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Original Article

Excessive Leukotriene B4 in Nucleus Tractus Solitarii Is Prohypertensive in Spontaneously Hypertensive Rats

Hidefumi Waki,* Emma B. Hendy,* Charles C.T. Hindmarch, Sabine Gouraud, Marie Toward, Sergey Kasparov, David Murphy, Julian F.R. Paton

Abstract—Inflammation within the brain stem microvasculature has been associated with chronic cardiovascular diseases. We found that the expression of several enzymes involved in arachidonic acid-leukotriene B4 (LTB4) production was altered in nucleus tractus solitarii (NTS) of spontaneously hypertensive rat (SHR). LTB4 produced from arachidonic acid by 5-lipoxygenase is a potent chemoattractant of leukocytes. Leukotriene B4-12-hydroxydehydrogenase (LTB4-12-HD), which degrades LTB4, was downregulated in SHR rats compared with that in Wistar-Kyoto rats. Quantitative real-time PCR revealed that LTB4-12-HD was reduced by 63% and 58% in the NTS of adult SHR and prehypertensive SHR, respectively, compared with that in age-matched Wistar-Kyoto rats (n=6). 5-lipoxygenase gene expression was upregulated in the NTS of SHR ($\approx$50%; n=6). LTB4 levels were increased in the NTS of the SHR, (17%; n=10, $P<0.05$). LTB4 receptors BLT1 (but not BLT2) were expressed on astroglia in the NTS but not neurons or vessels. Microinjection of LTB4 into the NTS of Wistar-Kyoto rats increased both leukocyte adherence and arterial pressure for over 4 days (peak: +15 mm Hg; $P<0.01$). In contrast, blockade of NTS BLT1 receptors lowered blood pressure in the SHR (peak: −13 mm Hg; $P<0.05$) but not in Wistar-Kyoto rats. Thus, excessive amounts of LTB4 in NTS of SHR, possibly as a result of upregulation of 5-lipoxygenase and downregulation of LTB4-12-HD, can induce inflammation. Because blockade of NTS BLT1 receptors lowered arterial pressure in the SHR, their endogenous activity may contribute to the hypertensive state of this rodent model. Thus, inflammatory reactions in the brain stem are causally associated with neurogenic hypertension. (*Hypertension*. 2013;61:XXX-XXX.) ● **Online Data Supplement**
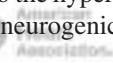
Key Words: brain stem ■ hypertension ■ inflammation ■ sympathetic nervous system

High blood pressure (hypertension) is a major contributor to stroke, heart attacks, and kidney disease. It has escalated to pandemic proportions (0.9 billion currently) and is expected to rise further to 1.4 billion by 2025.[1] The most common form of human hypertension is neurogenic hypertension characterized by excessive sympathetic activity that not only increases vascular resistance and cardiac output, which raises blood pressure, but also damages end organs, causing stiffening of arteries and the chambers of the heart.[2] The finding that this pathological rise in sympathetic activity precedes the onset of hypertension in humans[3] suggests that it is an early event in the disease process, therefore making it an important therapeutic target. However, the remarkable statistic that $\approx$23% of hypertensive patients who take multiple antihypertensive (polypill) medication are resistant to therapy[4] emphasizes the need to discover new ways to control excessive sympathetic nerve activity to bring blood pressure under control.

It is likely that immune cells communicate with the brain across the blood-brain barrier and vagal afferents and that the autonomic nervous system modulates the immune system.[2–7] Such cross-talk occurs during systemic infection, stress, and cardiovascular disease and results in alterations in animal behavior, autonomic vasomotor tone, and ventilation, for example. Evidence is accumulating for immune-to-brain signaling in a number of pathophysiological conditions including hypertension.[2,5] In this regard, leukocyte counts in spontaneously hypertensive rat (SHR) are 50% to 100% higher than that in controls, and leukocyte–endothelial interactions are abundant in the SHR.[8] Vascular inflammation in the SHR was associated with elevated expression of interleukin-1β, IL-6, and TNF-α.[9–12] Angiotensin II promotes leukocyte–endothelial interactions contributing to vascular inflammation,[13] whereas candesartan decreases inflammatory cytokines.[13,14] T cells play an important role in vascular inflammation in hypertension,[15] angiotensin II infusion– and DOCA salt–induced hypertension, whereas the T-cell modulating agent, mycophenolate mofetil, can prevent hypertension.[16,17] Immune-to-brain signaling involves the release of proinflammatory cytokines from intraluminar and

extravasated leukocytes and from microglial cells activated by leukocytes.[2] It is speculated that inflammatory molecules may also diffuse across the blood-brain barrier to effect neuronal activity and synaptic function. At the level of the nucleus tractus solitarii (NTS), a major region that governs both the sensitivity of the baroreceptor reflex and the set point of arterial pressure and the structure studied in this study, we described a unique pattern of expression of cytokines and chemokines within the NTS of the SHR relative to the normotensive rat.[18] Subsequently, we have found functional roles for the chemokine (C-C motif) ligand 5 (Ccl5 or Regulated on activation, normal T-cell expressed and secreted) and interleukin 6 in the NTS in modulating arterial pressure and baroreceptor reflex function, respectively.[19,20] Further, we reported an increase in a chemoattractant protein in the NTS of the SHR, which is called junctional adhesion molecule-A.[21] When overexpressed in normotensive rats, junctional adhesion molecule-A induced leukocyte adhesion in the brain stem microvasculature and induced mild hypertension in a normotensive animal.[21]

Given these previous findings and the importance and power of immune-to-brain signaling, this study has sought to determine whether other molecules are involved in mediating leukocyte adhesion in the brain stem of hypertensive human and SHR. Here, we used microarray screening of the NTS from SHR and Wistar-Kyoto (WKY) rats and identified a major difference in arachidonic acid metabolism in the SHR, including a downregulation of leukotriene B4 12-hydroxydehydrogenase (LTB4-12-HD), the enzyme that degrades leukotriene B4 (a powerful chemoattractant of leukocytes).[22] We further demonstrate that the NTS of the SHR has elevated levels of LTB4 and that this has functional consequences for its hypertensive state.

Methods

Procedures were carried out according to the UK Home Office guidelines on animals (Scientific Procedures) Act 1986. They were also approved by the University of Bristol's Animal Ethic Committee. All animals were housed individually, given normal rat chow and drinking water ad libitum, and kept on a 12-hour light/12-hour dark cycle. Human brain tissue studies were approved by Frenchay Hospital (Bristol) ethics committee.

