Hypertension-Linked Pathophysiological Alterations in the Gut

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Rationale: Sympathetic nervous system control of inflammation plays a central role in hypertension. The gut receives significant sympathetic innervation, is densely populated with a diverse microbial ecosystem, and contains immune cells that greatly impact overall inflammatory homeostasis. Despite this uniqueness, little is known about the involvement of the gut in hypertension.

Objective: Test the hypothesis that increased sympathetic drive to the gut is associated with increased gut wall permeability, increased inflammatory status, and microbial dysbiosis and that these gut pathological changes are linked to hypertension.

Methods and Results: Gut epithelial integrity and wall pathology were examined in spontaneously hypertensive rat and chronic angiotensin II infusion rat models. The increase in blood pressure in spontaneously hypertensive rat was associated with gut pathology that included increased intestinal permeability and decreased tight junction proteins. These changes in gut pathology in hypertension were associated with alterations in microbial communities relevant in blood pressure control. We also observed enhanced gut–neuronal communication in hypertension originating from paraventricular nucleus of the hypothalamus and presenting as increased sympathetic drive to the gut. Finally, angiotensin-converting enzyme inhibition (captopril) normalized blood pressure and was associated with reversal of gut pathology.

Conclusions: A dysfunctional sympathetic–gut communication is associated with gut pathology, dysbiosis, and inflammation and plays a key role in hypertension. Thus, targeting of gut microbiota by innovative probiotics, antibiotics, and fecal transplant, in combination with the current pharmacotherapy, may be a novel strategy for hypertension treatment. (Circ Res. 2017;120:312-323. DOI: 10.1161/CIRCRESAHA.116.309006.)

Key Words: autonomic nervous system ● gut microbiota ● hypertension ● inflammation ● intestines

T he Centers for Disease Control estimates that hypertension contributes to ≈1000 American deaths each day.1,2 Hypertension is the most modifiable risk factor for cardiovascular disease and stroke and also plays an important role in obesity, diabetes mellitus, and metabolic syndrome.3 Despite advances in blood pressure (BP) control, ≈15% of hypertensive patients remain resistant to available lifestyle modifications and pharmacotherapy.4,8 Neurogenic components have been implicated as a cause of resistant hypertension, as evidenced by measurements of sympathetic outflow and norepinephrine overflow.4,8 Specifically, altered autonomic nervous system activity and associated increases in peripheral and neuroinflammation have been implicated in the pathogenesis of resistant hypertension.8,10,11 Our recent evidence, demonstrating increased peripheral and neuroinflammation in hypertension as a result of autonomic influences on bone marrow (BM) activity, further supports this concept.12 However, the specific mechanism of autonomic dysregulation in hypertension pathophysiology remains elusive.

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Novelty and Significance

**What Is Known?**
- Increased sympathetic activity, inflammation, and gut dysbiosis in hypertension.
- Gut houses largest number of immune cells and trillions of microorganisms.
- The central nervous system-immune system communication is altered in hypertension.

**What New Information Does This Article Contribute?**
- Hypertension is associated with increased gut wall permeability, altered tight junction proteins and fibrosis, decrease in goblet cells and shorter villi, and microbial dysbiosis.
- Angiotensin-converting enzyme inhibitor, captopril, which lowers blood pressure in spontaneously hypertensive rat, reverses gut pathology.
- Sympathetic nerve activity and tight junction proteins, but not gut wall pathology and microbial composition, are altered in prehypertensive animals.

Abundant evidence reveals that both sympathetic nervous system and inflammation are key players in the development and establishment of hypertension. The gastrointestinal tract, which houses the largest number of immune cells in the body and contains trillions of microorganisms, is emerging as an important organ in the maintenance of normal body homeostasis. Despite this, the involvement of gut in blood pressure control and hypertension is not known. This coupled with recent evidence of gut microbial dysbiosis in hypertension led us to test the following hypothesis in this study: sympathetic nervous system impacts gut physiology to maintain blood pressure homeostasis; its increased activity is associated with increased gut wall permeability, pathology, and dysbiosis in hypertension. We demonstrate that gut wall was significantly thicker, more permeable, fibrotic, and had less numbers of goblet cells and stunted villi in hypertensive animals. These changes in the gut were associated with increase in the sympathetic nerve activity to the gut. Angiotensin-converting enzyme inhibitor treatment lowered blood pressure and attenuated gut pathology. Further investigation into the mechanism of this proposed dysfunctional sympathetic–gut communication is necessary to evaluate the potential of gut and microbiota as novel targets with the use of pre- and probiotics as combination therapy for drug-resistant hypertension.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>ACE</th>
<th>angiotensin-converting enzyme</th>
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<tr>
<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<td>BP</td>
<td>blood pressure</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>SHR</td>
<td>spontaneously hypertensive rat</td>
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<td>WKY</td>
<td>Wistar–Kyoto</td>
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It has become evident in recent years that a symbiotic relationship exists between the gut and its microbiota for regulation of both local gut and systemic immunity. Imbalances in this host–gut microbiota eubiosis are potentially of major pathophysiological consequence to the host. For example, imbalances in this symbiotic relationship have been associated with diverse diseases, such as obesity, nervous system disorders, chronic kidney disease, inflammatory bowel disease, and cardiovascular disease. However, the involvement of the host–gut relationship in hypertension has yet to be investigated in detail despite the fact that (1) the gut is the most highly innervated peripheral organ, with significant numbers of the motor fibers to the gut identified as sympathetic nerves. It is pertinent to note that elevated sympathetic nervous system activity is a hallmark of both animal and human hypertension. (2) Autonomic nervous system influences gut permeability, inflammatory state, and microbial communities in inflammatory bowel disease and other diseases; (3) gut microbial products, such as short chain fatty acids, lipopolysaccharides, and neurotransmitters influence both the immune and vascular systems; and (4) studies from our group and others have demonstrated a link between gut dysbiosis and hypertension. This evidence, taken together, led us to the following hypothesis: increased sympathetic activity to the gut alters gut pathology in such a way that is conducive to increased gut permeability, altered inflammatory status, and microbial dysbiosis impacting BM production of proinflammatory cells. Thus, we propose that hypertension is, at least in part, a result of pathophysiological changes in the gut. The present study was designed to provide experimental evidence to support or refute this hypothesis.

