Neurohormonal Modulation of the Innate Immune System Is Proinflammatory in the Prehypertensive Spontaneously Hypertensive Rat, a Genetic Model of Essential Hypertension

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Rationale: Inflammation and autonomic dysfunction contribute to the pathophysiology of hypertension. Cholinergic stimulation suppresses innate immune responses. Angiotensin II (Ang II) induces hypertension and is associated with proinflammatory immune responses.

Objective: Our goal was to define the innate immune response in a model of genetic hypertension and the influences of cholinergic stimulation and Ang II.

Methods and Results: Studies were conducted on 4- to 5-week-old prehypertensive spontaneously hypertensive rats (SHRs) and age-matched normotensive control, Wistar Kyoto (WKY) rats. Isolated splenocytes were preexposed to nicotine or Ang II before Toll-like receptor (TLR) activation. Culture supernatants were tested for cytokines (tumor necrosis factor-α, interleukin [IL]-10, and IL-6). TLR-mediated cytokine responses were most pronounced with TLR7/8 and TLR9 activation and similar between WKY rats and SHRs. Nicotine and Ang II enhanced this TLR-mediated IL-6 response in prehypertensive SHR splenocytes. In contrast, nicotine suppressed the TLR-mediated IL-6 response in WKY rats, whereas Ang II had no effect. In vivo, nicotine enhanced plasma levels of TLR7/8-mediated IL-6 and IL-1β responses in prehypertensive SHRs but suppressed these responses in WKY rats. Flow cytometry revealed an increase in a CD161+ innate immune cell population, which was enhanced by nicotine in the prehypertensive SHR spleen but not in WKY.

Conclusions: There is a pronounced anti-inflammatory nicotinic/cholinergic modulation of the innate immune system in WKY rats, which is reversed in prehypertensive SHRs. The results support the novel concept that neurohormonal regulation of the innate immune system plays a role in the pathogenesis of genetic hypertension and provide putative molecular targets for treatment of hypertension. (Circ Res. 2012;111:1190-1197.)

Key Words: hypertension | toll-like receptor | nicotine | angiotensin II | interleukin-6 | innate immunity
angiotensin II (Ang II) activates peripheral sympathetic nerve activity and enhances splenic cytokine gene expression, suggesting that Ang II may contribute to the development of hypertension by stimulating a neurally mediated proinflammatory immune response in the spleen. In addition to its direct renal effects.

The SHR has been used as a genetic model for essential hypertension because it develops hypertension as it ages. Hypertension in SHRs begins at about 6 weeks of age, and at 4 to 5 weeks of age the SHRs are in the prehypertensive period. Given the autonomic dysfunction and immunologic abnormalities present in the SHR, it serves as an ideal model for the investigation of neurohormonal regulation of innate immune responses. Although the adaptive immune system has been implicated in hypertension, the role of the innate immune system remains undefined. We hypothesized that there is a dysregulation of innate immune responses before the development of hypertension in SHRs. In the present work, we use Toll-like receptor (TLR) ligands to activate the innate immune response. These TLRs detect pathogen-associated molecular patterns and damage-associated molecular patterns and induce cytokine production and release that trigger the activation of the acquired/adaptive immune system. The modulation of the cytokine response to TLR activation by nicotine and Ang II was contrasted in young Wistar Kyoto (WKY) normotensive rats and the young prehypertensive SHRs. In the WKY rats, nicotine suppressed the cytokine response, but in SHR rats, nicotine, as well as Ang II, significantly enhanced the cytokine response. We propose that this abnormal proinflammatory modulation of the innate immune system which precedes the onset of hypertension contributes significantly to its development in the SHR.

Methods

Please see the Online Data Supplement for detailed Materials and Methods.

Animals

Male WKY rats and SHRs (Charles River Laboratories) were used at 4 to 5 weeks of age.

TLR Activation

Several Toll receptors recognize specific extracellular ligands and induce nuclear factor-κB activation in immune cells and the release of inflammatory cytokines. Responses to the TLR ligand activation were measured in supernatants of cultured splenocytes in vitro, as well as in blood in vivo.

Spentocyte Cultures

Splenocytes from 4- to 5-week-old animals were stimulated in vitro with known TLR ligands in the presence or absence of preexposure to nicotine or Ang II. Culture supernatants were collected, and IL-6, TNFα, and IL-10 were measured by ELISA.

In Vivo Studies

Subcutaneous osmotic minipumps were implanted and infused either saline or nicotine over 24 hours (15 mg/kg). At 20 hours, animals received an intraperitoneal injection of either saline or the TLR7/8 ligand (Clo97). Serum was collected from each animal, and IL-6, IL-1β, TNFα, IL-10, and IL-17 were measured at 24 hours.

Flow Cytometry

Splenocytes were stained with fluorochrome-conjugated antibodies to CD3, CD4, CD8, CD45R, CD11c, CD161a, and major histocompatibility complex-II and analyzed by flow cytometry.

