Evidence for Prohypertensive, Proinflammatory Effect of Interleukin-10 During Chronic High Salt Intake in the Condition of Elevated Angiotensin II Level

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See Editorial Commentary, pp 700–702

Abstract—IL-10 (interleukin-10) has been suggested to play a protective role in angiotensin II (AngII)–induced cardiovascular disorders. This study examined the role of endogenous IL-10 in salt-sensitive hypertension and renal injury induced by AngII. Responses to chronic AngII (400 ng/min per kilogram body weight; osmotic minipump) infusion were evaluated in IL-10 gene knockout mice fed with either normal salt diet (0.3% NaCl) or high salt (HS; 4% NaCl) diet, and these responses were compared with those in wild-type mice. Normal salt diets or HS diets were given alone for the first 2 weeks and then with AngII treatment for an additional 2 weeks (n=6 in each group). Arterial pressure was continuously monitored by implanted radio-telemetry, and a 24-hour urine collection was performed by metabolic cages on the last day of the experimental period. Basal mean arterial pressure was lower in IL-10 gene knockout mice than in wild-type (98±3 versus 113±3 mm Hg) mice. Mean arterial pressure responses to normal salt/HS alone or to the AngII+normal salt treatment were similar in both strains. However, the increase in mean arterial pressure induced by the AngII+HS treatment was significantly lower in IL-10 gene knockout mice (15±5% versus 37±3%) compared with wild-type mice. Renal tissue endothelial nitric oxide synthase expression (≈3-folds) and urinary excretion of nitric oxide metabolites, nitrate/nitrite (1.2±0.1 versus 0.2±0.02 μmol/L/24 hours) were higher in IL-10 gene knockout mice compared with wild-type mice. These results indicate that an increase in nitric oxide production helps to mitigate salt-sensitive hypertension induced by AngII and suggest that a compensatory interaction between IL-10 and nitric oxide exists in modulating AngII-induced responses during HS intake. **(*Hypertension*. 2017;70:839-845. DOI: 10.1161/HYPERTENSIONAHA.117.09401.)** ● **Online Data Supplement**

Key Words: angiotensin II ■ blood pressure ■ interleukin-6 ■ interleukin-10 ■ nitric oxide

It is generally known that the anti-inflammatory cytokines effectively downregulate the generation of the proinflammatory cytokines.[1] The anti-inflammatory cytokine IL-10 (interleukin-10) exerts immune downregulating action on the generation of proinflammatory cytokines, such as IL-6 (interleukin-6) and TNF-α (tumor necrosis factor-α).[2,3] Endogenous IL-10 has been suggested to play a role as a key mediator of vascular protection in atherosclerosis and diabetes mellitus.[4,5] It is also suggested that IL-10 serves a protective role in angiotensin II (AngII)–induced hypertension and its cardiovascular complications.[6,7] AngII chronically induces salt-sensitive hypertension (SSH) and also activates multiple inflammatory mechanisms leading to injury of various organs, including the kidney.[8] However, the role of endogenous IL-10 formation in the pathogenesis of AngII-induced SSH and associated renal injury is not yet clearly defined.[9]

Although high salt (HS) intake alone induces no or minimal changes in blood pressure, it exaggerates the hypertensive and renal injury responses to elevated AngII levels.[8,10] The mechanisms for such exaggerated responses remain unresolved.[9] Although HS diet alone does not alter systolic blood pressure, it causes mesangial expansion and kidney fibrosis without eliciting cell proliferation.[8] However, an HS diet given to chronic AngII-infused rats leads to greater levels of systolic blood pressure along with marked exacerbation of mesangial expansion, kidney fibrosis, and tubular epithelial cell proliferation.[11] These findings could indicate the involvement of proinflammatory cytokines, particularly TNF-α in these renal inflammatory responses.[12] AngII infusion in rats subjected to an HS diet causes increases in inflammatory cell infiltration in the tubulointerstitial areas.[11] Thus, it is suggested that the production of TNF-α induced by the elevated AngII level is involved in the exaggerated hypertensive and renal injury responses to combined AngII and HS intake.[9] However, as a modulator of the proinflammatory cytokines, the contribution of anti-inflammatory cytokines, particularly the role of IL-10, has not been assessed in such conditions in any previous studies.[2,9]

