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Systemic Leukotriene B4 receptor Antagonism Lowers Arterial Blood Pressure and Improves Autonomic Function in the Spontaneously Hypertensive Rat

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Abstract

Accumulating evidence indicates an association between hypertension and chronic systemic inflammation in both human hypertension and experimental animal models. Previous studies in the spontaneously hypertensive rat (SHR) supports a role for leukotriene B4 (LTB_4), a potent chemoattractant involved in the inflammatory response. However the mechanism for LTB_4 mediated inflammation in hypertension is poorly understood. Here we report in the SHR, increased brainstem infiltration of T cells and macrophages plus gene expression profiling data showing that LTB_4 production, degradation and downstream signalling in the brainstem of the SHR are dynamically regulated during hypertension. Chronic blockade of the LTB_4 receptor 1 (BLT1) receptor with CP-105,696, reduced arterial pressure in the SHR compared to the normotensive control and this reduction was associated with a significant decrease in low and high frequency spectra of systolic blood pressure, and an increase in spontaneous baroreceptor reflex gain (sBRG). These data provide new evidence for the role of LTB_4 as an important neuroimmune pathway in the development of hypertension and therefore may serve as a novel therapeutic target for the treatment of neurogenic hypertension.

Introduction

Hypertension has been linked with chronic inflammation in pre-clinical(Rodríguez-Iturbe *et al.*, 2005; Guzik *et al.*, 2007; Harrison *et al.*, 2010; Marvar *et al.*, 2010; Barhoumi *et al.*, 2011; Mattson *et al.*, 2013; Singh *et al.*, 2015) and clinical studies,(Dalekos *et al.*, 1997; Chae *et al.*, 2001; Bautista *et al.*, 2005) however whether inflammation and sources of inflammation are a cause or consequence is much debated. The spontaneously hypertensive rat (SHR) is known to have an activated inflammatory system(Schmid-Schonbein *et al.*, 1991; Harwani *et al.*, 2012; Li *et al.*, 2015*a*; Dange, 2015) and we have shown that there is enhanced leukocyte and adhesion molecule accumulation in the brainstem microvasculature (Waki *et al.*, 2007; 2008; 2010; Xu *et al.*, 2012). Leukocytes release cytokines and chemokines that affect neuronal activity (D'Arcangelo *et al.*, 2000; Gosselin & Rivest, 2007; Jun *et al.*, 2012) and have effects on arterial pressure regulation at the level of the nucleus tractus solitarii (NTS) (Takagishi *et al.*, 2010; Gouraud *et al.*, 2011) and the paraventricular nucleus (PVN) (Kang *et al.*, 2009; Shi *et al.*, 2010; Colombari *et al.*, 2010; Sriramula *et al.*, 2013). At present however, the mechanism for how immune cells infiltrate the brainstem and impact cardiovascular autonomic control is unknown.

Leukotriene B4 (LTB_4) is a metabolic product of arachidonic acid synthesis via the actions of cytosolic phospholipase A_2 (PLA_2), 5-lipoxygenase (5-LO), and LTA4 hydrolase (Yokomizo *et al.*, 2001). LTB_4 is been shown to be associated with various cardiovascular disease related pathologies including atherosclerosis (Aiello, 2002), obesity (Li *et al.*, 2015*b*), ischaemic stroke (Bevan *et al.*, 2008) and pulmonary hypertension (Tian *et al.*, 2013). In addition, auto-immune diseases such as multiple sclerosis, (Kihara *et al.*, 2010) rheumatoid arthritis (Alten *et al.*, 2004) and asthma (Busse & Kraft, 2005) have demonstrated LTB_4 dependent pathology. In the SHR, we have previously shown that LTB_4 levels are increased in the NTS, a major

brainstem region that governs both the sensitivity of the baroreceptor reflex and the set point of arterial pressure and that acute blockade of LTB_4 receptors in the NTS lowers blood pressure (Waki *et al.*, 2010; 2013).

LTB_4 is a known major inflammatory mediator and potent chemoattractant for leukocytes enhancing their interaction with endothelial cells (Murray *et al.*, 2003; Yokomizo, 2011). Traditionally, LTB4 is considered to be one of the most potent chemotactic and activating factors for neutrophils (Saiwai *et al.*, 2010). As a chemoattractant for inflammatory cells, LTB_4 triggers adherence and aggregation of leukocytes to the endothelium and recruits granulocytes and macrophages to the site of inflammation. LTB_4 can also be released by brain microglial cells (Matsuo *et al.*, 1995), which have been show to be important for the neurogenic component of experimental hypertension. Morevoer, LTB_4 can also act as a strong chemoattractant for T cells (Tager & Luster, 2003*a*; Goodarzi *et al.*, 2003; Medoff *et al.*, 2005; Lone & Taskén, 2013), which are implicated in the pathogenesis of experimental and human hypertension (Trott & Harrison, 2014) (Waki *et al.*, 2013).

The effects of LTB_4 are mediated by binding to the high-affinity LTB_4 receptor (BLTR), which is expressed on inflammatory cells such as neutrophils, eosinophils, and macrophages, and more recently have been found on T cells (Tager & Luster, 2003*b*). LTB_4 has two receptors, the high affinity BLT1 receptor and the lower affinity BLT2 receptor (Tager and Luster, 2003). BLT1 receptors mediate leukocyte chemotaxis whereas BLT2 receptors mediate neutrophil secretion (Showell et al., 1996). These are both G-protein coupled receptors. Here, we have focused on BLT1 receptors because of their higher affinity for LTB4 and association with inflammatory diseases. BLT1 receptors are expressed primarily by neutrophils and vascular smooth muscle cells (Back et al., 2005), but have also been found on endothelial cells (Qiu et al.,

2006), dorsal root ganglion (Andoh and Kuraishi, 2005) and, most recently in the NTS (Hendy E., Waki H. & Paton J.F.R. – unpublished). Thus, antagonising BLT1 receptors would be expected to reduce the pro-inflammatory effects of LTB_4. Moreover, in preclinical disease models, LTB_4 receptor antagonists have been shown to inhibit the recruitment of immune cells to sites of inflammation (Aiello, 2002; Medoff *et al.*, 2005; Spite *et al.*, 2011; Tian *et al.*, 2013) and BLT1 antagonists are continuing to be evaluated in clinical studies for the treatment of inflammatory diseases (Díaz-González *et al.*, 2007). Therefore, the primary aim of the current study was to determine whether chronic systemic inhibition of BLT1 receptors reduces blood pressure and restores autonomic balance in the SHR.

Methods

Procedures were carried out according to the UK Home Office guidelines on animals (Scientific Procedures) Act 1986 and approved by the University of Bristol's Animal Ethics Committee. The Thomas Jefferson University Institutional Animal Care and Use Committee approved procedures for studies conducted at Thomas Jefferson University.

Procedures for real-time PCR: Methods previously used for sample acquisition were followed (DeCicco *et al.*, 2015). Briefly, male, Wistar Kyoto (WKY/NHsd) rats and spontaneous hypertensive rats (SHR/NHsd) obtained from Harlan Laboratories were housed 1 per cage in the Thomas Jefferson University (TJU) animal facility. Facilities were maintained at constant temperature and humidity with 12/12 hour light cycles. The three time points of interest, prehypertension, hypertension onset, and chronic hypertension, correspond to rat age 6-7 weeks, 10-12 weeks and 16 weeks, respectively. At each time point rats were sacrificed and brainstems

were removed and sectioned into 275 um transverse sections. Bilateral microdissected punches of the NTS from one animal were combined into a single sample. Total RNA was extracted with the miRNeasy extraction kit (Qiagen,Valencia, CA). A standard BioMark™ protocol was used to pre-amplify cDNA samples for 12 cycles using TaqMan® PreAmp Master Mix per the manufacturer's protocol (Applied Biosystems, Foster City, CA). qPCR reactions were performed using a 48.48 BioMark™ Dynamic Array (Fluidigm, South San Francisco, CA) enabling quantitative measurement of multiple genes with assay replicates and samples under identical reaction conditions. Each mRNA level was measured in multiple reaction chambers on a single BioMark array (n=6 or 7 technical replicates). The PCR was performed for 30 cycles (15s at 95°C, 5s at 70°C, 60s at 60°C). The primers are listed in Table S1. Ct values were calculated using the Real-Time PCR Analysis Software (Fluidigm). Software-designated failed reactions as well as reactions with melt curves not at the appropriate temperature were discarded from subsequent analysis.