Results

Cytokine Release From Splenocytes in Response to TLR Activation Is Pronounced With TLR7/8 and -9 Equivalent in SHRs and WKY Rats

We first asked whether the native innate immune response of the SHRs differed from the normotensive WKY rats. Splenocytes were stimulated with 3 graded doses of the ligands for TLR2, TLR3, TLR4, TLR5, TLR7/8, and TLR9, as listed in the legend of Figure 1. Similar levels of TNFα (Figure 1A), IL-10 (Figure 1B), and IL-6 (Figure 1C) were induced in WKY and SHR splenocytes in response to TLR activation. The most robust responses were seen with TLR7/8 and TLR9 activation. Nicotine and Ang II alone did not induce cytokine secretion from either WKY or SHR splenocytes (Figure 1).

Modulation of TLR-Induced IL-6 Secretion by Nicotine and Ang II Is Markedly Proinflammatory in SHRs

Although nicotine and Ang II did not directly induce the secretion of cytokines, exposure of splenocytes to nicotine (10 μmol/L) and Ang II (1 μmol/L) for 2 hours before the addition of TLR ligands resulted in significant modulation of TLR-induced IL-6 secretion. In WKY splenocytes, nicotine preexposure suppressed TLR9-mediated IL-6 secretion (Figure 2A). In contrast, nicotine exposure of SHR splenocytes resulted in a dramatic increase in the release of IL-6 (Figure 2B) in response to TLR7/8 and TLR9. Ang II exposure of WKY splenocytes did not alter TLR-mediated IL-6 release (Figure 2C) but led to a significant increase in TLR-mediated IL-6 release in SHR splenocytes, similar to the effect of nicotine (Figure 2D).

Based on the robust responses to activation of TLR7/8 and TLR9, we focused on these receptors in subsequent experiments (Figure 3). We continued to see uniformly suppressive effects of nicotine on TLR responses in WKY splenocytes (Figure 3A) and uniformly enhanced effects of both nicotine and Ang II in SHR splenocytes (Figure 3B and 3D). Ang II exposure did not appreciably alter the TLR responses in WKY splenocytes (Figure 3C).

In Vivo Effect of Nicotine and Ang II on TLR7/8-Mediated Cytokine Production: Inhibitory in WKY Rats and Proinflammatory in SHRs

We asked whether the contrasting nicotine effects seen in vitro would be reproduced in vivo. We found that subcutaneous infusion of nicotine alone or Ang II alone demonstrated a trend
of increased serum levels of IL-6 and IL-1β in both WKY rats (Figure 3E and 3G) and SHRs (Figure 3F and 3H). We also found that the serum levels of both IL-6 and IL-1β in response to TLR7/8 activation with Clo97 (intraperitoneally) were markedly suppressed by the subcutaneous infusion of nicotine in WKY rats (Figure 3E) and conversely significantly enhanced in SHRs (Figure 3F). Ang II did not alter the TLR responses of IL-6 and IL-1β in WKY rats (Figure 3G), but in SHRs the IL-1β response was significantly enhanced and the increase in IL-6 was not significant (Figure 3H). Thus, the anti-inflammatory

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The major finding of this study is that SHRs exhibit a proinflammatory innate immune response (measured by IL-6 and IL-1β) that is most pronounced with activation of TLR7/8 and TLR9. This population also remained low in WKY splenocytes (1.5%), yet it significantly enhanced the IL-1β response in SHRs (H). Significant differences between responses to Nic/TLR or Ang/TLR vs saline/TLR in both WKY rats and SHRs (P<0.01). The anti-inflammatory effects of nicotine in WKY splenocytes and proinflammatory effects of nicotine and Ang II in SHR splenocytes were thus confirmed in vivo.

Figure 3. Immunomodulation of interleukin (IL)-6 and IL-1β responses to Toll-like receptor (TLR) 7/8 and TLR9 by nicotine and angiotensin II (Ang II) in vitro and in vivo. Individual responses of isolated splenocytes from 4- to 5-week-old Wistar-Kyoto (WKY) rats (n=5) and spontaneously hypertensive rats (SHRs; n=5) are shown in upper panels A to D. In vivo changes in serum levels in response to subcutaneous infusions of nicotine or Ang II in WKY rats and SHRs are in the lower panels E to H. Numbers above each bar graph represent the number of animals (n) in the particular group. Splenocyte responses in vitro: Splenocytes were activated by ligands to TLR7/8 (Clo97, 1.0 μg/mL) and TLR9 (CpG, 10 μg/mL). IL-6 was measured in culture supernatants. A to D. Responses to activation of the TLRs with exposure to nicotine (10 μmol/L) in WKY and SHR splenocytes (A and B, respectively) and with exposure to Ang II (1 μmol/L) in WKY and SHR splenocytes (C and D, respectively). Responses of WKY splenocytes are in black and white (A and C) and those of SHR splenocytes are in red (B and D). The suppressive effect of nicotine on responses to activation of both TLRs in WKY splenocytes was significant (P<0.01), and Ang II had generally small and inconsistent effects in WKY splenocytes. In contrast, a significant augmentation of the responses to activation of both TLRs by nicotine and Ang II was consistently observed in SHR splenocytes (P<0.01). In vivo serum levels: The effects of subcutaneous infusions of nicotine (15 mg/kg) or Ang II (0.72 mg/kg) on IL-6 and IL-1β serum levels induced by the TLR7/8 ligand (Clo97, 1 mg/kg) intraperitoneally are shown in E through H. The infusions of saline, Nic, or Ang II were maintained for 24 hours. At 20 hours, animals received intraperitoneal injection of Clo97 or saline. Sera were collected at 24 hours and assayed for IL-6 and IL-1β. Nicotine (E and F) and Ang II (G and H) when given with intraperitoneal saline increased IL-6 and IL-1β in WKY rats and to a lesser extent in SHRs. In WKY rats, nicotine (subcutaneous) significantly suppressed IL-6 and IL-1β responses to intraperitoneal Clo97 (E). In contrast, in SHRs, nicotine significantly enhanced both IL-6 and IL-1β responses (F). Ang II had no effect on the responses in WKY rats (G), yet it significantly enhanced the IL-1β response in SHRs (H). Significant differences between responses to Nic/TLR or Ang/TLR vs saline/TLR in both WKY rats and SHRs (P<0.01). The anti-inflammatory effects of nicotine in WKY splenocytes and proinflammatory effects of nicotine and Ang II in SHR splenocytes were thus confirmed in vivo.

