
and hypertensive end-organ damage. Treg are known to limit target organ damage in
hypertension, since adoptive transfer of Treg has been shown to dampen AngII-induced cardiac13
and vascular14 damage. Moreover, depletion of Treg accelerates atherosclerosis in
hypercholesterolemic mice.15 Increased proportions of TEM are associated with the development
of atherosclerosis in humans and mice.37,38 In addition to the significant effect on TEM, our data
suggest that the propionate effect is also Treg-dependent, since Treg depletion abrogated the
propionate effect on systemic and cardiac inflammation, as well as on cardiac fibrosis.
Interestingly, we observed an increase in splenic Treg frequencies in response to AngII along
with an increase in Th17 cells, suggesting Treg counterbalance the AngII-elicited effector Th17 response. This observation is supported by a study showing increased plasma levels of the anti-inflammatory cytokine IL-10 in AngII-infused mice. Likewise, serum IL-10 is increased in hypercholesterolemic mice and human atherosclerotic lesions show substantial IL-10 expression. Enrichment of Treg in nonlymphoid tissues in response to inflammatory stimuli or injury can be observed in various contexts, likely as a compensatory response. Most importantly, propionate treatment in our study preserved the balance of Th17 and Treg in AngII-infused mice. Furthermore, Treg depletion abrogated the anti-inflammatory effect of propionate, indicated by increased Th17 and TEM frequencies.

The current picture of SCFA signaling to host cells is complex, since both the interaction with G protein-coupled receptors (Gpr) and olfactory receptor 78, as well as HDAC inhibitory properties have been described. Propionate is known to bind to Gpr41 and Gpr43, and independent studies have shown that either Gpr41 or Gpr43, but also HDAC inhibition may account for the propionate effect on immune cells. Direct propionate signaling to non-immune cells such as cardiomyocytes may also play a role, since inhibition of HDAC activity is known to inhibit cardiomyocyte hypertrophy. To distinguish effects mediated by immune cells from direct effects on cardiomyocytes, we tested the ability of propionate to inhibit AngII-induced hypertrophy of rat primary neonatal cardiomyocytes in comparison to the established HDAC inhibitor TSA. However, propionate failed to inhibit cardiomyocyte hypertrophy. These data together with the abrogation of the propionate effects by Treg depletion suggest that T cells substantially contribute to the protective effect of propionate. Further studies are warranted to dissect other potentially protective pleiotropic effects of propionate.
Our data demonstrate that in addition to beneficial immunomodulatory effects, propionate moderately reduced blood pressure in both models. Endothelial dysfunction is associated with the development of essential hypertension. In isolated mesenteric arteries of AngII-infused WT mice and in isolated perfused kidneys of AngII-infused ApoE<sup>−/−</sup> mice, we could show that chronic C3 treatment improved endothelium-dependent vasodilation. Earlier reports described acute vasodilation in response to propionate and other SCFA. Similarly, we observed a C3-induced vasodilation in isolated perfused kidneys, albeit only at supra-physiological concentrations. Propionate was previously shown to activate Gpr41 located in the vascular endothelium, mediating the vasodilating effect of propionate, although the downstream signaling cascades are less clear. In isolated mesenteric rings, C3 failed to improve endothelial function in a short-term experiment. Nevertheless, we cannot exclude that chronic Gpr41-mediated anti-hypertensive effects of propionate on the endothelium may have contributed to target organ protection. However, blood pressure reduction was observed only towards the late phase of AngII infusion, suggesting rather a chronic effect of propionate on hypertension, thereby arguing against a direct endothelium-dependent effect.