**Methods**

All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Full details of all experimental protocols are presented in the Methods section in the Online Data Supplement.

**Results**

Alterations in Gut Pathology in Rat Models of Hypertension

First, we investigated whether the integrity of the gut epithelial barrier is altered in the spontaneously hypertensive rat (SHR) model using various techniques: (1) oral fluorescein isothiocyanate-dextran for epithelial permeability, (2) an innovative Multi-Scale Indention System to measure gut wall stiffness, and (3) Western blotting for tight junction proteins. Both young prehypertensive and adult SHR with established hypertension along with age-matched, normotensive Wistar–Kyoto rats (WKY) were used to investigate gut associations with BP (Figure 1A). We observed a 2.1-fold increase in fluorescein isothiocyanate-dextran accumulation in adult SHR plasma (Figure 1B; \( P<0.05 \)), whereas no difference was observed between young WKY and prehypertensive SHR. These data provide evidence to support the proposal that adult SHR with established hypertension have a leaky intestinal barrier. This enhanced permeability has
Figure 1. Permeability and stiffness of small intestine and colon are increased in adult spontaneously hypertensive rat (SHR). A, Mean arterial blood pressure (MAP) of young Wistar–Kyoto rats WKY (WKY-y), young SHR (SHR-y), and adult WKY and SHR. B, Intestinal mucosa-to-blood permeability assessed by fluorescein isothiocyanate (FITC)-dextran 4-kDa concentration in the plasma revealed increased FITC-dextran concentration in adult, but not in young, SHR (n=4–7 per group). C and D, Effective modulus measured by a Multi-Scale Indentation System is increased in both small intestine and proximal colon of WKY-y, SHR-y, and adult WKY, SHR (n=4–5 per group). E–H, Western blotting for tight junction proteins in small intestine (E and F) and proximal colon (G and H) in WKY-y, SHR-y, and adult WKY, SHR. *P<0.05, SHR vs all other groups; #P<0.05, WKY-y vs SHR-y; **P<0.05, SHR vs WKY.
been associated with an increase in tissue stiffness (effective modulus). Accordingly, using the Multi-Scale Indention, we observed a 2.7- and 3.0-fold \((P<0.05)\) increase in stiffness in the small intestine and proximal colon, respectively, in the adult SHR when compared with age-matched WKY (Figure 1C and 1D). However, no change was observed between young WKY and prehypertensive SHR. Next, we compared tight junction proteins by Western blotting. Levels of Ocln (occludin), Tjp1 (tight junction protein 1), and Cldn4 (claudin 4) were decreased by 46.3\% \((P<0.05)\), 22.5\% \((P<0.05)\), and 45.4\% \((P<0.05)\), respectively, in the small intestine of adult SHR compared with that of adult WKY (Figure 1E and 1F). Comparable decreases were observed in Ocln, Tjp1, and Cldn4 of the proximal colon in adult SHR (Figure 1G and 1H). Interestingly, the proximal colon from prehypertensive SHR also demonstrated significant decreases in Ocln 68.6\%, \(P<0.05\); Tjp1 76.9\%, \(P<0.05\); Cldn4 69.0\%, \(P<0.05\); and Cgn 23.1\%, \(P<0.05\) compared with age-matched WKY controls (Figure 1G and 1H). Similarly, the small intestine of prehypertensive SHR showed a 24\% decrease in Ocln \((P<0.05)\), 36.9\% in Tjp1 \((P<0.05)\), 34.7\% in Cldn4 \((P<0.05)\), and 39.9\% in Cgn \((P<0.05)\) (Figure 1E and 1F). These observations support the suggestion that decreases in gut tight junction proteins may precede increases in gut permeability, stiffness, and high BP.

To investigate whether gut permeability is compromised in another model of hypertension, we evaluated the angiotensin II (Ang II)–infusion rat model of hypertension (Online Figure IA). An increase in plasma fluorescein isothiocyanate-dextran (Online Figure IB) was observed in Ang II–infused rats that was comparable to adult SHR, suggesting enhanced gut permeability. However, mean effective modulus was not significantly changed in this model (Online Figure IC and ID).
Figure 3. Antihypertensive treatment with angiotensin-converting enzyme-inhibition (ACEi) has beneficial effects on gut pathology. A, Mean arterial blood pressure (MAP) of vehicle and captopril (ACEi)-treated Wistar-Kyoto rats (WKY) and spontaneously hypertensive rat (SHR; n=6–8 per group). B, Intestinal mucosa-to-blood permeability assessed by fluorescein isothiocyanate (FITC)-dextran 4-kDa concentration in the plasma revealed ACEi reverses increased permeability in the SHR. C and D, Mean effective modulus measured by a Multi-Scale Indentation System in both small intestine and proximal colon of vehicle and captopril-treated WKY and SHR. E–H, Fibrotic area is decreased by ACEi in SHR (U). I–L, Cross sections of the small intestine of WKY and SHR show protected tunica muscularis layer in ACEi-treated SHR (V). M–P, The number of goblet cells per 100 epithelial cells was not improved by ACEi in SHR (W). Q–T, Villi lengths were improved by ACEi in SHR (X). *P<0.05 SHR vs all other groups; #P<0.05 SHR-ACEi vs SHR; +P<0.05 WKY-ACEi vs SHR-ACEi;  φP<0.05 SHR vs WKY; ^P<0.05 SHR-ACEi vs WKY and WKY-ACEi.
Protein levels of tight junctional proteins were also reduced in both small intestine and proximal colon of Ang II–infused rats (Online Figure IE and IH). For example, Tjp1 decreased by 48% (P<0.01) and 46% (P<0.05) and Cgn by 43% (P<0.01) and 49% (P<0.001) in small intestine and proximal colon, respectively. Occludin was decreased by 55% (P<0.01) only in the proximal colon. Although the differences between the SHR and Ang II–infusion models are evident, these observations suggest that both rat hypertension models exhibit an increase in gut permeability and decrease in tight junction proteins.