Activated Macrophages in the SHR Are Increased by Nicotine
Based on the clearly opposite effects of nicotine on IL-6 secretion in WKY rats and SHRs, both in vitro and in vivo, we asked whether there was a difference in the immune cell subpopulations in SHRs versus WKY rats and used flow cytometry to answer this question.

Although there was no difference in the distribution of CD3+/CD8+, CD3+CD4+, CD11b+, or CD45R+ immune cells, we found a discordance in the composition of CD3+/CD8α/β and CD3+/CD8αdim cell populations, with an increase in the latter in SHR compared with WKY splenocytes (Figure 4). Further analysis of the CD3+/CD8αdim cells was made by assessing CD161a, a marker of activated macrophages. We found a CD161a population that was more dominant in SHR (4.6%) compared with WKY splenocytes (1.1%) and expanded in response to nicotine to 6.4%, whereas the WKY population remained at 1.1%. Another marker of activated macrophages (CD3+/CD8α/β CD161a+) was also increased in SHR splenocytes and expanded further from 3.7% to 6.0% in response to nicotine (Figure 4). This population also remained low in WKY splenocytes (1.5%), despite the presence of nicotine (Figure 4). Hence, there seems to be a population of activated macrophages (CD161a+) present in the SHR splenocytes, but not in the WKY splenocytes, before the development of hypertension, which may account for the proinflammatory enhancement of the TLR-induced increase in IL-6 and IL-1β levels.

Effect of Nicotine on Myeloid Dendritic Cells in WKY Rats
Preliminary findings reported in the Online Figures II to IV indicate suppression of a major histocompatibility complex-II+ CD11b+ myeloid dendritic cell population by nicotine exclusively in WKY splenocytes but not in SHR splenocytes, which may account for the anti-inflammatory effects of nicotine.

Discussion
The major finding of this study is that SHRs exhibit a proinflammatory innate immune response (measured by IL-6 and IL-1β) before the development of hypertension, which suggests its contribution to the development of hypertension. It is most pronounced with activation of TLR7/8 and TLR9.
Nicotine, a ligand for cholinergic receptors, is anti-inflammatory in the normotensive control WKY rats but is paradoxically proinflammatory in SHRs. An accentuated proinflammatory response to Ang II is also seen in SHRs but not in WKY rats. Changes in innate immune cell populations with exposure to nicotine correlate with the anti-inflammatory effects of nicotine in WKY rats and proinflammatory effects in young SHRs.

In the Discussion section, we will address the following: (1) the contribution of the innate immune system in human hypertension and SHRs; (2) the functional relevance of immune responses to nicotine and angiotensin to the survival advantage of parasympathetic activity and decreased parasympathetic activity has been observed in young SHRs. Previous studies18 and preliminary results from our laboratory19 have shown that SHRs are normotensive at 4 to 5 weeks of age. In the present study, we show that activation of isolated splenocytes with several TLR ligands induced increases in IL-6, TNFα, and IL-10, which are comparable in the prehypertensive SHR with those of normotensive WKY rats. Also, in vivo TLR activation caused increases in IL-6 and IL-1β blood levels in both WKY rats and SHRs. More importantly, however, an enhanced proinflammatory response to TLR activation similar to that seen in patients with essential hypertension was unmasked in our prehypertensive SHRs by nicotine and Ang II in vitro and in vivo.

In contrast, in the WKY rats, nicotine that had been reported to suppress inflammatory responses by activating α7 nicotinic acetylcholine receptors (nAChRs)20 suppressed the increase in IL-6 in vitro and the increases in IL-6 and IL-1β in vivo. Ang II had no effect on the cytokine response to TLR activation in WKY rats.