Importantly, an increased occurrence of ventricular tachycardia can be observed in hypertensive patients with left ventricular hypertrophy, which is prognostically relevant. Spatial redistribution of gap junction proteins is characteristic of pathological electrical remodeling and has been described in the human hypertrophic myocardium. We show that propionate improves electrical remodeling with a reduced susceptibility to programmed ventricular tachycardia <em>in vivo</em>. Our observation is confirmed by attenuated cardiac gap junction remodeling in propionate-treated mice, as indicated by a reduced lateralization of Cx43 in
cardiomyocytes. Similarly, beneficial effects were observed earlier in AngII-infused mice after adoptive Treg transfer.\textsuperscript{13}

In conclusion, propionate treatment protects from cardiac damage and reduces atherosclerosis in experimental hypertension. Target organ protection by propionate is at least partially dependent on Treg, although other pleiotropic effects likely contribute to this result. Our data provide further experimental evidence for the importance of microbiota-derived SCFA in promoting host cardiovascular health. Hypertension and atherosclerosis account for a substantial proportion of worldwide cardiovascular morbidity and mortality. Propionate could be important in improving cardiovascular health, since both atherosclerosis and hypertensive cardiac remodeling were significantly reduced upon propionate treatment in our study. Interestingly, several subsets of gut bacteria are capable of producing propionate\textsuperscript{45}, some of which were shown to be less abundant in experimental hypertension\textsuperscript{46} and hypertensive patients\textsuperscript{17}. Consequently, oral supplementation with propionate or its precursors may be beneficial in hypertensive individuals to prevent damage to target organs. Current hypertension guidelines recommend lifestyle modifications prior to the initiation of any pharmacological anti-hypertensive treatment.\textsuperscript{47} Dietary augmentation of propionate is an affordable intervention, and our observations suggest that this could be a novel approach to prevent hypertensive damage to target organs.
Authors

Hendrik Bartolomaeus\textsuperscript{1,2,3,4,5}; András Balogh, MD, PhD\textsuperscript{1,2,3,4,5}; Mina Yakoub, MSc\textsuperscript{6}; Susanne Homann, PhD\textsuperscript{7}; Lajos Markó, MD, PhD\textsuperscript{1,2,3,4,5}; Sascha Höges\textsuperscript{6}; Dmitry Tsvetkov, MD\textsuperscript{1,2,8}; Alexander Krannich, PhD\textsuperscript{3}; Sebastian Wundersitz\textsuperscript{1,2,4}; Ellen G. Avery, MSc\textsuperscript{1,2,3,4,5}; Nadine Haase, PhD\textsuperscript{1,3,4,5}; Kristin Kräker, MSc\textsuperscript{1,3,4,5}; Lydia Hering, PhD\textsuperscript{6}; Martina Maase, PhD\textsuperscript{9}; Kristina Kusche-Vihrog, PhD\textsuperscript{9}; Maria Grandoch, MD\textsuperscript{7}; Jens Fielitz, MD\textsuperscript{1,4,10}; Stefan Kempa, PhD\textsuperscript{3,11}; Maik Gollasch, MD, PhD\textsuperscript{1,2,12}; Zhaxybay Zhumadilov, MD, PhD\textsuperscript{13}; Samat Kozhakhmetov, PhD\textsuperscript{13}; Almagul Kushugulova, MD, PhD\textsuperscript{13}; Kai-Uwe Eckardt, MD\textsuperscript{12}; Ralf Dechend, MD\textsuperscript{1,2,3,4,5,14}; Lars Christian Rump, MD\textsuperscript{6}; Sofia K. Forslund, PhD\textsuperscript{1,2,3,5,15}; Dominik N. Müller, PhD\textsuperscript{1,2,3,4,5,*}; Johannes Stegbauer, MD\textsuperscript{6,*}; Nicola Wilck, MD\textsuperscript{1,2,3,4,5,12*}

Affiliations

\textsuperscript{1}Experimental and Clinical Research Center, a cooperation of Charité-Universitätsmedizin Berlin and Max Delbrück Center for Molecular Medicine, Berlin, Germany; \textsuperscript{2}Charité-Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany; \textsuperscript{3}Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany; \textsuperscript{4}DZHK (German Centre for Cardiovascular Research), partner site Berlin, Germany; \textsuperscript{5}Berlin Institute of Health (BIH), Berlin, Germany; \textsuperscript{6}Department of Nephrology, Medical Faculty, University Hospital Düsseldorf, Heinrich-Heine-University Düsseldorf, Germany; \textsuperscript{7}Institute of Pharmacology and Clinical Pharmacology, Medical Faculty, University Hospital Düsseldorf, Heinrich-Heine-University Düsseldorf, Germany; \textsuperscript{8}Department of Pharmacology and Experimental Therapy, Institute of
Acknowledgments