Next, we investigated whether the observed increased permeability is associated with alterations in gut pathology in the SHR. Figure 2A through 2P depicts changes in the SHR small intestine compared with normotensive WKY rats, in both young and adult rats. We observed an 1.8-fold increase in fibrotic area in the adult SHR (Figure 2A through 2D and 2Q; P<0.05). An increase in the muscularis layer of the SHR small intestine was also observed (Figure 2E through 2H and 2R; P<0.05). We also observed a 57% decrease in numbers of goblet cells in adult SHR (Figure 2I through 2L and 2S). Figure 2M through 2P shows that villi in the small intestine of adult SHR were much shorter and appeared stunted, compared with WKY. None of these pathological alterations were observed in young prehypertensive SHR when compared with age-matched WKY. The chronic Ang II–infusion rat model of hypertension was used to confirm these intestinal pathological changes. Similar to adult SHR, we observed increases in fibrotic area (Online Figure IIA through IIC), an increase in muscularis layer (Online Figure IID through IIIF), no difference in villi length (Online Figure IIG through IIII), and a modest decrease in goblet cells (Online Figure IIJ through IIIL).

**Effects of Angiotensin-Converting Enzyme Inhibition (Captopril) on Gut Pathology in the SHR**

We investigated the effects of captopril, an effective anti-hypertensive medication in SHR, to further explore the relationships between gut pathology and high BP. Adult WKY and SHR were treated with captopril for 4 weeks, which significantly reduced mean arterial pressure in SHR (Figure 3A; 170±5 mm Hg water versus 119±7 mm Hg captopril; P<0.01), with only a slight reduction in mean arterial pressure in WKY (110±9 mm Hg water versus 85±5 mm Hg captopril; P=0.071). Angiotensin-converting enzyme (ACE)-inhibition–related BP reduction was associated with a decrease in gut permeability in the SHR (Figure 3B; 1273±212 ng/mL versus 685±124 ng/mL; P<0.05), whereas no change was observed in WKY. Although effective modulus seems to be reduced in both the small intestine and proximal colon of captopril-treated SHR (Figure 3C and 3D), the values did not reach significance. We found a complete reversal in both fibrotic area (Figure 3E through 3H and 3U; P<0.001) and thickness of the muscularis layer (Figure 3I through 3L and 3V; P<0.01) with ACE-inhibition–related decreased BP in SHR. Although this did not prevent the reduction in goblet cell numbers (Figure 3M through 3P and 3W), the villi length increased by 55% with ACE-inhibition reduced BP in treated SHR (Figures 3Q through 3T and 3X; P<0.01). These data suggest that ACE inhibition–related decreased BP has beneficial effects on hypertension-linked gut pathology.

**Increase in Gut Inflammation in Hypertension**

Real time-PCR revealed that Cd68 (Figure 4A) and Cd3e (Figure 4B) gene expression, representative of macrophages and T cells, respectively, was elevated in both the small intestine (1.8- and 2.7-fold, respectively) and proximal colon (1.9- and 6.2-fold, respectively) in the SHR (P<0.05). An increase in inflammatory status was further confirmed by levels of cytokine mRNAs (Figure 4C and 4D). The mRNA levels of interleukin 1β and high mobility group box 1 were 75% and 57% higher, respectively, in the SHR small intestine (P<0.05; Figure 4C). In the proximal colon, we observed a 70% and 108% increase in interleukin 1β and tumor necrosis factor-α mRNA levels in the SHR (Figure 4D). No differences in high mobility group box 1 levels were observed in the colon between the 2 strains.

Next, we investigated the mRNA levels of 3 proposed receptors for high mobility group box 1: toll-like receptor 2, toll-like receptor 4, and receptor for advanced glycation end-products, in view of accumulating evidence that gut high mobility group box 1 plays a critical role in systemic

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**Figure 4. Inflammatory cells, cytokines, and their receptors are increased in the spontaneously hypertensive rat (SHR) small intestine and colon.** A and B. Small intestine and proximal colon of SHR show increased CD68 and CD3 mRNAs. C and D. Further mRNA revealed increased interleukin (IL)-1β, high mobility group box 1 (HMBG1), toll-like receptor (TLR) 4, and receptor for advanced glycation end-products (RAGE) in the small intestine, and (E and F) increased IL-1β, tumor necrosis factor (TNF)-α, TLR2, TLR4, and RAGE in the proximal colon. *P<0.05, **P<0.01 vs WKY (n=5–7 per group).
and gut inflammation\(^3\) (Figure 4E and 4F). Furthermore, these receptors are implicated in inflammation induced by bacterial products.\(^3\) We observed increases in mRNAs for both toll-like receptor 4 (46\%) and receptor for advanced glycation end-products (303\%) in the small intestine (Figure 4E) and colon (35\% and 135\%, respectively; Figure 4F), whereas toll-like receptor 2 was increased 130\% only in the SHR colon (Figure 4F; \(P<0.05\)).