Real-time PCR data analysis: The gene expression data was normalized relative to the expression of *Eif4e* within sample as a reference (ΔC_t). The reference-normalized data were analyzed using a two-way ANOVA considering strain and stage as two independent factors (Strain: SHR and WKY; Stage: prehypertension, onset and chronic hypertension). For visualization, the reference-normalized gene expression data was further normalized to the WKY data at the onset timepoint ($\Delta\Delta C_t$).

Flow Cytometric Analysis of Circulating Immune Cells: Circulating inflammatory cells were analyzed using flow cytometry as previously described (McBryde *et al.*, 1AD; Marvar *et al.*,

2010; Marvar & Harrison, 2012). Antibodies (BD Biosciences) used for staining were as follows: FITC anti-CD45 (30-F11); APC anti-CD4 (GK1.5); PerCP anti-CD8 (53–6.7) and APC anti-CD3e (145-2C11) and eflour-660 anti-CD11b/c. All antibodies were diluted (1.5ul / 100ul) in FACS buffer (0.5% bovine serum albumin in PBS). After immunostaining, cells were resuspended in FACS buffer and analyzed immediately on a LSR-II flow cytometer with DIVA software (Becton Dickinson). Data were analyzed with FlowJo software (Tree Star, Inc.). All samples were normalized to 1x 10^6 cells and the percentage of the total events collected out of the 1x 10^6 cells were analyzed.

Flow Cytometric Analysis of tissue-infiltrating immune cells: FACS analysis of T cells in brainstem homogenates was performed as previously describe(McBryde *et al.*, 2009). To analyze leukocytes in the brainstem, tissue was digested using collagenase type IX (125u/ml); collagenase type IS (450U/ml) and hyaluronidase IS (60U/ml) dissolved in 20 mM HEPES-PBS buffer for 30 minutes at 37^o C, while constantly agitated. The dissolved tissue was then passed through a 70 m sterile filter (Falcon, BD), yielding a single cell suspension. An additional step was applied for brain tissue using a 30% /70% percoll gradient to separate out the mononuclear cell layer. Cells were then washed twice with FACS buffer then counted. 1x 10^6 cells were stained and analyzed using multicolor flow cytometry. Antibodies (BD Biosciences) were then used for staining as described above. After immunostaining, cells were re-suspended in FACS buffer and analyzed immediately on a LSR-II flow cytometer with DIVA software (Becton Dickinson). Data were analyzed with FlowJo software (Tree Star Inc., Ashland, Oregon, USA) and an initial gate was applied to exclude cell debris from analysis and CD45 positive cells were identified as leukocytes within the tissue cell suspension and T cells were identified with anti-

CD3 antibodies and the HIS36 monoclonal antibody which reacts with a molecule expressed on the surface of rat macrophages.

Drug administration: CP-105,696 a specific high affinity antagonist for BLT1 receptors in rat (Souza *et al.*, 2000) was kindly provided by Pfizer Inc., Groton, Connecticut, USA. Previous studies have shown that CP-105,696 reduced inflammation, vascular permeability, neutrophil and monocyte accumulation in the rat (Souza *et al.*, 2000; Aiello, 2002). Based on pilot studies we determined an effective dose of CP-105,696 of 74 mg/kg/day which is comparable to a previous study where up to 100 mg/kg/day was used in mice to reduce atherosclerotic disease (Aiello, 2002). CP-105,696 was prepared in dimethyl sulfoxide (DMSO) with a final concentration of 0.8% and well below concentrations used in other studies (15-20%) where no change in cardiovascular variables were reported (Sampey *et al.*, 1999; Pĭtre *et al.*, 1999). CP-105,696 was administered in the drinking water over a 21 day period. Vehicle control consisted of 0.8% DMSO in the drinking water. Subgroups of rats were treated with CP-105,696 for a shorter period (5 days) for evaluation of effects on acute changes in circulating leukocytes and related surface expression markers. However, we excluded the inclusion of a Wistar-vehicle group from analysis because we saw no change in blood pressure in the Wistar-CP-105,696 group. Blood pressure was our primary endpoint therefore we did not consider additional interrogation of these variables.

Radio-telemetry recordings: All animals (24-26 weeks of age) were housed individually, allowed normal rat chow and drinking water *ad libitum*, and kept on a 12 h light/12 h dark cycle. Age matched male SHR and Wistar rats (24-26 weeks old) were allowed to adapt for 1 week (standard laboratory rat chow with water *ad libitum)* before implantation of a radio-telemetry

device for recording of arterial pressure (Data Sciences International, Arden Hills, MN, USA) as previously described (Waki *et al.*, 2007). The blood pressure of each rat was recorded for a three-day baseline period prior to CP-105,696 administration. CP-105,696, or vehicle control (0.8% DMSO was given in the drinking water for 21 days. The three groups of rats were studied: (i) SHR + CP-105,696 (ii) SHR + vehicle; (iii) Wistar rats + CP-105,696. Telemetry data were acquired and both the spectra of systolic blood pressure and inert-pulse interval and spontaneous cardiac baroreceptor reflex gain (sBRG) analyzed using Hey-Presto software as previously described (Waki *et al.*, 2006). Arterial blood pressure was recorded for a minute interval every hour for 24 h during the entire experimental period. 24 hour, plus separate light (12 hours) and dark phase (12 hours) telemetry data was analyzed.

Autonomic and Cardiovascular Function analysis: Our analysis program contained a fast Fourier transform (FFT) function for power spectral analysis of PI and BP variability and a function for spontaneous baroreflex gain (sBRG) based on a time-series technique (Waki *et al.*, 2006). The magnitude of power was integrated in the very low-frequency (VLF) band (0-0.27 Hz), the low-frequency (LF) band (0.27-0.75 Hz) and the high-frequency (HF) band (0.75–3.3 Hz). Cardiovascular data including systolic blood pressure and heart rate were expressed as either as a change from baseline or peak change that was defined as the peak change value from baseline.

Statistical analysis: Statistical analysis was performed using Graphpad Prism v4.02. Data are expressed as mean ± SEM and values of $p < 0.05$ were considered statistically significant. Statistical tests were performed using 2-way ANOVA with repeated measures and Bonferroni

post hoc test. Significance of the peak difference in systolic blood pressure between groups as well as flow cytometry analysis was determined by an unpaired two-tailed Students t-test. 2-factor ANOVA was used for qPCR statistical analysis. For qPCR analysis, significance of gene expression differences between SHR and WKY at each time point was evaluated by a *post hoc* Tukey Honest Significant Difference approach using *aov* and *Tukey HSD* functions in the core statistical package of the R platform (DeCicco *et al.*, 2015).