NTS Transcriptomic Analysis and Data Handling

Affymetrix 230 2.0 Gene chips were used. Tissue from NTS was microdissected from brain slices from 11- to 13-week-old age-matched adult male inbred WKY rats and SHR. Five replicates were made for each rat strain. For further details, see the online-only Data Supplement.

Quantitative RT-PCR of Whole NTS and Primer Sequences

See the online-only Data Supplement.

Isolation of Microvasculature from the Medulla Oblongata and Quantitative RT-PCR

See the online-only Data Supplement.

Quantitative RT-PCR of Human Brain Stem

Fresh frozen brain stem tissue was thawed and transected coronally at the level of the NTS, which was identified as a distinct translucent structure in the dorsomedial medulla. At the level of the area postrema, a 2- to 3-mm-diameter (1- to 2-mm-thick) piece of NTS was cut out using a scapel under a binocular microscope. Subjects were male, and either had a medical history of uncomplicated essential hypertension

(>140/90 mm Hg; n=3) or were normotensive (n=4). For RNA extraction and primer sequences used, see the online-only Data Supplement.

LTB4 Content in Medulla Oblongata of WKY and SHR

See the online-only Data Supplement.

NTS Microinjection and Immunohistochemistry for Leukocytes

See the online-only Data Supplement.

BLT1 Receptor Immuncytochemistry in SHR

See the online-only Data Supplement.

Blood Pressure Responses to NTS Microinjections in (1) Anaesthetized and (2) Conscious Rats

Procedures were as we have described previously[21,23] and given in full in the online-only Data Supplement.

Data Analysis

Group data were expressed as mean±SEM. To evaluate time-dependent changes of cardiovascular variables by injecting LTB4 or the BLT1 receptor antagonist into the NTS, we used repeated-measures ANOVA and the Bonferroni post hoc test. An unpaired *t* test was also used for comparisons between 2 groups (eg, comparison of gene expression levels). Differences were considered significant if $P<0.05$.

Results

Catalogue of Gene Expression in the NTS

We present here lists of genes that, with a high degree of statistical confidence, represent comprehensive descriptions of the RNA populations expressed in the NTS of WKY (15 402 probesets, S1) and SHR (13 618 probesets, S2); see http://www.vasopressin.org/#/data-bank/3755442 for full details. Our genetic data were also submitted to the NCBI gene expression and hybridization array data repository (GEO); the GEO accession number is: Series GSE8796.

Combination of these lists provides a basis from which statistical testing was conducted. Of 15 870 probesets that were considered to be present in all the independent microarrays from both SHR and WKY NTS, 85 were significantly regulated differentially by greater than 1.5-fold (54 downregulated and 31 upregulated). We identified a clear downregulation of LTB4-12-DH (or prostaglandin reductase 1) in the NTS of SHR. This drove the hypothesis that this pathway may contribute to the hypertensive phenotype of SHR. Hence, first we validated the array result using RT-PCR, second we investigated the expression of other components of this pathway, and third, we proceeded to establish its functional significance to blood pressure control.

RT-PCR Analysis of Expression of the Components of the Arachidonic Acid Signaling Cascade in SHR and WKY Rats

NTS

We confirmed the array data by showing that *LTB4-12-HD* gene expression level was significantly lower in both young and adult SHR than in age-matched WKY (adult WKY and SHR: 1.13±0.25 versus 0.42±0.04, respectively, n=6 for each strain, $P<0.05$; young WKY and SHR, 1.07±0.20 versus 0.45±0.10, respectively, n=6 for each strain, $P<0.05$; Table

Table. Gene Expression in Rat RVLM, PVN, and Brain Stem Blood Vessels

Enzyme	RVLM (15 Weeks Old)		PVN (15 Weeks Old)		Rat Brain Stem Blood Vessels (3 Weeks Old)	
	WKY (n=6)	SHR (n=6)	WKY (n=5)	SHR (n=5)	WKY (n=4)	SHR (n=4)
5LOX	1.01+0.1	1.39+0.09*	…	…	…	…
LTA4H	1.01+0.1	0.98+0.09	…	…	…	…
LTB4-12-HD	1.04+0.1	0.17+0.03†	1.03+0.13	0.73+0.11*	1.01+0.1	0.49+0.08*

RVLM indicates rostral ventrolateral medulla, PVN, paraventricular nucleus of the hypothalamus; WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rats; 5LOX, 5 lipoxygenase; LTA4H, leukotriene A4 hydrolase; LTB4-12-HD, leukotriene B4-12-hydroxydehydrogenase.

Expression of target genes relative to β-actin gene in each sample was derived using the comparative ($2^{-\Delta\Delta CT}$) method.[16] Fold differences were calculated against the average of age-matched WKY rats.

*$P<0.05$, †$P<0.01$.

and Figure 1A). The level of *5LOX* (5 lipoxygenase) gene was significantly higher in the NTS of both young and adult SHR compared with age-matched WKY rats (adult WKY and SHR: 1.02±0.08 versus 1.52±0.23, respectively, n=6 for each strain, $P<0.05$; young WKY and SHR, 1.02±0.10 versus 1.47±0.09, respectively, n=6 for each strain, $P<0.05$; Figure 1A). The level of *LTA4H* (leukotriene A4 hydrolase) gene expression was not different between SHR and WKY (adult WKY and SHR: 1.01±0.07 versus 1.09±0.05, respectively, n=6 for each strain, $P<0.05$; young WKY and SHR, 1.05±0.14 versus 0.98±0.15, respectively, n=6 for each strain; Figure 1A). Note that both the rostral ventrolateral medulla and hypothalamic paraventricular nucleus also exhibited lower levels of LTB4-12-HD in SHR versus WKY rats (Table).

Brain Stem Blood Vessels

Consistent with brain stem tissue, there was also a reduction of *LTB4-12-HD* gene in isolated microvessels extracted from the brain stem of 3-week-old rat strains (SHR, 0.49±0.08 versus WKY 1.01±0.05; n=4, $P<0.05$; Table).

Human Hypertensive NTS Tissue.