Our previous studies have demonstrated that hypertension impacts BM activity by influencing proliferation, mobilization, and differentiation of hematopoietic stem cells.\(^3\) Thus, this cellular response contributes to increased peripheral and neuroinflammation in hypertension.\(^3\) Thus, we sought to determine whether altered BM cell activity contributes to increases in proinflammatory cells in the gut in hypertension using the chronic Ang II–infusion rat model. Enhanced green fluorescent protein (GFP) Sprague–Dawley BM chimeric rats were generated and subjected to a 4-week Ang II infusion (200 ng/kg per minute).\(^3\) This infusion resulted in a significant increase in mean arterial pressure (systolic, 95±2 mm Hg versus Ang II infusion, 150±3 mm Hg; \(P<0.001\)). The hypertensive response to Ang II infusion in enhanced GFP-chimeric rats was comparable to naive Sprague–Dawley rats.\(^3\) We observed significant numbers of green fluorescent cells in the small intestine and colon of both the saline and Ang II–treated rats (Online Figure III). However, levels of these BM-derived enhanced GFP\(^+\) cells were 2.2- and 1.3-fold higher in small intestine and colon, respectively, of Ang II–treated rats compared with saline. Double staining with inflammatory markers documented an increase in CD3\(^+\) T cells, CD68\(^+\) macrophages, and Iba1\(^+\) macrophages in Ang II–treated rats (Online Figure IV). Quantification revealed increases of 103\% in CD3\(^+\) T cells, of 278\% in CD68\(^+\) macrophages, and of 101\% in Iba1\(^+\) macrophages in the small intestine of Ang II–treated rats. Similar increases were observed in the colon of the Ang II–treated animals. In contrast, no significant changes in numbers of cells double-stained for neurons (NeuN antibody) and smooth muscle cells (smooth muscle actin-\(\alpha\) antibody) were observed comparing the 2 groups (data not shown). Taken together, these data indicate that the increase in inflammatory cells in hypertension is, in part, a result of mobilization of BM cells to gut.

**Gut Perfusion Is Decreased in Hypertension**

We compared the blood perfusion of the gut in WKY and SHR using Laser Spackle Contrast Imaging\(^3\) and observed a significant decrease in mesenteric blood flow normalized to BP, in a section of small intestine in anesthetized adult WKY and SHR (Figure 5). This interesting observation is consistent with reports indicating that decreased gut blood flow is associated with proinflammatory gut pathophysiology.\(^4\)

**Altered Microbial Community in Hypertensive Rat Gut**

The above data indicate that hypertension is associated with increases in gut permeability and inflammatory status. These findings led us to investigate whether there are also differences in gut bacterial taxa between the normotensive WKY and SHR. Our previous data indicated a significant increase in the ratio of the Firmicutes to Bacteroidetes (F/B ratio, an indicator of gut microbial dysbiosis) phyla in the gut of SHR versus WKY.\(^2\) Here, we examined whether this shift in F/B ratio is associated with the SHR strain or the development of hypertension in the SHR strain. We found that the F/B ratio in prehypertensive SHR is not different from age-matched WKY (Figure 6A). This finding supports the suggestion that the BP increase in the SHR is closely associated with the development of gut dysbiosis. Of the total number of genera represented in the gut microbiota, members of 25 most abundantly represented genera were compared in the prehypertensive SHR and the age-matched WKY using a heat map (Figure 6B). The heat map did not show any clustering between SHR and WKY, indicating that the abundance levels of selected genera in prehypertensive SHR were not significantly different from the age-matched WKY.

Fecal bacterial analysis was done to determine specific bacterial genera that may be associated with the observed changes in permeability and inflammation in the adult SHR. We observed a significant increase in Gram-positive Streptococcus...
Figure 6. Gut microbial community is altered in hypertensive spontaneously hypertensive rat (SHR). A, Comparison of microbiota composition between young Wistar–Kyoto rats (y-WKY) and SHR (y-SHR). The Firmicutes/Bacteroidetes ratio (F/B ratio) was calculated as a biomarker of gut dysbiosis. B, Heat map indicating the genus-level changes in the young WKY and SHR groups. Each green or red box represents 1 animal (WKY-y green, SHR-y light red). The relative abundance of the bacteria in each genus is indicated by a gradient of color from blue (low abundance) to red (high abundance). C, Red circles indicate taxa enriched in the SHR, green circles indicate taxa enriched in WKY rats, whereas yellow circles indicate no difference between the groups. Each circle represents 1 bacterial taxa with circle size being proportional to abundance/effect size. Gamma on the bottom right in light green is an abbreviation for gammaproteobacteria (n=5–6 per group).
genus and Streptococcaceae family in the gut of the SHR, both belonging to the order of Lactobacillales (lactic acid–producing bacteria) and the phylum of Firmicutes (Figure 6C, in red). This was consistent with changes we had previously observed in the Ang II–infusion hypertensive rodent model.22 Furthermore, we observed a significant decrease in the Bifidobacterium genus and the related Bifidobacteriaceae family of Actinobacteria phyla (Figure 6C, in green), a Gram-positive bacterium with probiotic properties. 35,36 We also observed a decrease in several bacterial genera of the Bacteroidetes phyla. These changes begin to pinpoint specific bacterial genera with implications in inflammation and hypertension.

Dysfunctional Autonomic–Gut Communication in Hypertension

Activity of the splanchnic sympathetic nerve innervating gut in young 4-week-old normotensive rats and SHR was determined using a decerebrated artificially perfused rat preparation (Figure 7A) as established previously.17 This in situ preparation revealed classic phrenic nerve inspiratory (I) and expiratory (E) phases used to determine respiratory uncoupling. Note the typical shift in the peak of sSNA burst from the E phase in the NT (left, red arrow) to the I phase of PNA in the HT (right, red arrow). C and D, Intra-arterial bolus KCN injection produced a ≈25% higher sSNA burst from baseline (pre-KCN) in the HT compared with NT rats (n=5 per group). *P<0.05 baseline HT vs post-KCN HT. PP indicates pulse pressure.