We thank Gabriele N’diaye, Ilona Kramer, May-Britt Köhler, Jana Czychi, Martin Taube, Dr. Sabine Bartel, Christina Schwandt for technical assistance and Thomas Hünig for the provision of the PC61 antibody. N.W., D.N.M., J.S. and H.B. designed the study. H.B., N.W., A.B., L.M., E.G.A., N.H. and K.K. performed experiments in WT mice, analyzed and interpreted the data. M.Y., S.Ho., S.Hö., L.H., M.M., K.K-V., M.G. and JS performed experiments in ApoE−/− mice, analyzed and interpreted the data. A.Ku., S.K., Z.Z., R.D., K.U.E., L.C.R. and S.F.K. helped with data analysis and interpretation. S.W. and J.F. performed and analyzed neonatal cardiomyocyte experiments. S.K. performed and analyzed GC-MS measurements. A.K. performed statistical analyses. N.W., H.B., J.S. and D.N.M. wrote the manuscript with input from all authors.
Sources of Funding

N.W. is participant in the Clinician Scientist Program funded by the Berlin Institute of Health (BIH). M.Y. M.G. L.C.R. S.Ho. and J.S. were supported by DFG (German Research Foundation, IRTG 1902). J.F. received funding from the DZHK (German Centre for Cardiovascular Research, 81Z5400153) and the DFG (FI 965/5-2).

Disclosures

None

References


Figure Legends

Figure 1. Propionate provides beneficial modulation of effector T cells in AngII-infused wild-type NMRI (WT) mice. (A) AngII infused WT mice were treated with sodium propionate (C3) or sodium chloride as a control, starting two weeks prior to AngII infusion. Saline-infused mice served as non-hypertensive control group (sham). (B) Survival curves of AngII infused WT mice treated with C3 or control. WT AngII n=36, WT AngII+C3 n=31, *p<0.05 by log-rank test. (C) After 14 days of AngII, splenocytes were analyzed for CD4+ effector memory (CD44+CD62L-) and naïve (CD44-CD62L+) subsets. Left panels show representative flow cytometry plots, quantification in % of CD4+ cells to the right. WT Sham n=8, WT AngII n=8, WT AngII+C3 n=9. (D) Restimulated splenocytes were analyzed for IL-10 and IL-17A by flow cytometry. Representative flow cytometry plots left, quantification in % of CD4+ to the right. WT Sham n=10, WT AngII n=5-6, WT AngII+C3 n=5-6. (E) Quantification of FoxP3+ CD25+ and RORγt+ in CD4+ splenocytes. Representative flow cytometry plots left, quantification in % of CD4+ to the right. WT Sham n=7-8, WT AngII n=10, WT AngII+C3 n=9. *p<0.05, **p<0.01, one-way ANOVA and Tukey’s post hoc for C-E.

Figure 2. Propionate provides beneficial modulation of effector T cells in AngII-infused ApoE−/− mice. (A) AngII infused ApoE−/− mice were treated with C3 or sodium chloride, starting 5 days prior to minipump implantation. (B) Survival curves of AngII infused ApoE−/− mice treated with C3 or control. n=30 per group, ***p<0.001 by log-rank test. (C) After 28 days of AngII infusion, splenocytes were analyzed for CD4+ effector memory (CD44+CD62L-) and naïve (CD44−CD62L+) subsets. Representative flow cytometry plots left, quantification in % of...
CD4+ to the right, ApoE+/− AngII n=15, ApoE+/− AngII+C3 n=19. (D) Quantification of FoxP3+ CD25+ and RORγt+ in CD4+ splenocytes. Representative flow cytometry plots left, quantification in % of CD4+ to the right, ApoE+/− AngII n=12, ApoE+/− AngII+C3 n=15. *p<0.05, **p<0.01, by one-tailed t-test.