As with the SHR:WKY rat difference, a similar difference was found in human NTS where hypertensive subjects had lowered expression levels of LTB4-12-HD compared with controls (hypertensives: 0.42±0.05 versus control: 1.02±0.10, n=4; $P<0.01$).

Levels of LTB4 in the Medulla Oblongata of SHR and WKY Rats

When the LTB4 quantity was normalized to the total protein concentration, the concentration of LTB4 was higher in the SHR versus WKY rats (WKY: 7.95 pg LTB4/mg protein, SHR: 9.94 pg LTB4/mg protein, n=10, $P<0.01$; Figure 1B and the online-only Data Supplement). Thus, either the production of LTB4 is increased or its degradation decreased in the NTS of SHR relative to WKY rats.

BLT1 and BLT2 Receptor Expression in the NTS

mRNA expression of LTB4 receptors indicated the presence of BLT1 receptors in the NTS of both SHR and WKY rats (Figure 2). BLT2 receptor expression was hardly detectable (Figure 2A). At the protein level, BLT1 receptors were detected immunocytochemically in the NTS of SHR and found to be colocalized with cells that were immunopositive for GFAP but not NeuN or RECA (Figure 2B). Colocalization with GFAP was relatively dense and supports substantial numbers of BLT1 receptors on astrocytes.
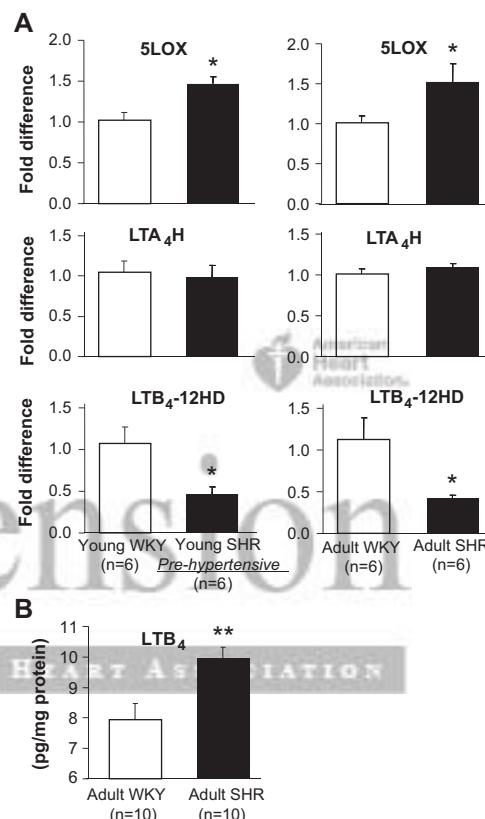
Figure 1. Altered arachidonic acid metabolism in the nucleus tractus solitarii (NTS) of SHR and (Wistar-Kyoto) WKY rats. **A**, 5LOX mRNA was upregulated in both adult and prehypertensive spontaneously hypertensive rats (SHR) compared with age-matched WKY. In comparison, LTB4-12-HD mRNA expression level was reduced in both adult and prehypertensive SHR compared with age-matched WKY. In contrast, the level of LTA₄H mRNA expression was not different between SHR and WKY. The reduced expression levels of LTB4-12-HD mRNA were associated with an increase in LTB4 content within the brain stem of SHR (**B**). *$P<0.05$, **$P<0.01$.

LTB4 in the NTS of WKY Rats: Acute and Chronic Effects on Cardiovascular Control

Acute

In normotensive anesthetized WKY rats, a depressor site was located in the NTS as determined by glutamate microinjection (range: −30 to −60 mm Hg drop in arterial pressure). Within a 1-hour observation period, neither arterial pressure nor HR changed after LTB4 microinjection into this NTS depressor

site (SBP, before: 105±7 and after: 107±8 mm Hg; HR, before: 323±23 and after: 322±25 bpm; n=6). After 1 hour, glutamate still produced a quantitatively similar fall in arterial pressure, suggesting that the structure had maintained its viability.

Chronic

In conscious telemetered WKY rats, baseline levels of SBP, HR, and sBRG were 113±3 mm Hg, 365±6 bpm, and 0.92±0.08 ms/mm Hg, respectively. After NTS microinjection of LTB4, the maximum increment of SBP was +15±3 mm Hg relative to preinjection levels; this occurred 2 days after the injection ($P<0.001$, Figure 3A) and remained above baseline levels for postinjection days 3 ($P<0.05$) and 4 ($P<0.05$). The peak mean arterial pressure response increased from 93±3 to 107±4 mm Hg ($P<0.05$). In contrast, HR and sBRG did not change ($P>0.1$). Three days post LTB4 injection, the low frequency+very low frequency (LF+VLF) power of SBP variability increased from 7.40±0.34 to 10.69±1.29 mm Hg ($P<0.05$) and the change in the LTB4 injection group was higher than that in the control group (3.30±0.99 versus 0.22±0.74 mm Hg,[2] $P<0.05$; Figure 3B), indirectly suggesting raised sympathetic vasomotor activity. Consistently, LF SBP increased from 0.20±0.3 to 0.87±0.3 mm Hg ($P<0.05$). Both the changes in LF:HF and HF power of HR variability were not different between LTB4 injection and control groups ($P>0.1$). Post hoc analysis at the conclusion of the experiment (ie, day 6 postinjection) failed to find leukocyte adhesion (ie, CD4 immunoreactivity) in the NTS, suggesting that any inflammatory response was either undetectable or transient and resolved by the time arterial pressure had returned to control levels.

Chronic Blockade of BLT1R in the NTS of WKY and SHR on Cardiovascular Control

In conscious rats fitted with radio transmitters, resting SBP was 171±8 (SHR, U75 group), 179±5 (SHR, acsf), and 121±4 mm Hg (Wistar, U75302). SBP decreased maximally by −13±5 mm Hg (n=5; $P=0.05$) after NTS microinjection of U75302 in SHR. This reduction in SBP persisted for up to 6 days relative to baseline and SHR treated with acsf (Figure 3C; $P<0.05$). Mean arterial pressure fell from 134±5 to

115±3 mm Hg ($P<0.05$). In SHR treated with BLT1 receptor blocker, LF+VLF power of SBP decreased significantly relative to baseline (from 4±0.2 to 3.3±0.2 mm Hg, $P<0.05$; Figure 3D). This reduction remained significant for up to 6 days, thereby accompanying the pressure fall. LF SBP also fell (4.0±0.2 to 3.3±0.2 mm Hg; $P<0.05$). No such changes were observed in the SHR acsf and Wistar rat groups (Figure 3D). None of the 3 rat groups showed any significant change in heart rate, heart rate spectra, or sBRG. In control SHR microinjected with acsf, a rise of 12±1 mm Hg was noted (n=4; $P<0.05$), whereas no change was observed in Wistar rats receiving U75302.