Figure 7. Elevated splanchnic sympathetic nerve activity in the spontaneously hypertensive rat (SHR). A, In situ decerebrated artificially perfused rat preparation (schematics on the left) reveals respiratory uncoupling of the B, splanchnic sympathetic nerve activity (sSNA; red trace) in the hypertensive (HT) SHR compared with normotensive (NT) control rats. Phrenic nerve activity (PNA, black trace) inspiratory (I) and expiratory (E) phases were used to determine respiratory uncoupling. Note the typical shift in the peak of sSNA burst from the E phase in the NT (left, red arrow) to the I phase of PNA in the HT (right, red arrow). C and D, Intra-arterial bolus KCN injection produced a ≈25% higher sSNA burst from baseline (pre-KCN) in the HT compared with NT rats (n=5 per group). *P<0.05 baseline HT vs post-KCN HT. PP indicates pulse pressure.
confirmed augmented paraventricular nucleus–gut connectivity in both adult SHR and Ang II–infusion hypertension models.

Discussion

Our study demonstrates important changes in the gut in 2 rodent models of hypertension, suggesting a gut link with hypertension. The major findings are as follows: (1) increased permeability of gut epithelial barrier and inflammatory state is associated with BP elevation; (2) enhanced sympathetic neuronal communication between the paraventricular nucleus and gut; and (3) a shift in gut microbial genera is likely associated with the pathophysiological and immune status of the gut and high BP. Together, these changes could be important in the initiation and establishment of hypertension.

Additional significance of our study is the association of gut pathology and microbial dysbiosis with hypertension, and data from prehypertensive SHR are relevant in support of this concept. They show that changes in sympathetic nerve activity and gut tight junction proteins precede changes in gut pathology, dysbiosis, and hypertension. This view is further supported by ACE-inhibition–induced BP lowering in the SHR, which reversed gut pathology. It is pertinent to note that sympathetic tone in SHR is also attenuated by ACE-inhibition. Therefore, one cannot deduce whether changes in the gut pathology are BP or sympathetic activity driven or dependent on both. Further experiments would be needed to resolve this. Our data suggest that prohypertensive signals are perceived in the paraventricular nucleus and transmitted via central neural pathways to increase sympathetic activity and norepinephrine release to the gut. This could lead to alterations of gut junction proteins resulting in permeability changes, dysbiosis, and pathology. Chronic elevation of tyrosine hydroxylase in the gut of SHR, a key enzyme in norepinephrine generation, is consistent with this contention. In addition, gut dysbiosis and lactate-producing bacterial populations are increased in hypertensive rats. These bacteria also generate a variety of neurotransmitters themselves (eg, serotonin, dopamine, and histamine) that may potentially be involved in BP regulation. Furthermore, carbohydrates fermented by Streptococcus predominately yield lactic acid as a major end-product. Lactic acid can potentially contribute to the modulation of the immune system in both gut and circulation. Support for this view comes from our experimental findings that (1) the lactic acid–producing bacterial population is increased in the SHR and Ang II–infusion rat hypertension model, (2) increased inflammation in the small intestine and colon of hypertensive animals was associated with both the increase in F/B ratio and hypertension development, and (3) increased peripheral inflammation is associated with hypertension.

The increase in gut inflammatory cells in hypertension observed in this study could be a result of activation of resident immune cells or their infiltration from the BM. Although the resident immune cells undergo regular turnover, our data indicate that increased recruitment of BM inflammatory cells into the gut plays an important role in hypertension-associated gut inflammation in addition to the resident immune cells. The mechanism of this increase remains to be elucidated, but it could be a result of stimulation and recruitment of inflammatory cells by a shift in bacterial release of short chain fatty acids and direct autonomic nervous system–mediated

Figure 8. Higher pseudorabies virus (PRV)-green fluorescent protein (GFP) retrograde labeling from the small intestine to the PVN is associated with increased tyrosine hydroxylase (TH) immunoreactivity in the small intestine of the spontaneously hypertensive rat (SHR) compared with Wistar–Kyoto rats (WKY). A and B, GFP staining reveals robust PRV retrograde labeling from small intestine to the PVN in the SHR compared with WKY. C, Retrograde labeling in WKY is enhanced after chronic Ang II infusion. D and E, Representative images of TH immunoreactivity in WKY and SHR small intestinal tissue. F, Quantification of TH staining revealed increased immunoreactivity in the small intestine of SHR (n=3 per group). *P<0.05 SHR vs WKY.
regulation of BM inflammatory cell activity, as shown previously.12 Interestingly, gut microbiota reportedly promote Ang II–related hypertension, as germ-free mice are protected from Ang II–induced vascular dysfunction and hypertension,52 an effect suggested to be mediated by a dampened immune response.

The brain can also influence intestinal homeostasis by gut motility and mucus secretion. Involvement of mucus in mucosal immune responses is well recognized.13 Our data suggest that this is highly likely.52,53 (3) It is pertinent to recognize the well-established role of the afferent parasympathetic arm in the paradigm of gut–brain axis: implications for hypertension and related therapeutics. Circ Res. 2016;118:1327–1336. doi: 10.1161/CIRCRESAHA.116.307709.