The results indicate that there is an abnormal proinflammatory state of the innate immune system in the prehypertensive SHR, which is provoked by nicotine and Ang II, when TLR 7/8 and TLR9 are activated, and may thereby contribute to the development of hypertension in this model. We also note an anti-inflammatory effect of nicotine in the WKY rats, which is likely mediated by α7 nAChRs.

**Functional Relevance of Inflammatory Responses to Nicotine and Ang II to the Survival Advantage of Parasympathetic Stimulation and Angiotensin-Converting Enzyme Inhibition in Cardiovascular Disease**

Autonomic dysfunction with increased sympathetic nerve activity and decreased parasympathetic activity has been correlated with increased cardiovascular mortality21 and the development of hypertension.22-25 The attempt to restore autonomic balance with direct carotid sinus and vagal stimulation has shown promise in the treatment of hypertension and heart failure.26 Classically, autonomic dysfunction has referred to the imbalance of neural activity between the parasympathetic and the sympathetic nervous systems. Recently, the neurotransmitters of the autonomic nervous system have been shown to not only be produced by, but also exert effects on, non-neuronal cells, expanding the conceptual importance of autonomic dysfunction.27

There is direct sympathetic innervation of immunologic organs.28 Activation of adrenergic receptors has been shown to induce a proinflammatory immune response.29,30 In contrast, it has been shown that the vagus nerve stimulation exerts an anti-inflammatory effect in the gut in a murine model for sepsis.15

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**Inflammation and Innate Immunity in Human Hypertension and SHRs**

Circulating inflammatory cytokines have been detected in the sera of hypertensive patients; furthermore, amounts of IL-6 and IL-1β in whole blood of patients with essential hypertension were exaggerated in response to TLR4 activation with lipopolysaccharide, consistent with the activation of monocytes.21 Similarly, the genetically hypertensive adult SHRs have elevated blood levels of innate immune cytokines (IL-6 and IL-1β) compared with WKY rats.22 Furthermore, the systemic inhibition of nuclear factor-κB in SHRs decreases renal inflammation and results in significant reductions of systolic blood pressure.

Our results focused on the prehypertensive (normotensive) young SHRs. Previous studies18 and preliminary results from our laboratory19 have shown that SHRs are normotensive at 4 to 5 weeks of age. In the present study, we show that activation of isolated splenocytes with several TLR ligands induced increases in IL-6, TNFα, and IL-10, which are comparable in the prehypertensive SHR with those of normotensive WKY rats. Also, in vivo TLR activation caused increases in IL-6 and IL-1β blood levels in both WKY rats and SHRs. More importantly, however, an enhanced proinflammatory response to TLR activation similar to that seen in patients with essential hypertension was unmasked in our prehypertensive SHRs by nicotine and Ang II in vitro and in vivo.

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**Figure 4. Effects of nicotine on CD8+CD161+ innate immune cells are discordant between Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) splenocytes.** Subpopulations of CD8 and CD161 are shown in WKY and SHR splenocytes after 48 hours in culture in media alone (A and B, respectively) and in media plus nicotine (10 μmole/L in C and D, respectively). After gating for CD3− cells, flow cytometry revealed an increase in the CD8dim/CD161+ and CD8−/CD161+ populations in SHRs (B), compared with WKY (A). Furthermore, an inductive effect of nicotine on the CD161+ immune cell populations is seen in SHRs (D) but not in WKY splenocytes (C). The markers indicate an increase in activated macrophages in SHRs, which is enhanced by nicotine in contrast to the suppressed values in WKY splenocytes. This suggests that the proinflammatory effect of nicotine in SHRs results from an increase in activated macrophages (CD161+) not seen in WKY splenocytes. Results presented are representative of 3 individual experiments.
Vagal nerve stimulation also exerts an immunomodulatory effect on a subset of splenic T lymphocytes, which produces and secretes acetylcholine (Ach) in response to adrenergic input from the splenic nerve.26,31,32 The results of the present study suggest novel mechanisms by which autonomic regulation of immune responses may increase cardiovascular mortality or be protective and beneficial.

Cholinergic Anti-Inflammatory Immunomodulation: Role of α7 nAChRs

Our data show that there is an anti-inflammatory modulation in the normotensive WKY rats by nicotine, which in preliminary results is blocked by α-bungarotoxin, a blocker of α7 nAChR (Online Figure I). The paradoxically enhanced proinflammatory modulation of the TLR response by nicotine in SHRs was not altered by α-bungarotoxin and cannot be attributed to decreased expression of α7 nAChR in SHRs. It has been shown that the expression of α7 nAChRs is similar in prehypertensive SHRs and age-matched WKY rats.33 Interestingly, there are reports of decreased expression of α7 nAChRs in the central nervous system of the stroke-prone SHRs34 and in older (≥20-week) hypertensive SHRs.33 Furthermore, α7 nAChR agonists decrease the inflammatory end-organ damage in the hypertensive SHR,33 and recent studies in α7 nAChR knockout mice show an enhanced inflammatory end-organ response in the 2-kidney/1-clip model of hypertension.35 There is also evidence that T cells in the WKY rats and SHRs produce Ach and express ACh receptors36 and that circulating, thymic, and splenic levels of Ach in the very young SHRs are elevated but decreased with aging up to 20 weeks compared with the WKY age-matched controls.35

Nicotinic Proinflammatory Immunomodulation in SHRs

Although α7 nAChRs are potent suppressors of the inflammatory response and can protect the end organs from hypertensive damage, many other nAChR subunits36 and G protein-coupled receptors may be involved in proinflammatory responses. In a recent study,37 it was reported that nicotine and Ang II activate respective G protein-coupled receptors on vascular muscle cells and increase intracellular reactive oxygen species and cytokines (IL-6, 1FN-γ) that promote activation of AMP-kinase α2 and its nuclear translocation with the induction of transcription factors, such as matrix metalloproteinase 2.