BLT1 Receptor Modulation of Inflammation in the NTS

We found that LTB4 microinjection into the NTS of WKY rats produced inflammation as detected from elevated CD4 immunoreactivity after 2 days (Figure 4A; n=4). We confirmed our original finding of high endogenous CD4 immunoreactivity in the NTS of the SHR,[21] which was minimal in WKY rats (Figure 4B). However, blocking BLT1 receptors in the SHR failed to reduce endogenous CD4 immunoreactivity in the NTS of the SHR (Figure 4C; n=4), suggesting that maintaining inflammation is not dependent on continuous BLT1 receptor activation. Thus, these data indicate a potential role for LTB4 in evoking inflammation in the NTS of WKY rats, but that blockade of endogenous BLT1 receptor activity is insufficient to reduce CD4 immunoreactivity in the SHR.

Discussion

The novel findings of this study are that in both adult and young prehypertensive SHR, *LTB4-12-HD* gene was downregulated in the NTS, whereas *5LOX* gene was upregulated compared with age-matched WKY rats. Based on this, we predicted excessive amounts of the arachidonic acid metabolite LTB4 in the NTS of the SHR, which we have confirmed. We also described the presence of BLT1 receptors on glial cells in the NTS. We found that a single injection of LTB4 in NTS produced hypertension in normotensive rats, whereas BLT1 receptor antagonism lowered arterial pressure in the SHR but
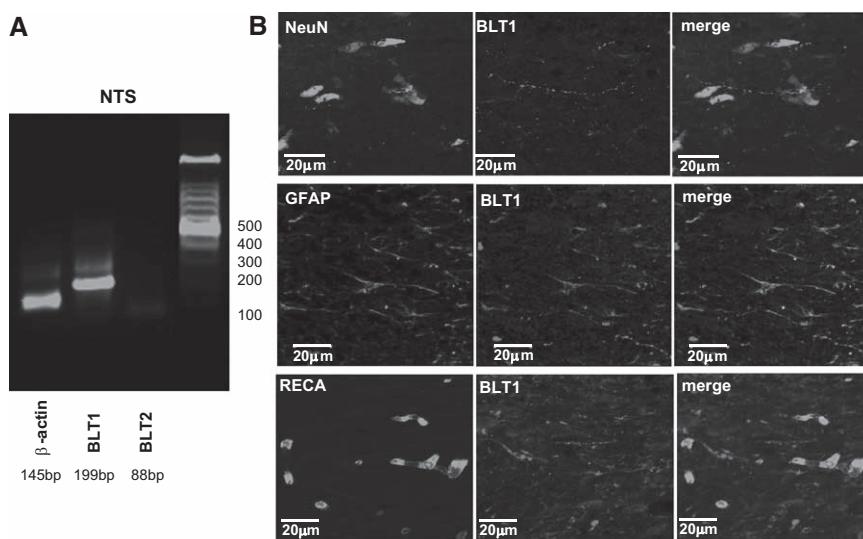
Figure 2. BLT1 receptors in the nucleus tractus solitarii (NTS). Gene expression of BLT1, but not BLT2 receptors, was identified in (Wistar-Kyoto) WKY and spontaneously hypertensive rats (SHR) (**A**, data from WKY rat). **B**, Immunofluorescence labeling indicated that BLT1 receptor expression was apparent on glial but not neuronal or endothelial cell types within the NTS of SHR. Glial cells were identified using antibodies against glial fibrillary acidic protein, NeuN for neurones, and RECA for endothelial cells.
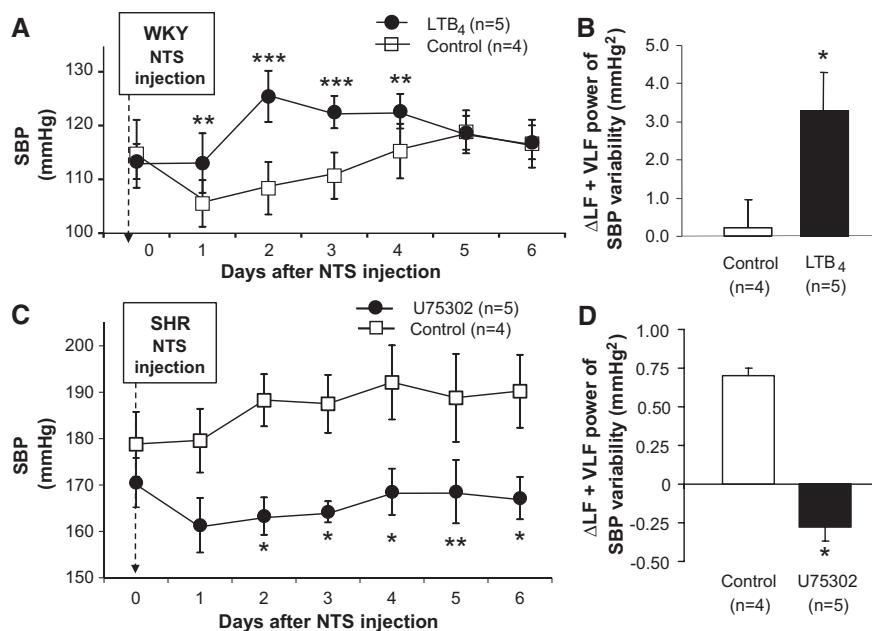
Figure 3. LTB4 and BLT1 receptor manipulation in nucleus tractus solitarii (NTS) evokes arterial pressure responses. **A**, Two to 4 days after NTS injection of LTB4, systolic blood pressure (SBP) was elevated significantly in Wistar-Kyoto (WKY) rats. **B**, Three days after LTB4 injection, low frequency+very low frequency (LF+VLF) power of SBP variability was increased, suggesting activation of vasomotor sympathetic activity. **C**, U75302 (to block BLT1 receptors) in NTS of spontaneously hypertensive rats (SHR) lowered both arterial pressure and the LF+VLF power of SBP variability. **D**, Differences are relative to control injections. *$P<0.05$, **$P<0.01$, and *** $P<0.001$.