Recently, we advanced a brain–gut–BM axis hypothesis and proposed dysfunctional brain–gut interactions in hypertension.7 Our current study, demonstrating an increased sympathetic drive associated with gut pathology and microbial dysbiosis, provides new evidence in support of that proposal. However, our findings generate questions for future investigation. For example, (1) does sympathetic activation precede or accompany changes in the gut? We favor the former scenario based on evidence that increases in sympathetic drive precede increases in BP in both animal models and human hypertension,10 and our data presented here showing elevated sympathetic drive is present before pathological changes in the gut. (2) Does microbial dysbiosis promote hypertension? Recent fecal transplantation studies suggest that this is highly likely.52,53 (3) It is pertinent to recognize the well-established role of the afferent parasympathetic arm in the paradigm of gut–brain axis.54,55 Evidence indicates that microbiota communication with the brain involves afferent vagal signaling pathways,56 and its activation modulates both neurochemical and behavioral effects. A recent report has described the role of the vagal anti-inflammatory pathway in modulation of intestinal muscularis layers and resident macrophages.57 Considering that vagal activity is dampened in rodent models of hypertension,10 it is possible that the shift in the balance of sympathetic and parasympathetic influences in the gut, rather than exaggerated sympathetic drive alone, contribute to hypertension-associated gut pathophysiology, dysbiosis, and immune system activation. Finally, therapeutic implications of this study for human hypertension must be investigated and clinical study (NCT 02188381) is underway.

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Disclosures
None.

References

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SUPPLEMENTAL MATERIAL

Hypertension-Linked Pathophysiological Alterations in the Gut

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Short title: Gut pathology in hypertension

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Methods
Animal Models
All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee and complied with the standards stated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were housed in a temperature-controlled room (22°C to 23°C) with a 12:12-hour light-dark cycle, in specific-pathogen free cages, and had access to standard rat chow and water ad libitum.

Twenty-week old Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) (Charles River Laboratories) were used as the adult group, and four-week old WKY (young, WKY-y) and SHR (young, SHR-y) were used as the young group. In ACEi experiments, adult WKY and SHR animals received 85mg/day captopril in the drinking water for 4 weeks.

Eight-week-old Sprague Dawley (SD) rats were used for experiments involving angiotensin II (Ang II) infusion. Hypertension was established by chronic infusion of Ang II (200ng/kg/min) using mini osmotic pumps (ALZET 2004) implanted subcutaneously. Control animals received 0.9% saline in osmotic pumps.

Six-week-old SD (Charles River Laboratories) and SD-Tg (UBC-eGFP) (Rat Resource & Research Center strain 65; bred in-house) were used for bone marrow (BM) reconstitution experiments described below. This hemizygous transgenic strain contains a single enhanced green fluorescent protein gene under the control of human Ubiquitin-C promoter located at Chromosome 14.

Small intestine (medial ileum, ~3 cm) and proximal colon (~3 cm, most proximal to cecum) areas of the gut were isolated for all the studies.

Blood Pressure Measurement
Noninvasive blood pressure (BP) measurements were performed using the CODA System for Rats, following the manufacturer’s protocol (Kent Scientific Corp). Additionally, Millar catheterization was performed to measure the direct BP as previously described [PMID: 26498282]. Briefly, rats were anesthetized with the 2% isoflurane-oxygen mixture. By cannulating the right carotid artery, an impedance-micromanometer catheter (Millar Instruments, Houston, Texas) was introduced into the carotid artery. The catheter was interfaced to a PowerLab (ADInstruments, Colorado Springs, CO, USA) signal transduction unit.

FITC-Dextran Permeability Assay
Following 6 hours of fasting, animals were orally gavaged with 4kDa FITC-dextran (44mg/100g, Sigma Aldrich, catalog #FD4). Four hours after feeding, blood was collected for plasma analysis of FITC fluorescence. Data were analyzed using a standard curve of FITC-dextran dilution, as previously described¹.

Multi-Scale Indentation Technique
Small intestine and proximal colon samples were tested from WKY-y, SHR-y, adult WKY, adult SHR, and ACEi treated WKY and SHR. Rats were euthanized and a 2-2.5 cm section of colon was excised under approved procedures from the University of Florida Institutional Animal Care and Use Committee. Freshly isolated tissue samples were placed in cold media solution consisting of Dulbecco’s Modified Eagle Medium (DMEM) and 5% Fetal Bovine Serum (FBS) and tested within four hours of isolation.

Intestinal samples were cleaned initially by gently pushing feces out of the sample with a soft edge. Samples were then cut open longitudinally, rinsed in cold buffered saline, and placed flat on Petri
dishes, which served as the substrate during testing. Tissues were handled and placed to ensure that the inner lining of the intestine remained on top. Sample thickness was approximately 1.5 mm. All samples were prepared and tested at room temperature (approximately 22°C). Small aliquots (less than 1 ml) of the DMEM solution were added periodically during testing to prevent dehydration of the samples.

A custom cantilever-based indenter\textsuperscript{2, 3} was used to indent tissue samples and record forced relaxation over time. A piezoelectric stage (P-628.1CD, Physik Instrumente) displaced a soft titanium cantilever with a 3 mm-diameter rigid tip. A custom program in LabVIEW (National Instruments) was used to control indentation profile and to read deflection of cantilever tip with capacitive sensor (C8S-3.2-2.0 and compact driver CD1-CD6, Lion Precision) through a data acquisition card system (NI 9220 and cDAQ-9171, National Instruments). Samples were indented at a depth of 150 micrometers at an indentation rate of 10 micrometers/s and then the probe was kept in place for 120 seconds to allow for the tissue to fully relax. Each sample was indented at four different locations along the length of the tissue. Force-displacement data from the tests were then fitted to the SLS model.