Hence, there are clear indications of a cholinergic influence on the immune system and on end organs through α7 nAChRs, Ach production, or activation of other nicotinic receptors. The interaction may be protective or paradoxically inflammatory. Our results identify a significant proinflammatory innate immune system abnormality that precedes the development of hypertension in SHRs. Further characterization of the cellular and molecular determinants of that interaction needs to be examined.

Proinflammatory Immunomodulation by Ang II in SHRs

Ang II is clearly involved in the pathogenesis of hypertension through neural, renal, and vascular mechanisms. In addition, Ang II activates nuclear factor-κB, causing the expression of proinflammatory cytokines in vitro and in vivo.38 Inhibition of angiotensin-converting enzyme reduces cardiac inflammatory markers in SHRs by inactivating nuclear factor-κB.22 Angiotensin type 1 receptor blockade reduces lipopolysaccharide-induced innate immune responses in rat spleen,39 reverses renal inflammation,30 and also reduces vascular and circulating inflammatory mediators in SHRs.40 Although we found that both nicotine and Ang II had no direct effect on cytokine release from splenocytes in culture, their systemic infusions did increase serum levels of IL-6 and IL-1β in both WKY rats and SHRs. This proinflammatory response may be secondary to an increase in sympathetic nerve activity through a central action of Ang II or ganglionic effect of nicotine.

Our results shed light on a novel mechanism by which Ang II may be proinflammatory in a model of genetic hypertension. It induces a marked enhancement of secretion of IL-6 and IL-1β from splenocytes of prehypertensive SHRs in response to TLR activation. This proinflammatory response, similar to that elicited by nicotine, is immunomodulatory because Ang II alone did not induce secretion of detectable levels of cytokines. Interestingly, our preliminary results demonstrate that the angiotensin type 1 receptor blocker losartan not only prevents the proinflammatory effect of Ang II in SHRs but also reduces IL-6 release to levels below those seen in the absence of Ang II (Online Figure I). An inhibitory effect of losartan on inflammatory signaling mediated by peroxisome proliferator-activated receptor γ has been reported.41

Prominent Contribution of TLR in Activating Innate Immunity in Hypertension

A description of TLRs and their linkage to hypertension and the innate immune system is discussed in the Online Data Supplement.

Changes in Populations of Innate Immune Cells With Nicotine Differ in WKY Rats and SHRs

Dendritic cells and macrophages are members of the innate immune system and serve as antigen-presenting cells, thus activating T lymphocytes and playing a role in their differentiation. Natural killer cells are also important members of the innate immune system. We show that there is a more prominent CD3/CD161a+ cell population in the SHR splenocytes that appeared to be further induced by nicotine preexposure (Figure 4). We postulate that the expansion of this cell population may be mediated by IL-6, which is known to affect the differentiation of monocytes to macrophages.42 CD161a is a marker for natural killer cells, dendritic cells, and activated macrophages.43,44 Thus, it appears that enhanced activation of macrophages, which is not seen in WKY rats, would explain the proinflammatory effect of nicotine. We are currently exploring the distribution of the various innate immune cells, including dendritic cell subsets, natural killer cells, and, possibly, natural killer T cells. Preliminary results included in the Online Data Supplement (Online Figures II–IV) suggest that the anti-inflammatory effect of nicotine in WKY rats represents a suppression of a larger population of myeloid dendritic cells not seen in SHRs.

Conclusions

The results of the current study support the novel concept of neurohormonal modulation of the innate immune system as
a pathogenic mechanism in genetic hypertension before the onset of hypertension. Furthermore, the results begin to identify cellular targets of interest that may mediate these effects. We show evidence that the innate immune system is abnormally primed and sensitized in SHRs to be highly proinflammatory in response to putative cholinergic and angiotensinergic stimuli.

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Disclosures
None.

References

**Novelty and Significance**

**What Is Known?**
- Inflammation characterized by the presence of activated immune cells and production of proinflammatory cytokines has been linked to hypertension. Recent studies have implicated T lymphocytes in angiotensin II (Ang II)-induced hypertension.
- Activation of nicotinic cholinergic receptors inhibits innate inflammatory immune responses, whereas binding of Ang II to angiotensin type 1 receptors is proinflammatory.