**Figure 3. Propionate reduces aortic inflammation and atherosclerotic lesion burden in AngII infused ApoE−/−.** (A) Single cell suspensions from whole aortas of were analyzed for T helper (CD3+ CD4+) and CD8+ and macrophages (F4/80+) by flow cytometry. (B) Aortic CD4+ T cells were analyzed for CD4+ effector memory (CD44+ CD62L−) and naïve (CD44− CD62L+) subsets by flow cytometry. (C-D) Quantification of CD3+ and F4/80+ positive cells in sections of the brachiocephalic artery, respectively. (E) En face Oil Red O staining of whole aortas for the quantification of atherosclerotic lesion burden. Representative aortas shown left, quantification to the right. (F) The degree of stenosis in the brachiocephalic artery was determined in Movat-stained cross sections. Representative sections shown left (scale bar 100 µm), quantification to the right. (A-F) ApoE+/− AngII n=6, ApoE+/− AngII+C3 n=8. (G) Cardiac hypertrophy index (heart weight [mg]/body weight [g]) of AngII infused ApoE−/− mice treated with C3 or control, ApoE+/− AngII n=16, ApoE+/− AngII+C3 n=21. (H) Left ventricular cardiac fibrosis as analyzed by Sirius red staining. Representative photomicrographs shown left (scale bar 100 µm), quantification to the right, ApoE−/− AngII n=9, ApoE+/− AngII+C3 n=10. *p<0.05, **p<0.01 by one-tailed t-test or Mann-Whitney test.

**Figure 4. Propionate attenuates hypertensive cardiac damage in AngII-infused wild-type NMRI (WT) mice.** (A-E) Single cells were isolated from hearts of sham infused or AngII
infused WT mice treated with C3 or control and analyzed by flow cytometry for T helper cells (CD3+ CD4+), cytotoxic T cells (CD3+ CD8+) as well as macrophages (F4/80+). (A-B) show representative gatings and (C-E) show the respective quantifications. WT Sham n=8, WT AngII n=10, WT AngII+C3 n=9. (F) Analysis of CD4+ FoxP3+ and CD4+ RORγt+ cells in heart single cell suspensions. Representative flow cytometry plots left, quantifications to the right. WT Sham n=6, WT AngII n=6-7, WT AngII+C3 n=8. (G) Cardiac hypertrophy index (heart weight [g]/tibia length [m]), (WT Sham n=9, WT AngII n=10, WT AngII+C3 n=10), (H) left ventricular wall thickness (sum of IVSd and LVPWd) as measured by echocardiography, (WT Sham n=9, WT AngII n=8, WT AngII+C3 n=9), and (I) cardiac Nppb and (J) Mhy7 expression as measured by qPCR at the end of the treatment (WT Sham n=10, WT AngII n=6, WT AngII+C3 n=6). (K-M) Immunofluorescence analysis of cardiac left ventricular fibrosis using fibronectin (K), collagen I (L) and FSP-1 (M) antibodies (WT Sham n=5, WT AngII n=6, WT AngII+C3 n=7). Left panels show representative photomicrographs (scale bar 100 µm), quantifications to the right. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA and Tukey’s post hoc.

**Figure 5. Depletion of regulatory T cells abrogates the effect of propionate in AngII infused wild-type NMRI (WT) mice.** AngII infused WT mice received propionate treatment with i.p. injections of anti-CD25 (PC61) or IgG control. (A) Relative reduction in splenic CD4+ CD25+ Foxp3+ regulatory T cells at day 14 of AngII infusion compared to IgG control. (B) IL-17A production in CD4+ restimulated splenocytes measured by flow cytometry. (C) Splenic effector memory T cell (CD4+ CD44+ CD62L-) frequencies. (D-E) Analysis of CD4+ (D) and CD8+ (E) lymphocytes in heart sections using immunofluorescence. (F) Left ventricular wall thickness
(sum of IVSd and LVPWd) as measured by echocardiography. (G-I) Immunofluorescence analysis of left ventricular fibrosis using fibronectin (G), collagen I (H) and FSP-1 (I) antibodies. Left panels show representative photomicrographs (scale bars 100 µm), quantification to the right. (A, D-I) WT AngII+C3+IgG n=4, WT AngII+C3+PC61 n=4, (B-C) WT AngII+C3+IgG n=3, WT AngII+C3+PC61 n=4, *p<0.05 by one-tailed Mann-Whitney test.