Western Blot
Segments of small intestine and proximal colon were homogenized in radioimmunoprecipitation assay buffer. 50 µg protein was fractionated in 12%SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk solution in Tris-buffered saline (TBS) with 0.1 % Tween-20 for 1 h. Then the membranes were incubated either with primary antibody for multiple proteins including occluding (Ocln), tight junction protein 1 (Tjp1), claudin 4 (Cldn4), and cingulin (Cgn) overnight at 4 °C [Ocln rabbit monoclonal antibody (1:5000; Abcam), Tjp1 rabbit polyclonal antibody (1:100; Novus Biologicals), Cldn4 rabbit polyclonal antibody (1:100; Abcam), and Cgn rabbit polyclonal antibody (1:100; Novus Biologicals)]. Mouse monoclonal anti-beta actin antibody (1:10000; Abcam) was used to confirm equal loading. The membranes were then washed three times for 5 min in TBS-T and incubated with secondary antibody conjugated with horseradish peroxidase (anti-goat, anti-rabbit, and anti-mouse IgG 1:2500; GE Healthcare) for 1 h. Finally, the membranes were subjected to a chemiluminescence detection system and exposed to a photographic film.

Histological Analysis
Paraffin-embedded sections were stained with hematoxylin-eosin and Masson-trichrome to evaluate general morphology and collagen formation. Specifically, villus length, fibrosis, the number of goblet cells and thickness of tunica muscularis externa were quantified to assess intestinal pathology. To determine the expression and localization of eGFP\textsuperscript{*} and inflammatory cells (CD3\textsuperscript{*}, CD68\textsuperscript{*} and Iba1\textsuperscript{*}) in the gut, parafomaldehyde-fixed intestines were protected with sucrose, embedded in OCT, and serially sectioned on a cryostat. We performed antigen retrieval via Citrate Buffer Antigen Retrieval Protocol on the OCT sections and blocked sections with normal goat serum. The slides were incubated with the primary antibodies against eGFP (Aves Labs, GFP-1020) with CD3 (Abcam, ab16669), CD68 (Abcam, ab31630), Iba1 (Wako, 019-19741), or TH (Millipore, AB152) followed by incubation with Alexa Fluor conjugated secondary antibodies (Invitrogen).

RT-PCR
RNA was isolated from ileum and proximal colon using TRlzol Reagent (Ambion) per manufacturer’s protocols. Purity of RNA was evaluated spectrophotometrically by 260/280 ratio. Reverse transcription was accomplished using High Capacity Reverse Transcription kit (Applied Biosystems) and 500 ng
RNA. Real time RT-PCR was performed using Taqman Universal PCR Master Mix and Taqman Gene Expression Assay primers (Applied Biosystems): GAPDH (Rn01775763_g1), Il1b (Rn00580432_m1), Tnf (Rn01525859_g1), HMGB1 (Rn02377062_g1), TLR2 (Rn02133647_s1), TLR4 (Rn00569848_m1), RAGE (Rn01525753_g1). RT-PCR was run using StepOnePlus (Applied Biosystems) sequence detection system. All cDNA samples were assayed in duplicate. Data were normalized to GAPDH.

**Intestinal Blood Flow Measurements**

In six rats (three from each group), the blood flow in the mesenteric blood vessels in a section of small intestine was measured. Animals were anesthetized with isoflurane, placed in a supine position, and a short length of the small intestine was surgically isolated via laparotomy and placed on a platform warmed by circulating water. Measurements of blood flow were taken over a three minute period using a laser speckle contrast imager (LSCI; PeriCamPSI, PeriMed, Inc.) interfaced with a dedicated computer as described previously. The laser generator probe was positioned 15.0 cm above the intestinal surface. The LSCI sample rate was 53 samples/sec and the digitized blood flow values were stored in a data file. Blood flow (perfusion) values were expressed in arbitrary “perfusion units”. The blood flow data was averaged over the three-minute record.

**Fecal Analysis**

Rat fecal DNA was extracted by using ZR Fecal DNA MiniPrep (Zymo Research, Irvine, CA). Primers with adaptor sequences for Illumina Miseq (Illumina, Inc., San Diego, CA) were used to amplify the bacterial 16S ribosomal DNA V4-V5 region. PCR amplicons were purified (Qiagen, Madison, WI) and subsequently quantified by Qubit 2.0 Fluorometer (Invitrogen, Grand Island, NY) and KAPA SYBR FAST qPCR kit (Kapa Biosystems, Inc., Woburn, MA). DNA library was finalized with equal amount of amplicons for all data analysis.

Standard bioinformatics alignment comparison was utilized for data analysis. Paired end reads were demultiplexed according to a combination of forward and reverse indices. Additional quality filtering included exact match to sequencing primers and an average quality score of 30 or higher on each read. Prior to further analysis, each paired end read was stitched into one contiguous read using the FLASh (Fast Length Adjustment of Short reads) software tool. Reads that could not be joined were excluded from downstream analysis. All sequences passing filters were aligned against a Silva non-redundant 16S reference database (v108) and assigned taxonomic classifications using USEARCH at a 97% identity threshold, and dereplication to unique reference sequence-based Operational Taxonomic Units (refOTU) was performed using UCLUST at a 97% clustering threshold and summarized in a refOTU table. Additional alpha diversity measures and normalized per level taxonomic abundances were created using custom scripts written in R. Differentially significant features at each level were identified using linear discriminant analysis (LDA) along with effect size measurements (LEfSe) to generate the taxonomic cladogram.