not in normotensive rats. Although BLT1 receptor blockade was unable to reduce CD4 immunoreactivity in the NTS of SHR, LTB4 could induce inflammation in this brain stem region of normotensive rats. Our findings indicated that a high level of LTB4 in the NTS may have roles in both the development and maintenance of the hypertension in the SHR and that this is likely mediated via a BLT1 receptor signaling process involving astrocytes.
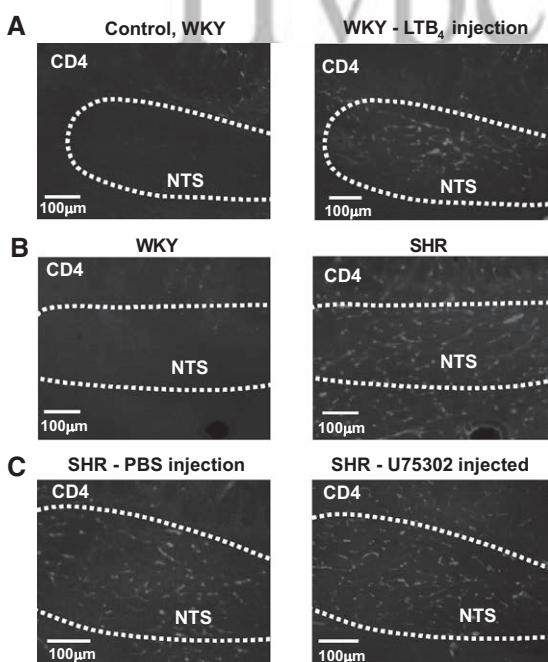
Figure 4. BLT1 receptor modulation of inflammation in the nucleus tractus solitarii (NTS). **A**, LTB4 microinjection into the NTS of a Wistar-Kyoto (WKY) rat induced CD4 immunoreactivity relative to rats injected with vehicle control. **B**, The NTS of the spontaneously hypertensive rats (SHR) is inflamed relative to WKY rats as indicated by the greater CD4 immunoreactivity. **C**, Blockade of BLT1 receptors in the NTS of the SHR did not affect the endogenous leukocyte accumulation.

Altered Arachidonic Acid-LTB4 Metabolic System in the NTS of SHR

5-lipoxygenase is a lipoxygenase present in the central nervous system.[24] Both its upregulation in the NTS of the SHR and downregulation of LTB4-12-HD, the enzyme that causes dehydrogenation of LTB4 to 12-oxo-LTB4, could cause the elevated levels of LTB4 that we found. Because this imbalance was seen in PHSHR, these changes are not secondary to the hypertension but occur early in the pathology and could therefore be involved in the establishment of hypertension. Although our sample number was low, the observation that LTB4-12-HD was lower in the NTS of human hypertensives lends both credence to the relevance of our rat transcriptomic data to human essential hypertension and the applicability of the animal model for understanding human neurogenic hypertension. These changes are unlikely to be unique to the NTS because LTB4-12-HD was also downregulated in the rostral ventrolateral medulla and hypothalamic paraventricular nucleus (Table). Thus, the magnitude of the blood pressure responses reported herein may well be amplified if the LTB4 signaling pathways are modulated in this additional brain stem site simultaneously.

Potential Sources of LTB4 in the NTS of the SHR

Generally, LTB4 synthesis is increased by inflammatory mediators including endotoxin, complement fragments, tumor necrosis factor, and interleukins.[25] In the brain, astrocytes[26] and oligodendrocytes[27] are both potential sources of LTB_4. However, because our data indicate reduced levels of LTB4-12-HD in isolated brain stem vessels, LTB4 may also be released from the endothelium and vascular muscle cells in the SHR. This is consistent with the evidence that both these cell types have been shown to synthesize this leukotriene.[28,29] If of endothelial origin, LTB4 could be released into the blood stream to attract leukocytes (and into the brain to activate glial cells). As confirmed in this study, and found previously,[21] there is leukocyte accumulation in the NTS capillaries of SHR

but not in that of WKY rats. This may, in part, be attributable to the greater adhesiveness of leukocytes in the SHR than in normotensive rats.[30] It may also be accentuated by the high level of endothelial junctional adhesion molecule-A expression in the NTS,[21] which has a binding site for leukocytes[31–33] but also attributable to the high level of LTB4 as detected in this study. The types of leukocytes will need to be identified in future studies. Because leukocytes can synthesize LTB4,[22] we cannot rule out their contribution to the raised levels of this LTB4 in the medulla oblongata of the SHR described herein. Given these multiple mechanisms for attracting leukocytes to the NTS in the SHR and that LTB4 production may be a product of an inflammatory process, it is perhaps unsurprising that blocking BLT1 receptors was ineffective in reducing CD4 immunoreactivity.

Potential Actions of LTB4 in the NTS of the SHR

LTB4 is one of the most potent chemoattractants and activators of leukocytes, and it has a primary role in inflammatory diseases.[22,34] We found that microinjection of the LTB4 into the NTS or normotensive rats induced leukocyte adherence. These data are consistent with the role of BLT1 receptor activation as a major stimulus driving leukocyte accumulation.[22,34–36] Because BLT1 receptor antagonism blocks neutrophil activation,[37] it may reduce the inflammatory response in the NTS. This is relevant because LTB4 stimulates the production of a number of proinflammatory cytokines that augment and prolong tissue inflammation. For example, it stimulates the release of monochemoattractant protein-1[38] (MCP-1) via the NF-κB pathway in human monocytes. Interestingly, we found that MCP-1 was higher in the NTS of the SHR,[18] but it is unclear whether this relates causally to the high LTB4 levels in the NTS of the SHR. LTB4 can increase levels of IL-6,[39] but this chemokine was downregulated in the NTS of the SHR,[18] and its expression was not altered after NTS injection of LTB4.[19] The latter support the notion of a specific type of inflammatory condition in the NTS of the SHR as proposed previously.[18] In contrast, LTB4 decreased the expression of Ccl5 in the NTS of normotensive rats.[19] Because endogenous Ccl5 was downregulated in the NTS of the SHR, it is tempting to speculate that this is triggered by the elevated levels of LTB4 in this nucleus, but this awaits confirmation. Ccl5 receptors have been associated with enhancing glutamate transmission and were previously found on NTS neurons.[19] Their activation resulted in a lowering of arterial pressure that was significantly more pronounced in the SHR than in the normotensive rat.[19] Because LTB4 lowers Ccl5 expression, it is suggested that the restraining effect of Ccl5 on arterial pressure may be depressed in the SHR, which could contribute to its hypertensive state. Ccl5 also contributes directly to monocyte-leukocyte activation and could further support the aforementioned actions of LTB4 and junctional adhesion molecule-A in white cell aggregation in the SHR. Because Ccl5 stimulates the production and release of specific proinflammatory arachidonic acid products, including LTB4 from monocytes,[40] we suggest that leukocyte aggregation in the NTS could lead to further LTB4 production through a positive feedback/wind-up mechanism.