Results

Leukotriene B4 associated gene expression levels in the nucleus tractus solitarii (NTS) during hypertension development in the SHR:

The expression levels of six genes involved in synthesis and metabolism of LTB4 as well as two LTB4 receptors were evaluated at different phases of hypertension development (Figure 1A). As shown in Figure 1B, at the onset and chronic phase of hypertension, two genes involved in the upstream steps of LTB4 production, arachidonate 5-lipoxygenase (*Alox5*) and arachidonate 5-lipoxygenase-activating protein (*Alox5ap*) showed a trend of higher mRNA transcript levels in SHR compared to WKY. However this failed to achieve statistical significance. In addition, leukotriene A4 hydrolase (*Lta4h*), which is responsible for the conversion of LTA4 into LTB4, showed a trend for a reduction at the onset and chronic phases compared to the WKY (Figure 1C). The expression levels of prostaglandin reductase 1 (*Ptgr1*) enzyme, which is responsible for the degradation of LTB4, was significantly reduced at the onset stage of hypertension and a significant overall strain difference across all three time points was determined by 2-way ANOVA (Figure 1C). Finally, there were no statistical differences in leukotriene B4 Receptors 1 and 2 (*Ltb4r* and *Ltb4r2*, respectively) expression over time (Figure 1D).

Enhanced immune cell infiltrates in the brainstem of the SHR and increased surface expression of $BLTR_1$ on circulating lymphocytes:

Prior evidence supports a pro-inflammatory phenotype in the SHR, in particular evidence that excessive inflammation of the brainstem and evidence suggests that LTB_4 may contribute to the hypertensive phenotype (Waki *et al.*, 2013). Moreover, the adaptive immune response has been shown to play a significant role in other models of experimental hypertension (Trott & Harrison, 2014). We therefore assessed brainstem infiltrates of adaptive (CD3+) and innate immune cells (macrophages) in the SHR and Wistar rats. As shown in figure 2B-E the percentage of $CD3^+$ cells ($t_{22} = 4.6$ $p<0.01$) and macrophages ($t_{22} = 3.1$ $p<0.01$) in the brainstem of the SHR are significantly greater than Wistar rats. Because BLT1 receptors are present on some lymphocytes, these cells may be a major target for LTB4 and our previous evidence demonstrates that within the NTS there are increased CD4+ cells in the SHR (Waki *et al.*, 2007; Xu *et al.*, 2012).

We next evaluated the surface expression levels of $BLTR_1$ on circulating CD4+ lymphocytes. As shown in figure 2F-G, the hypertensive group had a significantly greater percentage of CD4+ cells expressing the $BLTR_1$. These data provide further evidence that increased brainstem inflammatory cells and $BLTR_1$ expressing lymphocytes may contribute to the brainstem inflammatory phenotype in the SHR and associated autonomic dysfunction. Therefore, we next evaluated the *in vivo* anti-inflammatory effects of systemic blockade of the LTB_4 receptor BLT1.

BLT1 receptor antagonism (CP-105-696) reduces increased circulating levels of $CD11b^+$ cells in the SHR:

Through binding of the BLT1 receptor, LTB_4 can induce the expression of adhesion molecules such as $CD11b^+$ on polymorphonuclear leukocytes that can contribute to leukocyte

adherence to endothelial cells. LTB_4 can exert these effects primarily in neutrophils but also on T lymphocytes and macrophages, which ultimately promote accumulation of these cells at sites of inflammation, such as the brainstem. Previous studies have shown that the BLT1 receptor antagonist CP-105,696 inhibits the LTB4-mediated upregulation of $CD11b^+$ on neutrophils and monocytes (Aiello, 2002). In additional groups of rats, we therefore examined the short-term effects of CP-105,696 on monocyte $CD11b^+$ cell surface expression as well as the tissue homing marker $CD44^+$ (Salmi *et al.*, 2013) to determine whether the dose we administered was effective in reducing LTB4 mediated immune affects. As shown in Figure 3A, following 5 days of CP-105,696 treatment, the percentage of $CD11b^+$ cells in both SHR and Wistar was significantly reduced in addition to the expression levels of the tissue homing marker $CD44^+$ (Figure 3C). Notably, CP-105,696 did not affect other peripheral blood leukocyte populations including $CD4^+$ and $CD8^+$ populations of T cells, suggesting that this treatment was specific to monocytes (data not shown). Moreover, at baseline, $CD11b^+$ expressing leukocytes were significantly greater in the SHR compared to Wistar controls (Figure 3A-B), which may contribute to the dysregulation of the LTB_4 system in the SHR. Therefore, we next sought to determine whether systemic blockade of the LTB_4 receptor-BLT1 axis improves blood pressure and autonomic function in the SHR.

BLT1 receptor antagonism with CP-105,696 reduces blood pressure and heart rate in the SHR.

Baseline cardiovascular, autonomic and respiratory measures in all animals prior to receiving CP-105,696 are described in Table 1. The temporal profiles for average change in systolic blood (SBP) pressure and heart rate (HR) over the treatment period are shown in Figure 4A and C. In rats receiving CP-105,696, there was a significant 24-hour mean decrease in

change in SBP and HR starting on day 8 and this was well maintained until the end of the study (21 days). A significant peak reduction in HR from 296 ± 2 to 269 ± 9 BPM was observed on day 19 (Figure 4C). Peak delta responses in HR for all groups are shown in Figure 4B and D. The blood pressure and heart rate effects of CP-105,696 were greatest during the light phase, therefore this was the focus of our autonomic activity analysis (see below). There was no sustained changes in SBP or HR in SHR-Vehicle and Wistar-CP-105,696 rat groups.

Improved autonomic function in the SHR following BLT1 receptor antagonism with CP-105696:

To determine the effects of CP-105,696 on autonomic function in the SHR, we next analyzed the spectra of SBP and inter-pulse interval and spontaneous cardiac baroreceptor reflex gain (sBRG). As shown in Figure 5A the temporal profile in, sBRG(PI) in the SHR-CP105,696 group increased in the light phase from day three and remained significantly higher throughout the drug administration period (from 0.8 ± 0.04 to 1.1 ± 0.1 ms $mmHg^{-1}$). Peak response in sBRG(PI) is shown in Figure 5B. There was no change in the dark phase or in SHR-Vehicle and Wistar-CP-105,696 rat groups for either light or dark phases (Table 1).

As shown in the temporal profile in Figure 5C, the low and high frequency (HF/ LF) SBP was significantly reduced in the SHRs receiving CP-105,696 from days 11 onwards. This timing coincided with the fall in systolic blood pressure (Figure 4A). The decrease in LF SBP was intermittent in both the light and dark phases, while the SHR-Vehicle showed no persistent changes (Figure 5C). The peak reduction in LF SBP is represented in Figure 5D.

A significant reduction in HF SBP was observed in the SHRs that received CP-105,696. In the light phase, this persisted from day 11 to the end of the experiment (from 5.0 ± 0.2 $mmHg/Hz^{½}$ to 4.0 ± 0.2 $mmHg/Hz^{½}$ Figure 5E) and a similar reduction in HF (SBP) was

observed in the dark phase (from 5.6 ± 0.1 mmHg/Hz^{½} to 4.7 ± 0.1 mmHg/Hz^{½}; P<0.01). The peak changes are represented in Figure 5F. However, in the Wistar-CP-105,696 group, HF(SBP) decreased in the light phase, but not dark phase, becoming significantly lower than baseline from days 14-21 (from 4.7 ± 0.2 mmHg/Hz^{½} to 3.7 ± 0.2 mmHg/Hz^{½}; P<0.01). Moreover, consistent with the SHR-CP-105,696 exhibiting a prolonged reduction of HF (SBP), a persistent change in respiratory rate was observed in SHR-CP-105,696 group during the light phase (from 67 ± 1.5 to 54 ± 0.7 breaths per minute) (Data not shown).