**Decerebrate artificially perfused rat (DAPR) preparation and splanchnic sympathetic nerve activity (sSNA) recording**

SHR (HT) and Sprague Dawley (NT) rats were used for this preparation (male, 4-6 weeks old, 80-120 g). The rats were anesthetized, exsanguinated and decerebrated, and experimental set up was performed as previously described. Perfusion pressure, corresponding to the arterial pressure in situ, was measured using a pressure transducer connected to an amplifier. Simultaneous recordings of the phrenic nerve activity (PNA) and the lower splanchnic sympathetic nerve (sSNA) were obtained using glass suction electrodes. These were sampled at 5KHz (CED, Cambridge), amplified (20-50K), filtered
(3-30K) and monitored using Spike2 (CED). The perfusate flow (19-24 ml/min) was adjusted to produce a healthy eupneic pattern of PNA. Vasopressin (1.25-4 nM final concentration, Sigma-Aldrich, USA) was added to the perfusate to adjust perfusion pressure by increasing vascular resistance. In order to qualitatively characterize the sSNA, phrenic-triggered averaging was performed as previously described\textsuperscript{8-10}. This allowed for the classification of averaged sSNA signal into two respiratory-related phases: inspiration (I) and expiration (E), determined based on PNA activity\textsuperscript{8-10}. In this way, peak levels of sSNA during each respiratory phase can be compared across preparations. The immediate response of sSNA to peripheral chemoreceptor stimulation was tested by i.a. bolus injection of 100 µl of 0.03% potassium cyanide (KCN). This allowed for quantification of the maximum sSNA response, presented as percentage increase in sSNA during the 5s post-KCN injection, compared to the equivalent pre-KCN baseline timeline, as previously described\textsuperscript{11}.

**Pseudorabies viral (PRV) retrograde tracing from the small intestine to PVN**

Adult (male, 12 weeks old) WKY and SHR rats were used in this experiment. Some WKY were infused with Ang II (200 ng/kg/min) for seven days prior to the viral tracing injections. Pseudorabies Virus (PRV-152; Virus Center grant no. P40RR018604) tagged with GFP was a gift from Dr. J. Patrick Card, University of Pittsburgh, and was used as a retrograde tracer. The replication-competent virus was applied to the small intestine regions supplied by the mesenteric blood vessels (2 µl), and green fluorescence was examined in the autonomic brain regions four days later. Briefly, the surgical site was shaved and prepared with sterile scrub and the intestine was briefly exposed. PRV was applied onto the surface of the small intestine and spread by soft sterile paintbrush (PRV-152; 3 µl of 4.86 x 108 PFU/ml viral recombinants). The intestine was left exposed for one minute following the application, after which it was gently placed back into the abdominal cavity. The abdominal muscle was sutured, and the skin closed with surgical wound clips. Analgesics were administered prior to surgery and for 48 hours after, as needed. PRV-injected rats (SHR, WKY, and WKY+Ang II) were anesthetized with isoflurane and perfused with 200 ml heparinized saline, followed by 100 ml of 10% formaldehyde solution four days following the PRV injections. This time point was determined as optimal for green fluorescence expression by our previous study\textsuperscript{8}. The brains were collected and processed as previously described\textsuperscript{8}.

**Bone Marrow Chimeras**

Six-week-old SD rats were lethally irradiated (950 cGy of X-rays; service provided by University of Florida Animal Care Services) and reconstituted with age matched 1x10\textsuperscript{7} eGFP-SD whole BM cells. Following irradiation, animals were allowed to recover for 2.5 months before the initiation of experiments. During this time, they received 0.5168 mg/ml Baytril antibiotic (enrofloxacin; Bayer) in the drinking water, moist chow, and Nutri-Cal during the first 3 weeks. Subcutaneous injections of sterile 0.9% saline were performed as necessary to mildly dehydrated animals. BM reconstitution was confirmed by GFP+ FACS in blood mononuclear cells (MNCs).

**Data and Statistical Analysis**

All data are expressed as mean±SEM. 1-way ANOVAs and Bonferroni post-tests were used to allow multiple comparisons between different groups, and non-parametric tests (Kruskal-Wallis and Dunnet’s post-test) were used where necessary. Unpaired Student \textit{t} tests were used for comparisons between 2 groups where applicable, with \(p<0.05\) considered significant. GraphPad Prism 6 was used as the statistical software and for graph generation.
**Figures**

**Online Figure I:** Permeability and stiffness of small intestine and colon are increased in Ang II hypertension. **A,** MAP is increased in Ang II hypertension. **B,** FITC-dextran concentration is also increased in the plasma of Ang II-infused rats (n=4-8/group). **C-D,** Elastic modulus is not altered in the small intestine and proximal colon of Ang II-infused rats (n=4-8/group). **E-H,** Protein analysis of tight junction proteins in small intestine (**E, G**) and proximal colon (**F, H**) in saline and Ang II-infused rats. *p<0.05 vs. Saline.
Online Figure II: Intestinal pathology in Ang II infusion rat model of hypertension.
A-C, Cross sections of small intestine of saline and Ang II rats were stained with Masson’s trichrome to quantify the fibrosis (n=4/group) and D-F, stained with H&E to measure the thickness of tunica muscularis layer (n=4/group). G-I, Villi of the Ang II rats were shortened (n=4/group) and J-L, the number of goblet cells per 100 epithelial cells was decreased in Ang II rats (n=4/group). *p<0.05 vs. saline.
**Online Figure III:** Chronic Ang II infusion increases BM-derived GFP⁺ cells in small intestine and colon.

Representative images and quantification of GFP⁺ cells in small intestine (A-C) and proximal colon (D-F). *p<0.05 vs. WKY (n=4/group).
Online Figure IV: Chronic Ang II infusion increases BM-derived CD3⁺, CD68⁺, and Iba1⁺ cells in small intestine.
A-C, Representative images and quantification of CD3⁺/GFP⁺ BM-derived T-cells in the small intestine of Ang II-infused rats. D-F, Representative images and quantification of CD68⁺/GFP⁺ bone marrow-derived macrophages in small intestine of Ang II-infused rats. G-I, Representative images and quantification of Iba1⁺/GFP⁺ BM-derived myeloid cells in small intestine of Ang II-infused rats. *p<0.05 vs. WKY (n=3-6/group).
References