**What New Information Does This Article Contribute?**
- The innate immune system is abnormally proinflammatory in a genetic model of hypertension before the onset of hypertension.
- An anti-inflammatory effect of nicotine on spleenocytes isolated from control Wistar Kyoto rats (inhibition of interleukin-6 release) is reversed to a proinflammatory increase of interleukin-6 release from spleenocytes of young, prehypertensive spontaneously hypertensive rats (SHRs).
- Ang II evokes a proinflammatory response in prehypertensive SHRs but not in Wistar Kyoto rats.
- In vivo changes in serum levels of interleukin-6 and interleukin-1β matched the in vitro responses when nicotine and Ang II were infused subcutaneously in Wistar Kyoto rats and SHRs.
- α7 nicotinic receptors and type 1 Ang II receptors mediate the anti- and proinflammatory responses, respectively.
- Proinflammatory responses in SHRs are associated with a unique innate immune cell population (activated macrophages) that proliferates in response to nicotine in SHRs.

Little is known regarding regulation of the innate immune system in hypertension. This study shows that the innate immune system is abnormally primed and sensitized in SHRs to be highly proinflammatory, in response to both nicotine and angiotensin II, even before blood pressure increases. These results support the concept of neurohumoral modulation of the innate immune system as a pathogenic mechanism in the development of hypertension and could help in identifying new molecular targets for the treatment of hypertension.
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SUPPLEMENTAL MATERIAL

DETAILED MATERIALS AND METHODS

Animals

Male Wistar Kyoto (WKY) and male Spontaneous Hypertensive Rats (SHR) [Charles River Laboratories] at 4-5 weeks of age were used in the current study. At this age, the SHR are prehypertensive. Their awake systolic pressures by tail cuff average 96.5±6.7 mmHg (n=60) which is equivalent to the pressure of the normotensive WKY rats (95.7±3.9 mmHg, n=60) (Andresen et al, Circ Res. 1980, 47:821-828).

The use of all animals was in accordance with institutional guidelines. Animals were anesthetized with isoflurane and sacrificed by decapitation.

Splenocyte Cultures

Spleens were harvested from 4-5 week old WKY and SHR. A single-cell suspension was created by homogenization in serum free RPMI medium. Erythrocytes were lysed in a hypotonic buffer (150 mM ammonia chloride, 7.2 mM potassium carbonate, 0.6 mM EDTA, pH 7.2). Cells were washed and resuspended in complete RPMI (10% heat-inactivated fetal bovine serum, 0.1mM non-essential amino acids, 0.1 mM sodium pyruvate, 10 mM Hepes buffer, 100 μg penicillin/streptomycin, 0.2mM glutamine). Isolated splenocytes (200,000) were added to each well of a 96 well flat bottom culture plates. Splenocytes were cultured in the absence or presence of various TLR ligands (lipoteichoic acid, poly I:C, lipopolysaccharide, flagellin, Clo97, CpG 2395). To determine the effects of nicotine and Ang II on immune responses, splenocyte cultures were incubated in the presence of nicotine (10 μM) or Ang II (1 μM) or vehicle (medium) for two hours at 37°C/5% CO₂ prior to the addition of TLR ligands. Splenocytes were cultured for 48 hours and supernatants were collected and assessed for the presence of TNFα, IL-6, and IL-10 by ELISA (Invitrogen, Carlsbad, Ca).

In Vivo Studies

4-5 week old WKY and SHR were obtained and subcutaneous (SC) osmotic pumps (Alzet) were implanted under ketamine (91 mg/kg, i.p.) and xylazine (9.1 mg/kg, i.p.) anesthesia. Pumps infused either saline or nicotine (15 mg/kg) or Ang II (0.72 mg/kg) over 24 hours. At 20 hours, animals received an intraperitoneal (i.p.) injection of either saline or the TLR7/8 ligand (Clo97, 1 mg/kg). Serum was collected from each animal at the end of 24 hours and tested for IL-6, IL-1β, TNFα, IL-10, and IL-17 via sandwich ELISA (R&D Biosystems).

Immunofluorescence/Flow Cytometry

Splenocytes were isolated as described above. They were maintained in plain RPMI and washed twice. They were cultured for 48 hours in the presence or absence of nicotine. Flow cytometry was then performed. Cells were then filtered through a 70 micrometer nylon mesh to produce a single cell suspension. Cells (1 x 10⁶) were aliquotted into staining tubes and resuspended in staining buffer (PBS, 1% appropriate animal sera, 2% FBS). Fluorochrome conjugated antibodies (BD Biosciences), [Anti-rat CD45R antibody (Clone HIS24), Anti-rat CD3 (Clone G4.18), Anti-rat CD4 (Clone OX-35), Anti-rat CD8 (Clone Ox-8), Anti-rat CD161a (Clone 10/78), Anti-rat CD11bc (Clone Ox-42)] were added at pre-determined working dilutions and splenocytes were incubated 45 minutes at 4°C. Cells were washed and resuspended in staining buffer for analysis. They were
analyzed using a Becton Dickinson Laser Violet flow cytometer and data collected and analyzed in FACS Diva Software. Flow cytometry was performed on a Becton Dickinson LSR II device (Becton Dickinson, Heidelberg, Germany). For each acquisition, 30,000 events were recorded. Cellular debris and necrotic/non-viable cells were excluded by gating and staining with Hoechst 33258A.