Discussion

For the first time we have shown that mRNA expression levels of enzymes responsible for the production, degradation and down-stream signaling of LTB_4 demonstrates that some enzymes maybe dynamically regulated over the course of the development of hypertension in the SHR. Moreover, compared to normotensive controls, the SHR exhibit enhanced levels of infiltrating macrophages and T lymphocytes in brainstem homogenates and increased circulating $BLTR_1$ expressing lymphocytes. Importantly, chronic treatment with the BLT1 receptor antagonist CP-105,696 improved cardiovascular and autonomic measures in the SHR and reduced circulating inflammatory like cells. Overall these data support our previous findings (Waki *et al.*, 2011; 2013) and provide new genomic and *in vivo* physiological evidence for the involvement of the LTB_4-BLT1 axis as an important neuroimmune pathway in the development and maintenance of hypertension.

LTB_4 has been previously shown to be elevated in the NTS in both young (age 3 weeks) and adult (aged 15 weeks) SHRs(Waki *et al.*, 2013). To further understand the dynamic changes in the metabolic production of LTB_4 from arachidonic acid, we evaluated the expression of the

transcripts of key synthesis and degradation enzymes responsible for its production across stages of hypertension development in the SHR. The three time points of interest: prehypertension, hypertension onset, and chronic hypertension, correspond to rat age 6-7 weeks, 10-12 weeks and 16 weeks of age, respectively. The trend in *Alox5* and *Alox5ap* levels suggest an increase in production of LTA_4 in the first step of production of LTA_4 from 5-HETEs in the SHR. Typically, LTA_4 and 5-HETEs exist in much higher amounts than LTB_4 (Jakschik & Kuo, 1983). Our data demonstrate a trend for LKHA4 (*Lta4h*), the enzyme that converts LTA_4 into LTB_4 to be lower in SHR both at the hypertension onset and chronic hypertension stages. LKHA4 has been shown to be the rate-limiting enzyme in the production of LTB_4 (Jakschik & Kuo, 1983). Interestingly, in both SHR and WKY at the prehypertension age the *Lta4h* levels were not significantly different even though SHR showed marginally lower levels than in WKY. We note that the mRNA expression levels may not be fully indicative of the enzymatic activity of LKHA4 as there are additional posttranscriptional regulators of this pathway. For example, LKHA4 enzymatic activity is known to be inactivated by excess incubation with arachidonic acid and peroxy fatty acids (Jakschik & Kuo, 1983). It has previously been shown that oxidative stress precedes peroxy fatty acid formation in SHRs (Purushothaman *et al.*, 2011) and that oxidative stress is increased in the NTS of SHRs (Nozoe *et al.*, 2007).

LTB_4 degradation is controlled primarily through LTB_4-12-HD/PTGR1 (Jakschik & Kuo, 1983; Vitturi *et al.*, 2013). As shown in Figure 1C, *Ptgr1* expression is lower in SHR at the onset stages compared to WKY rats. This would suggest that the increase in LTB_4 levels previously described by Waki et al. (2013) is primarily due to low levels of its negative regulator, *Ptgr1*. It is also interesting to note that in the WKY rat at the hypertension onset and chronic hypertension stage, there is a trend for mRNA levels to be increased compared to SHR. Although speculative,

this may suggest that WKY rats can metabolize LTB_4 better than SHRs thus reducing their inflammatory state to maintain a normotensive state. Finally, despite the trend of a lower transcript expression levels, there were no statistical difference in the LTB_4 receptors, BLTR1 and BLTR2. However, we acknowledge the limitation of qPCR analysis for our study as relative expression does not necessarily predict changed activity of an enzyme. Future enzymatic assay studies are now required to more definitively determine dynamic changes in LTB4 enzyme activity over the course of hypertension. We speculate that in conjunction with the previously reported increase in LTB4 in NTS (Waki *et al.*, 2013), that a desensitization effect maybe occurring at the LTB_4 receptor in the NTS. Desensitization upon excess agonist exposure has been shown in many G-protein coupled receptors, and specifically in the case of BLT1 (Gaudreau *et al.*, 2002; Chen *et al.*, 2004). LTB_4 primarily exerts its inflammatory effects by interacting with its high-affinity receptor BLT1 receptor that is predominantly expressed on immune cells, including neutrophils, macrophages and effector T cells. (Tager & Luster, 2003*b*; Goodarzi *et al.*, 2003).

Our previous immunocytochemistry evidence demonstrated increased $CD4^+$ T cells within the NTS that may be accumulating due to the chemotactic actions of LTB_4 (Waki *et al.*, 2007; Xu *et al.*, 2012). CD4+ T cells may serve as an important immune cell type for the enhanced LTB_4 activity in the SHR. In support of these data, and in further quantifying brainstem immune infiltrates, our new data herein indicate that the SHR exhibits enhanced total $CD3^+$ T cells and macrophages; both these cell types are involved in the LTB_4 chemotactic inflammatory response. Moreover, we have shown that the SHR has an increased percentage of CD4+ cells expressing BLT1 receptor. Thus, a possibility is that hypertension promotes the expression of BLT1 receptor on T cells which leads to increased LTB4 – T cell mediated inflammation in the

brainstem. The mechanisms for this are unknown but highlight the complexity and specificity of the inflammatory state of the SHR (Waki *et al.*, 2008; DeCicco *et al.*, 2015). Despite these data, further immune cell phenotype analysis is required to determine the LTB_4 mediated immune cell interactions and associated inflammatory signals and adhesion molecules in tissue inflammation. In particular, additional investigation of BLTRs on subsets of T cells, such as CD8+ cells and their potential role in antigen presentation in hypertension needs further investigation.

We have previously shown that a localized injection of LTB_4 in the NTS evoked a pressor response and that BLT1 receptor antagonism within the NTS significantly lowers blood pressure as well as LF and VLF spectra of systolic blood pressure and these effects persisted for 6-days (Waki *et al.*, 2013). These data suggest a causal relationship for the LTB_4-BLT1 pathway within the NTS in modulating cardiovascular autonomic control. However, the peripheral effects of LTB_4 were not examined in this study. LTB_4 is a potent chemoattractant for circulating neutrophils (Canetti *et al.*, 2003; Grespan *et al.*, 2008) and these cells can also synthesize and secrete LTB_4, which plays an important role in their proliferative and migratory capacity (Afonso *et al.*, 2012). Previous studies have shown that the BLT1 receptor antagonist CP-105,696 inhibits the LTB4-mediated up regulation of $CD11b^+$ on neutrophils and monocytes (Aiello, 2002). Similarly, here we demonstrate that in the presence of the BLT1 antagonist (CP-105,696), $CD11b^+$ expression on leukocytes is significantly reduced. These data support the findings from Aiello et al. (2002) who demonstrated that CP-105,696 decreased $CD11b^+$ expression in $ApoE^{-/-}$ mice and subsequently reduced atherosclerotic lesions. Moreover, CP-105,696 treated rats, had a significant reduction in the tissue homing marker CD44+ cells expressed on leukocytes (Salmi *et al.*, 2013). Therefore, we next sought to evaluate the effects of systemic treatment of CP-105,696 on autonomic and cardiovascular indices in the SHR.

Following CP-105,696 administration there was a significant reduction in 24hr mean arterial pressure in the SHR compared to the normotensive Wistar rat. However, reductions in blood pressure and autonomic measures were greatest during the light phase and suggests that CP-105,696 impacted the modulation of the diurnal rhythm. Moreover, reductions in blood pressure in the SHR treated with CP-105696 coincided with significant reductions in the LF and HF power of SBP in both light and dark phases. Mechanistically, these data are suggestive of a possible decline in vasomotor sympathetic activity occurring at the time when blood pressure became reduced. Interestingly, the reduction in HF(SBP) was most pronounced and indicates a reduction in either the mechanical effect of respiration on SBP, which may be associated with the reduction in respiration rate seen in the SHR- CP-105,696 group, or reduced respiratory modulation of sympathetic activity, which significantly contributes to vasomotor tone in the SHR(Simms *et al.*, 2009; Moraes *et al.*, 2014; Briant *et al.*, 2015). Notably, the timing of this fall in arterial pressure also coincided with the effects of CP-105,696 on reducing the levels of circulating $CD11b^{+}$ expression after 5 days. Given these neuoro-immune effects of CP-105,696, it is also possible that this drug maybe impacting the bone marrow, which has been recently implicated in the development of neurogenic hypertension(Zubcevic *et al.*, 2011; Raizada & Jun, 2012). Overall, these data support that CP-105,696 is anti-hypertensive and this effect is mediated, in part, by the autonomic nervous system.