BLT1 Receptors and Potential Downstream Intercellular Signaling in the NTS

In this study, gene expression of BLT1 receptors predominated over BLT2 receptors in the NTS. BLT1 receptor is a G-protein–coupled receptor[22,41] and previously described on leukocytes.[42] Our immunofluorescence labeling suggested that BLT1 receptors were located on glial cells in the NTS. It is hypothesized that leukocyte accumulation in the NTS induced, in part, by LTB4 acting of BLT1 receptors, releases both cytokines and reactive oxygen species such as super oxide; these are established products from such cells[43,44] and known to activate central neurones including brain stem cardiovascular neurones.[45,46] Reactive oxygen species and some types of cytokines can cross the blood-brain barrier.[47] Whether leukocytes extravasate into the NTS was not confirmed,[21] and a role for LTB4 in diapedesis is controversial.[35,38,48] However, it is known that astrocytes are a potential source for cytokines and reactive oxygen species production.[49–51] On physical contact with leukocytes, astrocytes release MCP-1.[51] MCP-1 receptors are present on central neurones[52]; however, its functional role has yet to be identified in the NTS. Taken together, we propose that leukocyte accumulation, even if intraluminally, may well influence central neuronal circuits via cytokines, chemokines, and reactive oxygen species activation that originates from adhered white cells or LTB activation of glial cells via BLT1 receptors. This may dramatically alter neuronal function, leading to neurogenic hypertension. Because we have found that gene expression of LTB4-12-HD was downregulated in the rostral ventrolateral medulla and hypothalamic paraventricular nucleus, it remains to be established whether LTB4 also affects these other regions of the SHR, resulting in hypertension.

Perspectives

Our findings raise the intriguing and novel possibility that there is a link between high levels of leukocyte adhesion in the cardiovascular control regions of the brain with excessive levels of sympathetic nerve activity. We surmise that by antagonizing either leukocyte adhesion and reducing inflammation in the brain stem of the SHR, one might predict a reduction in the proinflammatory status, thereby alleviating the symptoms of hypertension. With the demonstration that BLT1 receptor activation can itself induce leukocyte adherence in the brain stem and trigger hypertension in normotensive rats, anti-inflammatory therapy may prove an effective antihypertensive strategy. Although anti-inflammatory drugs are generally ineffective antihypertensive agents, we argue that the inflammatory status of the brain stem is relatively specific.[18] Anti-inflammatory agents tested to date may be inappropriate or not cross the blood-brain barrier. However, minocycline given centrally restricts the pressor response induced by angiotensin II infusion and decreases the numbers of activated microglia and mRNAs for interleukin (IL) 1 β, IL-6, and tumor necrosis factor-α, but increase mRNA for IL-10 (anti-inflammatory) in the hypothalamus.[53] Because we found that *LTB4-12-HD* gene is also downregulated in the NTS of humans with a history of essential hypertension, we suggest that LTB4-12-HD itself might be an effective target for therapeutic intervention as proposed previously.[54]

Moreover, it may also be an early diagnostic indicator to predict whether a subject is likely to develop hypertension before it has manifested itself, especially because this gene was downregulated in the prehypertensive SHR. Such early diagnosis might stimulate anti-inflammatory approaches as an effective preventive approach to hypertension.

Disclosures

None.

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Novelty and Significance

What Is New?

- Leukotriene B4 (LTB4), a major chemoattractant for immune cells, is elevated in nucleus tractus solitarii of spontaneously hypertensive rats and human hypertensives.
- LTB4 receptor stimulation in nucleus tractus solitarii is prohypertensive, whereas their blockade is antihypertensive.
- LTB4 receptors exist on astrocytes in nucleus tractus solitarii.

What Is Relevant?

- The brain stem microvasculature of the spontaneously hypertensive rat is inflamed; this occurs before the onset of hypertension and may involve LTB4.
- Appropriate anti-inflammatory treatment may provide an antihypertensive strategy.

Summary

Arachidonic acid metabolism is altered in spontaneously hypertensive rat brain stem; this is associated with inflammatory reactions that seem to be causally related with neurogenic hypertension.

Hypertension

JOURNAL OF THE AMERICAN HEART ASSOCIATION

EXCESSIVE LEUKOTRIENE B4 IN NUCLEUS TRACTUS SOLITARII IS PRO-HYPERTENSIVE IN SPONTANEOUSLY HYPERTENSIVE RATS

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Short title: CNS leukotriene B4 and hypertension

Key Words: hypertension, inflammation, sympathetic nervous system, brain, baroreceptors

Supplemental Methods

NTS transcriptomic analysis and data handling
(i) Tissue extraction: Age-matched adult male inbred Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) (11-13 weeks old; Harlan Sera-lab, Loughborough, UK) were maintained in standardized temperature (22 ± 1°C), humidity (50 ± 5%) and diurnal conditions (10 hours light, 14 hours dark; lights on at 0700). Following at least 7 days acclimatization the SHR and WKY rats were sacrificed (between the hours of 1100 and 1300) and the tissue isolated and processed as described below. Rats were stunned and decapitated with a small animal guillotine (Harvard Apparatus, Holliston, MA, USA). The brain was rapidly removed from the cranium and placed in an ice-cold brain matrix (ASI Instruments, Warren, MI). Brainstem dissections were carefully trimmed, glued on a mounting block and submerged into the sectioning bath of a vibratome (Vibratome 1500, Vibratome, Bannockburn, IL, USA) containing ice-cold artificial cerebrospinal fluid (MgSO4 50mM; KH2PO4 50mM; KCl 200mM; NaHCO3 1M; NaCl 5M; CaCl2 100mM; D-glucose 20mM) gassed with carbogen (95% O2, 5% CO2) and two 500 μm thick sections (containing the NTS and AP) were cut. The sections were placed into Hibernate-A media (Brain Bits, IL) plus B27 supplement (Invitrogen, Paisley, UK) and the sub-postremal NTS separated from the area postrema (AP) with the aid of a microscope using a fine tip dissecting knife (Fine Science Tools, CA). Dissected tissue samples were immediately placed in an RNAse-free 1.5ml tube containing RNAlater® (Ambion, Huntingdon, UK) for storage (-20ºC) prior to further processing. The above procedures were carried out in RNase-free manner. RNA extraction *in vitro* transcription and microarray hybridisation to the Affymetrix 230 2.0 Gene chips were performed as previously described [1,2].