Statistics

Values were compared using one-way ANOVA and Tukey-Kramer comparison for significance with a P value <0.05.

RESULTS

Effect of α-bungarotoxin, and losartan on immunodulation by nicotine and Ang II.

In preliminary experiments, the inhibitory modulation of IL-6 release by nicotine in WKY was blocked by α-bungarotoxin, a known blocker of α7 nAChR (Online Figure 1).

The enhanced release of IL-6 in SHR was not altered by α-bungarotoxin but was markedly suppressed by losartan (Online Figure 1).

Flow Cytometry Immunofluorescence

Distribution of Immune Cell Subpopulation in Rat Spleens. Several surface markers indicated that the distribution of T and B lymphocytes, and monocytes were not different in WKY and SHR (Online Figure 2). Further definition of CD3- and CD8+ innate immune cells reveals a discordance between WKY and SHR (Online Figure 3).

Effect of Nicotine on Myeloid Dendritic Cells in WKY (Online Figure 4)

The MHCII and MHCII+ CD11bc+ cells were abundant (9.9%) and the CD11bc- cells were sparse (0.8%) in WKY; the reverse was seen in SHR, where the CD11bc+ were low (2.4%) and the CD11bc- were relatively high (14.6%) (Online Fig. 4). Moreover, nicotine significantly shifted the balance in WKY from the 9.9% CD11bc+, dropping it to 2.2%, whereas the CD11bc- increased from 0.8% to 16.9%. In the SHR, nicotine had a minimal effect on the distribution of CD11bc (Online Fig. 4). These results suggest that suppression of myeloid dendritic cells in WKY by nicotine accounts for the anti-inflammatory response.

DISCUSSION

Prominent Contribution of TLRs in Activating Innate Immunity in Hypertension

The immune system has been directly implicated in experimental models of hypertension. The studies to date have revolved primarily around the involvement of T-lymphocytes and the adaptive immune system in Ang II-induced hypertension [1]. Our work has focused on the innate immune system in genetic hypertension which involves activation of TLR.

Innate Immunity. The innate immune system is the major antigen-presenting activator of the cascade of adaptive immune responses and their positive feedback that underlies a very large number of pathologic processes, ranging from diabetes and hypertension to cancer.

Although originally it was thought that the innate and adaptive immune systems operate independently, recent developments clearly indicate a major role for the innate immune system in
the initiation and activation of an adaptive immune response. Thus, although the innate immune response per se may be of short duration, its effects on the adaptive immune system will result in a sustained and prolonged immunological consequence. Moreover, the innate immune response per se may be of short duration, its effects on the adaptive immune system will result in a sustained and prolonged immunological consequence. In addition, the secreted cytokines activate other innate immune cells as well as cytotoxic T cells, leading to a chronic sustained activation. The role of lipid-laden macrophages in atherosclerosis and the chronic innate immune response in autoimmune diseases are well established examples.

**Toll Receptor Activation Triggers Inflammatory Responses.** The detection of microbial pathogens leading to an anti-inflammatory response, or self-antigens that often trigger autoimmune diseases, is mediated through TLRs on immune cells.

TLRs share homology with the interleukin 1 (IL-1) receptor, possessing an intracellular Toll/IL-1 receptor domain. The extracellular domain is composed of leucine-rich repeat sequences containing the immunoglobulin-like domain of the IL-1 receptor. TLRs are classified as either extracellular (TLR-1, 2, 3, 4, 5, 6) or intracellular (TLR3, 7, 9), the latter expressed in endosomal compartments. Each TLR detects Pathogen Associated Molecular Patterns (PAMPs) associated with a class of pathogens. For example, TLR2 detects components of the cell wall of gram positive bacteria such as Lipoteichoic Acid (LTA); TLR3 binds single-stranded DNA (poly 1:C); TLR4 binds Lipopolysaccharide (LPS); TLR5 detects Flagellin (a component of flagellated bacteria); TLR 7/8 finds ssRNA as well as synthetic imidazoquinoline compounds (imiguimod and Clo97); and TLR9 detects unmethylated bacterial DNA and is activated by ODN2395 (CpG).

The selectivity of these TLR ligands is well established in the literature [2, 3]. The receptors, however, may be activated by several known and some unknown exogenous and endogenous ligands. The evidence for activation of the intended TLR pathway has been well documented in specific selective expression systems [2, 3].

In addition to the PAMPs, TLRs respond to several endogenous ligands including the self-antigens mentioned above that are referred to as DAMPs [4].

**Importance of TLR 7/8 and 9.** Our results are the first to demonstrate the relative effectiveness of several TLR ligands in releasing IL-6 from splenocytes of SHR and WKY rats. It was evident that responses to activation of TLR 7/8 and 9, which are intracellular receptors, were significantly greater than those of TLR 1, 2, 4, 5, and 6, which are extracellular. It is likely that TLR 7/8 and 9 are activated by an endogenous ligand triggering the inflammatory process. Other models of hypertension have also invoked an endogenous ligand that triggers the recruitment of T-lymphocytes [5].