From these data it is unclear whether the effects are due to changes in peripheral afferent sensory mechanisms that control arterial pressure, an effect on the brain and central control or at the level of the target organ per se or a combination of different sites. However, given that the depressor response was associated with reductions in LF(SBP) and increase in cardiac baroreceptor reflex gain it appears that part of the hypotensive response was mediated by an

action on cardiovascular autonomic activity. Moreover, based on its structure (CP-105,696 or (+)-1-(3S,4R)-[3-(4-phenylbenzyl)-4-hydroxy-chroman-7-yl] cyclopropane carboxylic acid) we cannot rule out CNS penetrance and the effects we have seen on the autonomic nervous support this contention. Therefore, we proposed that the build-up of adhered leukocytes in small microvessels of the brainstem may obstruct blood flow and cause focal ischemia providing a driver for enhanced sympathetic nerve activity.(Paton & Waki, 2009); whether these are cleared by CP-105,696 remains an open question.

Alternatively, cytokines being released by the adhered and activated leukocytes can result in increased neuronal stimulation, acting as neurotransmitters within the brain.(Rostène *et al.*, 2007) Interestingly, LTB_4 has been associated with increased pro-inflammatory cytokine release, namely IL-6 and IL-1ß and positively correlated with blood pressure in man (Dalekos *et al.*, 1997; Chae *et al.*, 2001; Taniyama & Griendling, 2003; Bautista *et al.*, 2005; Kim & Vaziri, 2005; Peterson *et al.*, 2006). NF-κB, a transcription factor involved in the up-regulation of several genes including chemokines and cytokines is responsive to both LTB_4, (Brach *et al.*, 1992; Huang *et al.*, 2004; Serezani *et al.*, 2011), angiotensin II (Hardy *et al.*, 2001; Virdis & Schiffrin, 2003) and ROS (Elks *et al.*, 2009). Moreover, LTB_4 can stimulate the release of IL-6 (Brach *et al.*, 1992) and MCP-1 (Huang *et al.*, 2004) via the NF-κB pathway and BLT1 receptor expression has been shown to be up-regulated in response to IL-1ß (Bäck *et al.*, 2005) thus indicating a positive feedback loop. Chronic pharmacological inhibition of the NF-κB pathway in young SHR prevented the onset of hypertension and blunted renal inflammation (Rodríguez-Iturbe *et al.*, 2005). Whether the hypotensive effect of antagonising the BLT1 receptors in the present study is due to reduced NF-κB activity or other pro-inflammatory cytokine mediated events remains to be determined. It is also interesting to speculate that dysregulation of the LTB_4

system in the SHR could contribute to excessive isoketal (F2-isoprostane pathways intermediates) formation which plays a role in mediating the inflammatory response in experimental and human hypertension (Kirabo *et al.*, 2014)

In summary, these data demonstrate that at the level of gene expression within the NTS, the metabolic regulation of LTB_4 changes dynamically during the development of hypertension and systemic BLT1 receptor inhibition effectively reduces blood pressure that includes a central action on autonomic activity. Overall, these data provide new evidence for the role of LTB_4 as an important neuroimmune pathway in the development and maintenance of hypertension and therefore may serve as an important biomarker and therapeutic target for the treatment of neurogenic hypertension.

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Disclosures

None

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Figure Legends

Figure 1. Dynamic changes in expression of LTB4 related genes over time during the development of hypertension. A) Summary of genes measured as they relate to Arachidonic Acid metabolism. Evaluation of six genes affecting LTB4 production (B, C-left panel), degradation (C-right panel) and signaling receptors (D) with reference to housekeeping gene Eif4e and WKY onset time point. * $p<0.05$, #Strain Difference Significant, 2-factor ANOVA, tukey post-hoc $p<0.05$. Of note for strain significant difference is: *AloxAP*, p-value = 0.076 and *Lta4h*, p-value = 0.077. Und, Undetermined Expression, n=3-4 per gene.

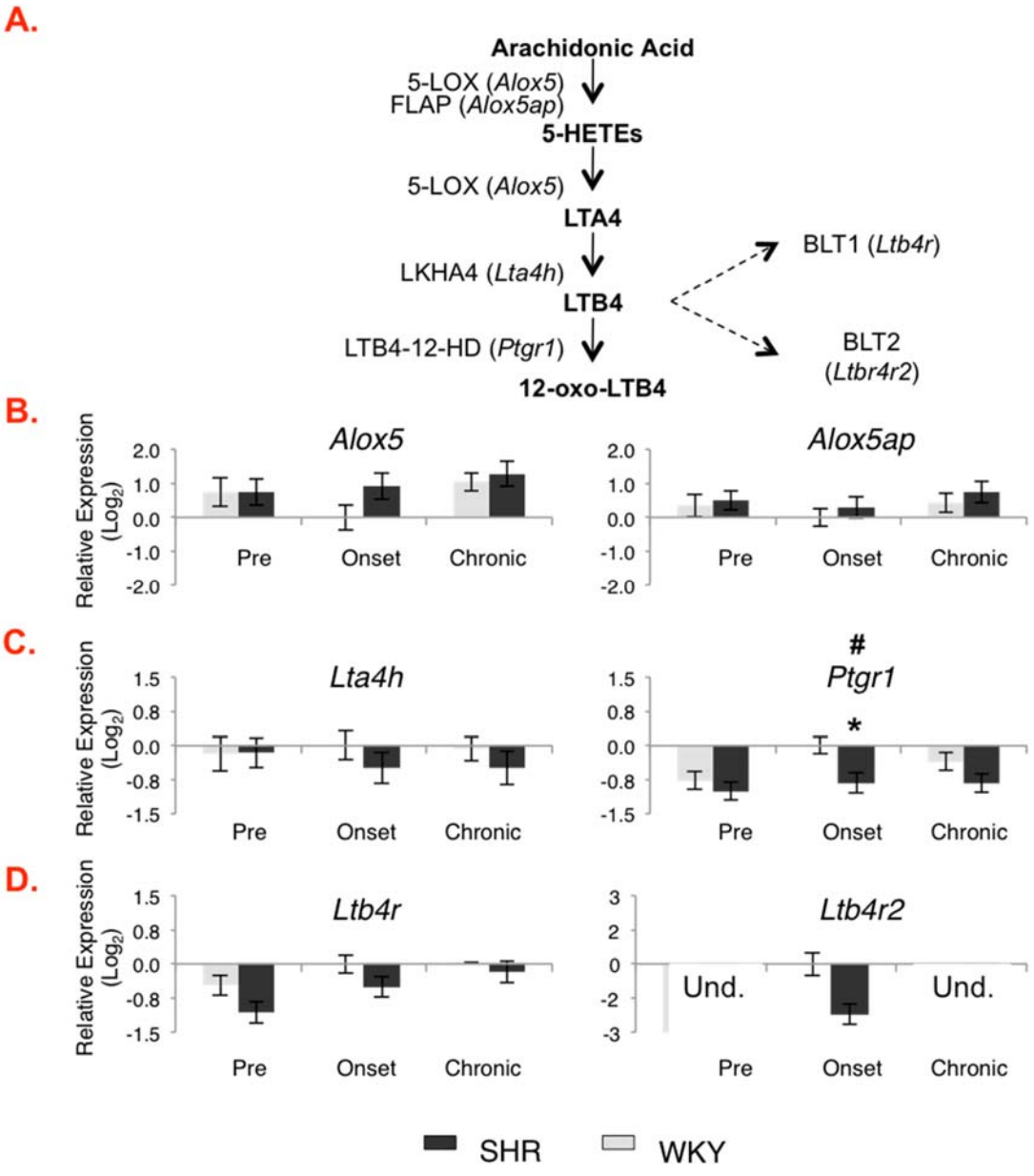
Figure 2. Increased lymphocyte brainstem infiltrates and increased expression circulating $CD4^+BLTR_1$ cells in the SHR. Flow Cytometry gating strategy (A). Percent total $CD45^+$ macrophages (HIS36) in the brainstem of SHR and Wistar rats (B) representative flow cytometry cell scatter plot (C). Percent total infiltrating $CD45^+CD3^+$ in the brainstem of SHR and Wistar (D) representative flow cytometry cell scatter plot (E). Increased percentage of circulating lymphocytes expressing the LTB4 receptor $BLTR_1$ (F) and corresponding representative flow cytometry cell scatter plot (G). Data are presented as mean ± SEM, *$p<0.05$