Figure 6. Propionate treatment shows a blood pressure-lowering effect confined to the second week of AngII infusion. (A-D) Systolic and diastolic blood pressure were measured continuously by radiotelemetry in AngII infused WT mice treated with C3 or control. (A, C) show smoothened curves over time for systolic and diastolic blood pressure, respectively. P-values by linear mixed model. (B, D) show systolic and diastolic pressures calculated as AUC in week 1 and week 2 of AngII infusion, respectively. n=4 per group. *p<0.05 by 2-way repeated measurement ANOVA and Sidaks’s post hoc.

Figure 7. Propionate reduces susceptibility to ventricular arrhythmias in AngII infused wild-type NMRI (WT) mice. In vivo programmed electrical ventricular stimulations were performed in AngII infused WT mice treated with C3 or control. (A) Representative original tracings showing the induction of ventricular tachyarrhythmia. Surface ECG, right ventricular (RV) and right atrial (RA) recording are shown. (B) Quantification of ventricular arrhythmias susceptibility. n=7 per group, *p<0.05 by Mann-Whitney test. (C) Immunofluorescent co-staining of connexin 43 (green) and N-cadherin (red) in cardiac cryosections from sham infused or AngII infused WT mice treated with C3 or control. WT Sham n=7, WT AngII n=5, WT
AngII+C3 n=7. Representative photomicrographs (scale bar 100 µm) and quantification of co-localization. P-values by one-way ANOVA and Tukey’s post hoc.
A

AngII minipump implantation
(1.44 mg/kg/d)

Randomization:
12 weeks old wild-type NMRI mice
C3 or control in drinking water

Collection of blood and organs for further analysis

B

Percent survival

WT AngII WT AngII+C3

AngII infusion in days

C

WT Sham

WT AngII

WT AngII+C3

CD44-FITC

CD62L-APC

CD44+ CD62L- [% of CD4+]

WT Sham

WT AngII

WT AngII+C3

CD44- CD62L+ [% of CD4+]

C3 or control in drinking water

AngII minipump implantation
(1.44 mg/kg/d)

Collection of blood and organs for further analysis

D

WT Sham

WT AngII

WT AngII+C3

IL-10-PB

IL-17A-APC

IL-10- IL-17A+ [% of CD4+]

WT Sham

WT AngII

WT AngII+C3

IL-10+ IL-17A- [% of CD4+]

E

WT Sham

WT AngII

WT AngII+C3

Foxp3-PerCP-Cy5.5

RORγt-APC

Foxp3+ CD25+ [% of CD4+]
AngII minipump implantation (0.72 mg/kg/d)
d-5
d0
d28

Randomization:
8 weeks old ApoE−/− C57BL/6 mice
C3 or control in drinking water

Collection of blood and organs for further analysis

ApoE−/− AngII
ApoE−/− AngII+C3

CD44− CD62L+
[% of CD4+]

CD44+ CD62L−
[% of CD4+]

Foxp3− RORγt+
[% of CD4+]

Foxp3+ CD25+
[% of CD4+]

**

AngII infusion in days

Percent survival

ApoE−/− AngII
ApoE−/− AngII+C3

CD44-FITC
CD62L-APC

C3 or control in drinking water

Collection of blood and organs for further analysis

Downloaded from http://ahajournals.org by on January 2, 2019
Downloaded from http://ahajournals.org by on January 2, 2019

Downloaded from http://ahajournals.org by on January 2, 2019
WT AngII+C3+IgG  WT AngII+C3+PC61
Fibronectin/Collagen I

WT AngII+C3+IgG  WT AngII+C3+PC61
CD4+ cells per heart section

WT AngII+C3+IgG  WT AngII+C3+PC61
CD8+ cells per heart section

WT AngII+C3+IgG  WT AngII+C3+PC61
FSP-1+ cells per HPF

WT AngII+C3+IgG  WT AngII+C3+PC61
Fibrosis width [µm] / vessel width [µm]
A

![Graph A showing systolic blood pressure.](image)

B

![Graph B showing systolic blood pressure AUC.](image)

C

![Graph C showing diastolic blood pressure.](image)

D

![Graph D showing diastolic blood pressure AUC.](image)