TLR7/8 and 9 are known to be highly expressed in innate immune cells of most species, including rats. A molecular target of activation of TLRs is the generation of NFκβ and increased expression and release of pro-inflammatory cytokines from innate immune cells, including monocytes, macrophages, and dendritic cells. Those, in turn, will activate the adaptive/acquired immune system.

Further, TLR 7/8 and TLR9 are known to play a role in experimental models of autoimmune renal disease, including glomerulonephritis, systemic lupus erythematosus, and diabetic nephropathy [6-8], and the inhibition of TLR7/8 or TLR7/8 plus TLR9 attenuates glomerulonephritis and lung injury in experimental lupus [6]. These diseases may be associated with the development of hypertension, even in the absence of renal insufficiency [8]. Thus, the pro-inflammatory activation of the innate immune system in SHR may be linked to renal inflammation and to the development of hypertension [6-8].
**IL-6 and IL-1-beta and Hypertension.** IL-6 has been shown to play a role in the development of chronic Ang II dependent hypertension [9] and to contribute to left ventricular hypertrophy, myocardial fibrosis, and diastolic dysfunction [10-12]. IL-6, in combination with IL-1β, leads to the development and maturation of TH17 cells and secretion of IL-17, which is believed to play a role in generation of autoimmunity in a variety of diseases, including diabetes and renal disease [13]. As such, the proinflammatory effect of nicotine on the SHR innate immune response and the antiinflammatory effect of activating α7 nAChR in WKY seen in vitro and in vivo may lead to the identification of cellular mediators and molecular targets for further treatment of hypertension and its end organ consequences.

**Genetic Susceptibility of the Innate Immune System in Hypertension.** The endogenous ligand that activates TLR 7/8 and 9 in innate immune cells of the prehypertensive SHR may be considered in the context of previous reports of the significance of weaning and cross fostering of SHR pups [14, 15]. The authors of these reports refer to a maternal environmental influence and a genetic susceptibility in SHR pups that initiates the expression of the hypertensive phenotype during the first two weeks of postnatal life prior to the age at which hypertension develops in SHR. It is conceivable that an antigen present in the fostering mother’s milk may trigger a gut immune response which, in addition to influencing the development of the microbiome, will influence several cascades of innate and adaptive systemic immune responses that result in the autonomic imbalance and accelerated development of sympathetic activity documented in the first postnatal week in SHR [16].

**Supplement References**


Online Figure I. Effects of α-bungarotoxin and Losartan on Immunomodulatory Effects of Nicotine and Ang II respectively. Isolated splenocytes from 5-week old WKY and SHR were pre-exposed to nicotine (Nic, 10 μmol/L) or Ang II (1 μmol/L) and then TLR7/8 or TLR9 were activated with their respective ligands (Clo97, 1 μg/ml, and CpG, 10 μg/ml) in the presence or absence of the respective blockers of α7 nAChR (α-bungarotoxin, α-BgTx, n=3) or AT1 receptors (Losartan, n=2). IL-6 was measured in culture supernatants (means ± SE).
Online Figure II. Distribution of Immune Cell Subpopulations in Rat Spleens.

Isolated splenocytes from 5-week old WKY (n=3) and SHR (n=3). Fluorochrome conjugated rat antibodies to CD11b/c (Panels A & E), CD3 and CD8 (Panels B & F), CD3 and CD4 (Panels C & G), and CD45R (Panels D & H) were used. Percentages of the total splenocyte population are shown for specific cell types. Data are representative of three separate experiments. Note discordance in Q1-3 (CD3-, CD8+) between SHR and WKY (F & B).
Online Figure III

**Online Figure III. CD3-/CD8+ Innate Immune Cells are Discordant Between WKY and SHR.**
The upper panels highlight the discordance in the composition of bright (blue) to dim (red) cell populations in Q1-3 (CD3-/CD8+) in SHR (Panels B) vs. WKY (Panels A). The larger lower panels contrast the relatively high percentages of dim vs. low percentage of bright CD3-/CD8+ cells, particularly in SHR compared to WKY. Percentages are of the total CD3- cell population. Values represent the mean of three separate experiments ± standard error of the mean. The ratio of the CD3/CD8$^\text{dim}$ to CD3-/CD8$^\text{bright}$ population in the SHR is significantly greater than that in the WKY (p<0.01).
Online Figure IV. Effects of Nicotine on Myeloid Dendritic Cells in WKY and SHR Splenocytes. Isolated splenocytes from 5-week old WKY (Panels A and C) and SHR (Panels B and D) stained with fluorochrome conjugated anti-rat CD11bc and anti-rat MHCII antibodies and cultured 48 hours (n=1). Percentages in each quadrant represent the percentages of the total parent cell populations. Upper panels (A and B) represent cells cultured in media only and lower panels represent cultures with nicotine (C and D). The dramatic reversal of MHCII+ cell populations from CD11bc+ to CD11bc- by nicotine in WKY but not in SHR suggests that a decrease in myeloid dendritic cells (CD11+) accounts for the antiinflammatory effect of nicotine in WKY.