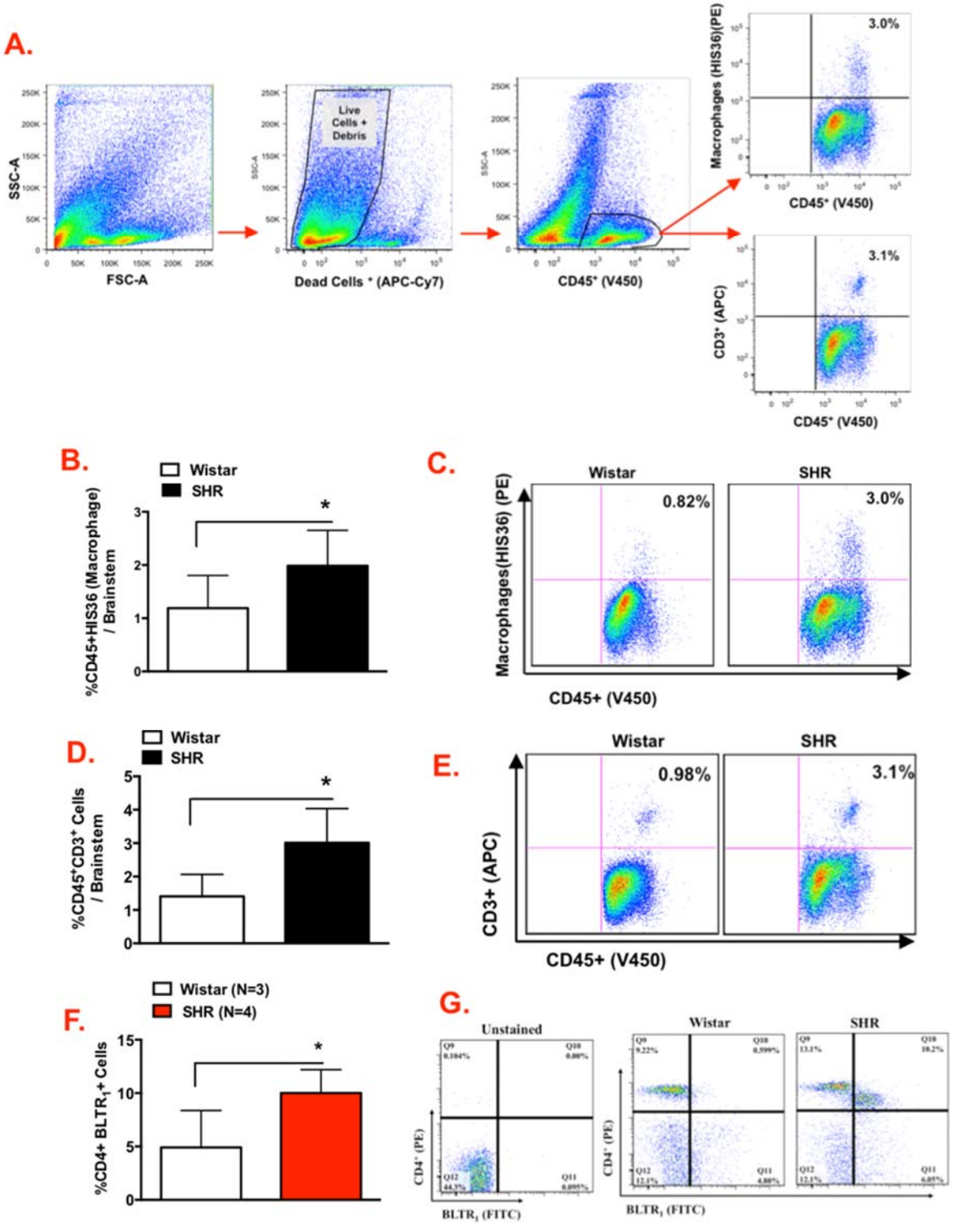
Figure 3. Increased circulating levels of $CD11b^+$ cells in the SHR are reduced with CP-105,696. Percent total circulating $CD11b^+$ cells in SHR and Wistar groups at baseline and following 5 days of CP-105,696 (a) Representative flow cytometry contour plot showing reduced circulating $CD11b^+$ following CP-105,696 (b). Percent total circulating $CD44^+$ leukocytes before

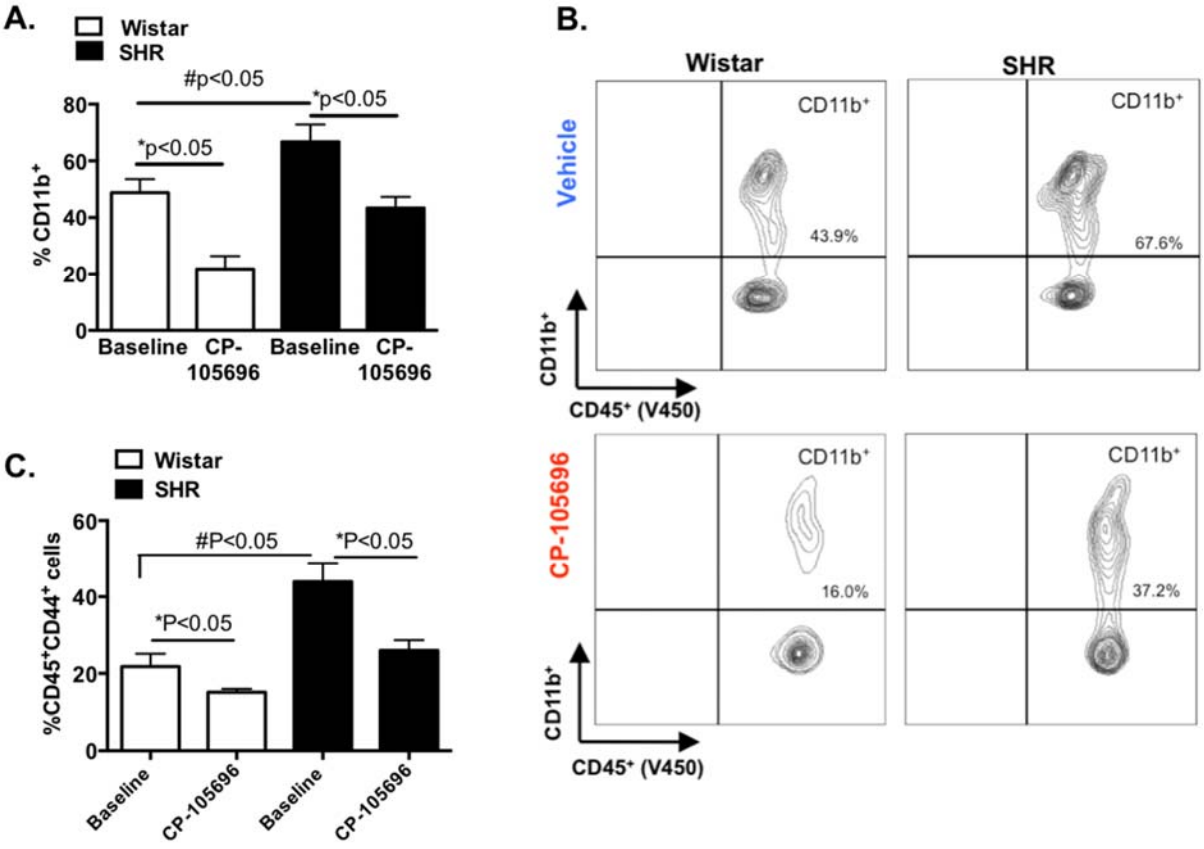
and after CP-105-696 in SHR and Wistar groups (c) n=5-6 per group; Data are presented as mean ± SEM, *p<0.05, #p<0.05 Baseline Wistar vs Baseline SHR.