(ii) Data handling: The raw CEL files were input into Genespring GX11 for normalisation using the MAS5 summarization algorithm and baseline transformation to the median of all samples. Data was filtered so that only probesets that are considered to be present in all the chips from at least one of the experimental groups (NTS-SHR or NTS-WKY) were available for statistical analysis. Within Genespring, a Welch *t*-test was performed which assumed unequal variance and included the Benjimini & Hochberg multiple testing correction, limiting those genes identified by chance to just 5% of those identified. Finally, a 1.5-fold difference cut-off was applied to the data.

Quantitative RT-PCR of whole NTS and primer sequences
(i) RNA extraction: Both three (SHR, n=6; WKY, n=6) and 15-18-week-old (SHR, n=6; WKY, n=6) male rats were humanely killed by cervical dislocation. The NTS was micro-dissected rapidly from each animal and homogenized in 400 μl TRIzol reagent (Invitrogen, Carlsbad, CA). To avoid contamination with genomic DNA, the RNA samples were treated with RNase-free DNase I (Roche Diagnostics GmbH, Mannheim, Germany). RNA purity was verified by performing PCR on samples not treated with reverse transcriptase. Other brain regions including the paraventricular nuclei (PVN) and rostral ventrolateral medulla (RVLM) were isolated from adult SHR and WKY rats and RNA from these tissues was extracted for comparison purposes. It should be noted that this procedure cannot isolate RNA from blood cells, indicating that the extracted RNA samples are predominantly from brain parenchyma.

(ii) Primers for rat tissue: β-actin, 5LOX, LTA4H, LTB4 12-HD and LTB4 receptors, BLT1 and BLT2 were tested in this study. Real-time RT-PCR reactions were carried out using a DNA Engine Opticon 2 system (MJ Research, Reno, Nevada, USA), QuantiTect Primer

Assay (Qiagen, Valencia, CA, USA) and The QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) as described previously[3,4]. Expression of target genes relative to control (ß-actin) was derived using the comparative ($2^{-\Delta\Delta CT}$) method[5] in each sample. Fold differences against average values of WKY were calculated. For 5LOX, LTA4H, and LTB4 12-HD, we used "QuantiTect Primer Assay (Qiagen, Valencia, CA, USA)". The sequences of these primers are held with the company. For BLT1 and BLT2 receptors, we designed our own primers. BLT1, forward: CAACTTCTTCTCCTGGTGGC, reverse: AAAAAGGGAGCAGTGAGCAA; BLT2, forward: TTTAGTTCCAGCGTCAACCC, reverse: AGAGTCGAGTGAGGAACCGA. Final product sizes were 199 and 88 bp for BLT1 and BLT2 receptors, respectively.

Isolation of microvasculature from the medulla oblongata and quantitative RT-PCR
Three-week-old male rats (SHR, n=4; WKY, n=4) were humanely killed by cervical dislocation. The methods described below for the isolation of an enriched fraction of brainstem micro vessels are based on the technique described previously[6]. Briefly, rats were anesthetized deeply using halothane (5%) followed by cervical dislocation and rapid decapitation. Brains are removed swiftly and the medullae dissected in ice cold artificial cerebrospinal fluid (ACSF; $MgSO_4.7H_2O$, 1.25 mM; KH_2PO_4, 1.25 mM; KCl, 3 mM; $NaHCO_3$, 25 mM; NaCl, 125 mM; $CaCl_2.2H_2O$, 2.5 mM; Glucose at 0.18 g/100 ml; bubbled with 95% O_2 and 5% CO_2). The tissue was homogenised on ice in 10 times volume of ice chilled ACSF. The homogenate was centrifuged 3 times at 15,000 times gravity for 15 min. Each time the supernatant was removed and replaced with fresh, ice cold ACSF. The final pellet was re-suspended in ice cold 0.25 M sucrose before being layered on top of a discontinuous sucrose gradient consisting of 8 ml 1.3 M sucrose and 8 ml 1.0 M sucrose. The samples were ultra-centrifuged at 58,000g for 35 min at 4°C. This resulted in a red colored pellet containing the fractionated micro vessels at the bottom of the tube. The vessels were placed in RNAse inhibiting solution RNAlater and stored at -20°C until RNA extraction (see above for details). Real-time RT-PCR reactions to assess LTB4 12-HD gene expression were carried out as described above.

Quantitative RT-PCR of human brainstem
Brainstem tissue containing cardiovascular control centers from deceased male subjects with a medical history of uncomplicated essential hypertension (>140/90 mmHg; n=3) was used to assess if LTB4 12-HD mRNA was abnormally expressed. RNA extraction from human tissue was as described above. Quantitative RT-PCR was performed to measure the gene expression level and compared with control subjects (i.e. no history of hypertension; n=4). The following primer sequences were used: β-actin (BC016045) forward (TGGAGAAAATCTGGCACCAC), reverse (GAGGCGTACAGGGATAGCAC); LTB4 12-HD (NM_012212) forward (CCACCCCCAGAGATTGTTATC), reverse (CTCCTTTCAGCATTCCCATA). The sizes of the PCR products amplified with the primers were β-actin, 190 bp and LTB4 12-HD, 195 bp, respectively. We previously confirmed that the primer efficiency is comparable between these genes. These brain samples were received from a human brain bank at Southmead Hospital, Bristol, UK.