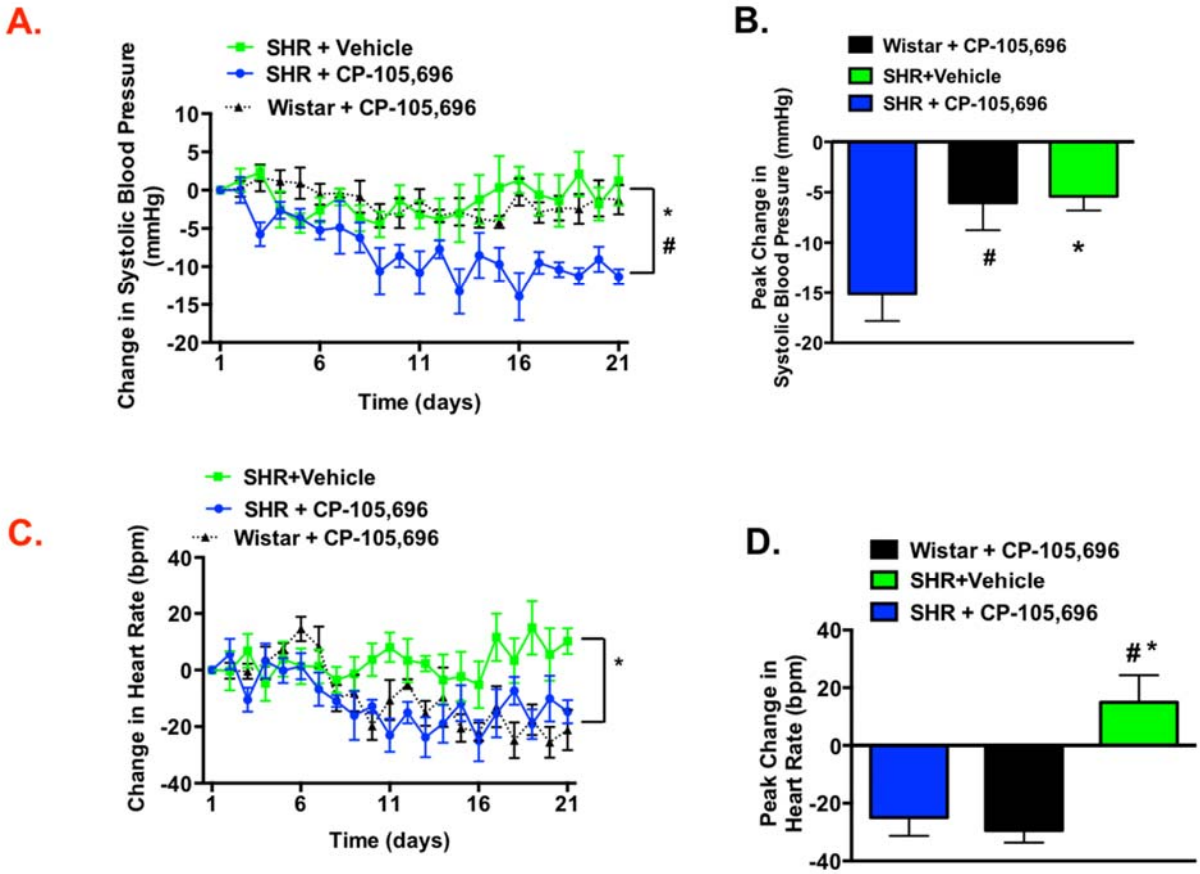
Figure 4. Chronic administration of CP-105,696 reduces blood pressure and heart rate in the SHR. Decreased change in systolic blood pressure (SBP) and heart rate from baseline in the SHR-CP group with no change in SHR vehicle and Wistar CP and vehicle (a,c). Peak change in SBP and heart rate in SBP SHR+CP-105,696 (n=5), SHR-Vehicle (n=5) and WKY-CP (n=5) groups are shown in panels b,d. n=5-6 per group; Data are presented as mean ± SEM, *p<0.05 overall main effect; post-hoc analysis #p<0.05 SHR-Vehicle vs SHR+CP-105,696.

Figure 5. Improved autonomic function in the SHR following BLT1 receptor antagonism with CP-105696. Increased change in spontaneous baroreceptor gain (sBRG) in the light phase of SHR+CP-105,696 vs SHR-vehicle groups (a) and peak changes in sBRG between groups in panel b. Decreased LF(SBP) and HF(SBP) in the light phase of the SHR-CP105,696 and Wistar CP and vehicle groups (c,e) and peak changes in LF(SBP) and HF(SBP) (d,f). n=5-6 per group; Data are presented as mean ± SEM, *p<0.05; Wistar vs SHR-Vehicle.


A.
Arachidonic Acid
5-LOX (Alox5)
FLAP (Alox5ap)
5-HETEs
5-LOX (Alox5)
LTA4
LKHA4 (Lta4h)
LTB4
LTB4-12-HD (Ptgr1)
12-oxo-LTB4
BLT1 (Ltb4r)
BLT2 (Ltbr4r2)
B.
Alox5
Alox5ap
C.
Lta4h
Ptgr1
#
*
D.
Ltb4r
Ltb4r2
Und.
Und.
Relative Expression (Log2)
Pre
Onset
Chronic
SHR
WKY


A.
SSC-A
FSC-A
Live Cells + Debris
Dead Cells * (APC-Cy7)
CD45* (V450)
Macrophages (HIS36)(PE)
3.0%
CD45* (V450)
CD3* (APC)
3.1%
CD45* (V450)
B.
Wistar
SHR
%CD45+HIS36 (Macrophage) / Brainstem
C.
Wistar
SHR
0.82%
3.0%
Macrophages(HIS36) (PE)
CD45+ (V450)
D.
Wistar
SHR
%CD45+CD3+ Cells / Brainstem
E.
Wistar
SHR
0.98%
3.1%
CD3+ (APC)
CD45+ (V450)
F.
Wistar (N=3)
SHR (N=4)
%CD4+ BLTR1+ Cells
G.
Unstained
Wistar
SHR
CD4+ (PE)
BLTR1 (FITC)


A.
Wistar
SHR
#p<0.05
*p<0.05
*p<0.05
% CD11b+
80
60
40
20
0
Baseline
CP-105696
Baseline
CP-105696
B.
Wistar
SHR
Vehicle
CD11b+
43.9%
CD11b+
67.6%
CD11b+
CD45+ (V450)
CP-105696
CD11b+
16.0%
CD11b+
37.2%
CD11b+
CD45+ (V450)
C.
Wistar
SHR
#P<0.05
*P<0.05
*P<0.05
%CD45+CD44+ cells
60
40
20
0
Baseline
CP-105696
Baseline
CP-105696