BLT1 receptor immuncytochemistry in SHR
Tissue from adult SHR rats (250-350 g, age >12 wks; n=3) was processed as described for fresh frozen tissue (see Supplemental text file). BLT1R antibody (Abcam; 1:200 dilution) were co-stained with glial cells (anti-glial fabrillary acidic protein (GFAP; Chemicon International; 1:1000 dilution) or neurones (anti-NeuN; Chemicon International; 1:2000 dilution). Alexa Fluor 488 conjugated streptavidin was used with the BLT1 receptor primary

antibody (Invitrogen; 1:500 dilution) whereas Alexa Fluor 594 (Invitrogen; 1:500 dilution) was used with GFAP and NeuN primary antibodies. All sections were mounted using Vectashield with the nuclear stain DAPI (1.5 µg/ml; Vector Labs) to allow co-localisation of the staining with cell nuclei.

NTS microinjection and immunohistochemistry for leukocytes
(i) Injections: This protocol was used to detect leukocyte adhesion 2 and 6 days following LTB4 microinjection into the NTS and 2 days after large microinjections of acsf to cause tissue damage and evoke an immune response. Rats (WKY and SHR, 15-week-old) were anaesthetized with ketamine (60 mg/kg) and medetomidine (250 µg/kg) intra-muscularly and placed in a stereotaxic head holder (SR-5; Narishige). Three 100nl bilateral microinjections of LTB4 (290 µM in saline, Cayman Chemical, Michigan, USA) or saline as a control (n=5 each) were made into the NTS at separate sites spanning ± 500 µm rostral/caudal to the calamus scriptorius and 350-500 µm from midline and 500-600 µm below the dorsal surface of the medulla. Each injection was made over 1min. For the large acsf microinjections 500nl was ejected rapidly over 2-3 s into a single site. Anaesthesia was reversed with atipamezole (1 mg/kg). After 2 (acsf injections) or 2 days (LTB4 injections), rats were killed humanely by cervical dislocation.

(ii) Fixed tissue: The brainstem was removed, fixed with 4% paraformaldehyde for at least 48 h, and transferred to phosphate buffered saline (PBS) containing 30% sucrose. Serial sections (40 µm) through NTS were obtained using a freezing microtome. The sections were rinsed in PBS, put in 10% serum with 0.3% Triton X-100 for 15 minutes at room temperature, rinsed again and incubated with a CD4 antibody (MRC OX-35, Abcam, dilution: 1:200 in PBS with 1% serum and 0.3% Triton X-100). After overnight incubation at 4 ℃ , the sections were rinsed in PBS and incubated with biotinylated goat anti-mouse IgG (1:500 dilution, Vector Laboratories, UK) for 1 hour. The sections were rinsed and then incubated in streptavidin conjugated Alexa-Fluor 594 (1:500 dilution, Molecular Probes, USA) for 1 hour. Finally, sections received a PBS wash before mounting in Vectashield (Vector Laboratories). Sections were photographed using a scanning laser confocal microscope (LSM 5 Pascal; Carl Zeiss).

(iii) Fresh frozen tissue: In an attempt to reduce non-specific background fluorescence, fresh frozen tissue was used. CD4, CD11b and CD45 staining was carried out on fresh frozen tissue. Following euthanasia, the brainstem was rapidly removed and frozen within a block of Tissue-Tek by floating the molds in 2-methylbutane (Sigma) cooled using a liquid nitrogen bath. Serial sections (15 µm) were cut using a cryostat and placed on Superfrost Plus slides. Sections were treated with ice cold 100% acetone for 2 min at -20°C. Slides were washed and blocked for 1 h at room temperature (10% NGS in 0.1 M PBS). Slides were incubated in primary antibody for 48 h at 4°C. Appropriate secondary antibodies were added for 1 h at room temperature.

Blood pressure responses to NTS microinjections
*(i) Anaesthetized rats***:** Male rats (WKY, 15-week-old, n=6) were anaesthetized with urethane (1.4 g kg^{-1}, i.p.), placed in a stereotaxic head holder and the caudal dorsal medulla exposed. A single bilateral microinjection of LTB4 (290 µM in saline; Cayman Chemical, Michigan, USA) was made from a micropipette (tip diameter 30-40 µm), at a depth of 600 µm ventral to the dorsal surface, ± 250 µm rostral/caudal to the calamus scriptorius and between 350-500 µm from the midline. The injected volume (100 nl) was measured by observing the movement of the meniscus through a binocular microscope fitted with a calibrated eye-piece graticule. Arterial pressure was monitored via radio-telemetry system

(see below) and data analysis was performed using the *Hey-Presto* system as described previously[7]. LTB4 was microinjected into a depressor site within NTS as characterized by prior injection of glutamate (0.2 M; 50 nl); the loci of the injections was identified by dye.

(ii) Conscious rats: Male rats (WKY and SHR, 15-weeks old, n=9 each) were anaesthetized with ketamine (60 mg/kg) and medetomidine (250 µg/kg) intra-muscularly. A radio transmitter (Data Sciences International; TA11PA-C40) was implanted to record arterial pressure from the abdominal aorta as described previously[3,4]. Anaesthesia was reversed with atipamezole (1 mg/kg). 9 days after the implantation animals were re-anaesthetised and three 100nl bilateral microinjections of LTB4 (290 µM) in WKY (n=5) or BLT1 receptor antagonist (U75302, 184 µM dissolved in acsf; Biomol) in SHR and Wistar rats (n=5 each) or saline/acsf as control (SHR; n=4) were made into the NTS at sites corresponding to those for LTB4 (see above). Each injection was made over 1 min. Data analysis was performed using the *Hey-Presto* system[7] 2-3 days before NTS microinjections and on 1 to 7 days after microinjections. On these days, arterial pressure was measured continuously for 5 min every hour from 9:00 am until 8:05 am on the following day. Heart rate (HR) was derived from the interpulse interval. Averaged systolic blood pressure (SBP) and HR were calculated. The spontaneous cardiac baroreflex gain (sBRG) was also determined from spontaneous changes in SBP and HR as described previously. Power spectral analysis of SBP and HR variability was also performed using a fast Fourier transform (FFT) algorithm as described[7]. 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). We assumed that the HF power of HR variability was mediated by cardiac parasympathetic tone whereas the LF:HF of HR variability is an index of cardiac sympathetic tone[7]. Similarly, the LF + VLF power of SBP variability is, in part, mediated by vasomotor sympathetic tone[7].

References for Supplemental File

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