A.
SHR + Vehicle
SHR + CP-105,696
Wistar + CP-105,696
Change in Systolic Blood Pressure (mmHg)
Time (days)
B.
Wistar + CP-105,696
SHR+Vehicle
SHR + CP-105,696
Peak Change in Systolic Blood Pressure (mmHg)
C.
SHR+Vehicle
SHR + CP-105,696
Wistar + CP-105,696
Change in Heart Rate (bpm)
Time (days)
D.
Wistar + CP-105,696
SHR+Vehicle
SHR + CP-105,696
Peak Change in Heart Rate (bpm)

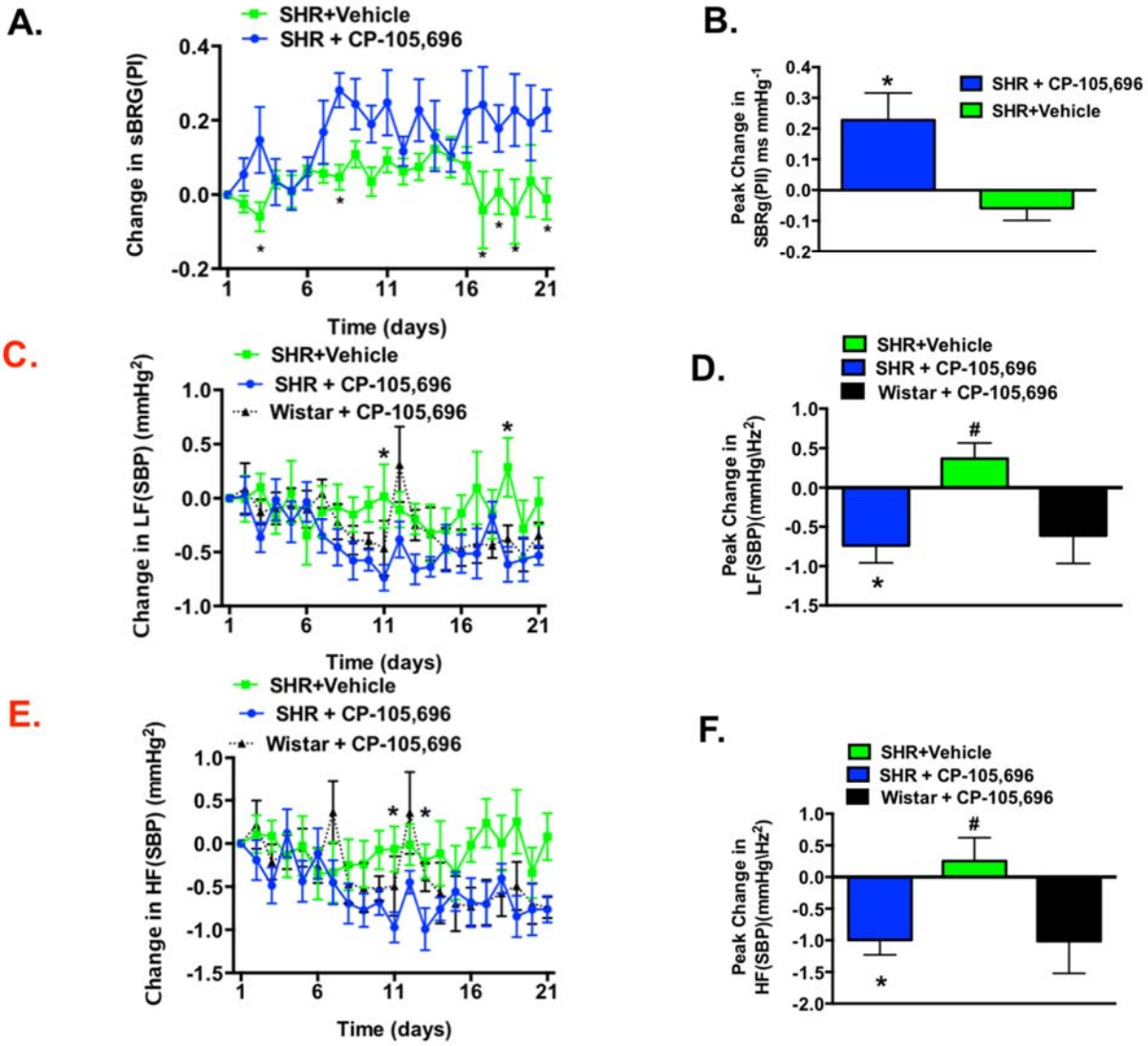

A.
SHR+Vehicle
SHR + CP-105,696
Change in sBRG(PI)
Time (days)
B.
SHR + CP-105,696
SHR+Vehicle
Peak Change in SBRg(PII) ms mmHg-1
C.
SHR+Vehicle
SHR + CP-105,696
Wistar + CP-105,696
Change in LF(SBP) (mmHg2)
Time (days)
D.
SHR+Vehicle
SHR + CP-105,696
Wistar + CP-105,696
Peak Change in LF(SBP)(mmHg\Hz2)
E.
SHR+Vehicle
SHR + CP-105,696
Wistar + CP-105,696
Change in HF(SBP) (mmHg2)
Time (days)
F.
SHR+Vehicle
SHR + CP-105,696
Wistar + CP-105,696
Peak Change in HF(SBP)(mmHg\Hz2)

Table 1: Mean Basal Cardiovascular and Autonomic Values for All Groups

Variable	SHR+CP105,696		SHR+vehicle		WKY+CP105,696	
	Light phase	Dark phase	Light phase	Dark phase	Light phase	Dark phase
SBP (mmHg)	181 ± 2	187 ± 2	183 ± 2	191 ± 2	120 ± 2	123 ± 2
MBP (mmHg)	143 ± 1	148 ± 2	143 ± 2	149 ± 2	93 ± 1	97 ± 1
DBP (mmHg)	125 ± 1	129 ± 2	124 ± 2	129 ± 2	80 ± 2	83 ± 2
HR (bpm)	296 ± 2	339 ± 5	290 ± 5	330 ± 3	304 ± 5	352 ± 6
Respiratory frequency (bpm)	67 ± 1.5	74 ± 2.1	60 ± 1.4	70 ± 1.5	90 ± 1.1	98 ± 1.7
sBRG ($ms.mmHg^{-1}$)	0.8 ± 0.04	0.7 ± 0.02	0.8 ± 0.04	0.6 ± 0.03	1.2 ± 0.05	0.9 ± 0.04
HF(PI) (ms^2)	19.6 ± 1.1	18.9 ± 0.6	17.6 ± 0.5	17.9 ± 0.5	17 ± 0.7	16 ± 0.9
LF/HF	0.6 ± 0.01	0.7 ± 0.02	0.6 ± 0.02	0.7 ± 0.02	0.6 ± 0.01	0.6 ± 0.01
LF(SBP) ($mmHg/Hz^2$)	3.1 ± 0.1	3.9 ± 0.1	3.0 ± 0.2	3.6 ± 0.1	2.7 ± 0.1	3.1 ± 0.1
VLF(SBP) ($mmHg/Hz^2$)	6.4 ± 0.1	7.1 ± 0.2	6.7 ± 0.2	7.3 ± 0.2	6.0 ± 0.2	6.4 ± 0.3
HF(SBP) ($mmHg/Hz^2$)	5.0 ± 0.2	5.6 ± 0.1	5.2 ± 0.2	5.7 ± 0.1	4.7 ± 0.2	5.0 ± 0.2