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In the present study, we hypothesized that endogenous IL-10 plays a protective role in hypertension and renal injury induced by HS intake in the elevated AngII condition by minimizing the production of proinflammatory cytokines, such as IL-6 and TNF-α. To examine this hypothesis, experiments were conducted to evaluate the changes in blood pressure, proinflammatory cytokine (TNF-α, IL-6) levels, and renal injury parameters in response to chronic administration of AngII in IL-10 gene knockout (IL-10KO) and wild-type (WT) mice that were fed either normal salt (NS)- or HS-containing diets. These experiments were primarily conducted to achieve 2 main aims: (1) to determine the extent of SSH and associated renal injury parameters (glomerular sclerosis and interstitial fibrosis) induced by AngII treatment in the condition of IL-10 deficiency, and (b) to determine the impact of endogenous IL-10 deficiency on TNF-α and IL-6 levels in response to HS/AngII treatment.

Materials and Methods

The experiments were performed in accordance with the guidelines and practices established by the Tulane University Animal Care and Use Committee. Male IL-10KO (B6.129P2-Il10tm1Cgn/J; stock no: 002251) mice and their genetic background WT control (C57BL/6J; stock no: 000664) mice (Jackson Laboratory, Bar Harbor, ME) were used in this study. These mice were housed in a temperature- and light-controlled room and allowed free access to standard diet (Ralston-Purina, St. Louis, MO) and tap water. The mice (8–9 weeks of age; ≈25 g body weight) were randomly divided into different groups depending on intake of NS diet (standard diet containing 0.3% NaCl) and HS diet (4% NaCl; Harlan-Teklad, Madison, WI), respectively, each group comprised of 6 animals. The groups are as follows:

1. NS-WT: WT mice given NS diet
2. HS-WT: WT mice given HS diet
3. NS-IL-10KO: IL-10KO mice given NS diet
4. HS-IL-10KO: IL-10KO mice given HS diet

These mice were fed NS or HS diets alone for 2 weeks before AngII infusion (400 ng/min per kilogram body weight; osmotic minipumps) for another 2 weeks. Blood pressure in these mice was continuously monitored by telemetry implantation. Urine was collected for 24 hours using metabolic cages only on the last day of the experimental period after the mice had been discontinued from the telemetry recordings. Urinary excretion of sodium and potassium was assessed by flame photometry.[13–15] Urinary excretion rate of nitric oxide (NO) metabolites, nitrate/nitrite ($U_{NOx}V$), and oxidative stress marker, 8-isoprostane ($U_{ISO}V$) were determined by colorimetry[10] and by enzyme immunoassay,[10,15] respectively. At the end of the experimental period, all the animals were euthanized, and blood samples were collected to assess the levels of TNF-α and IL-6 as conducted earlier.[13] The kidneys were also removed, and renal tissue homogenates were analyzed for TNF-α and IL-6 levels and eNOS (endothelial NO synthase) protein. The formalin-fixed paraffin-embedded kidney sections were analyzed for renal injury parameters, glomerulosclerosis using periodic acid–Schiff staining,[8,13,16] and interstitial fibrosis using Gomori trichrome staining.[14,17] Detailed experimental methods are available in the online-only Data Supplement.

Statistical Analyses

All results are expressed as means±SE. Statistical analysis was performed using Sigmastat software (Systat Software, Chicago, IL). Comparison of the responses within the same group and between the groups was conducted using the repeated measures ANOVA and Dunnett multiple comparisons test. $P \leq 0.05$ is considered as significant.

Results

Blood Pressure Response

The basal level of mean arterial pressure (MAP) was lower in IL-10KO mice than in WT mice (98±3 versus 113±3 mm Hg; $P<0.05$) as illustrated in Figure 1. HS intake alone for 2 weeks before AngII infusion did not cause any effects on MAP in either strain of mice (Figure 1). Chronic AngII infusion caused similar increases in MAP in NS-WT and NS-IL-10KO mice (absolute Δ, 26±6 versus 26±7 mm Hg; Figure 2A and percent Δ, 26±8% versus 23±5%; Figure 2B). However, AngII treatment in HS-IL-10KO mice induced a smaller increase in MAP (absolute Δ, 16±5 versus 40±3 mm Hg; Figure 2A and percent Δ, 15±5% versus 37±3%; Figure 2B) compared with that in HS-WT mice. These responses are calculated from the baseline MAP values on the day just before the start of AngII treatment. The reduction in AngII-induced MAP response in HS-IL-10KO is similar in magnitude both in absolute (Figure 2A) and in percent (Figure 2B) terms compared with that in HS-WT. It was noted that MAP started to increase on the third day of AngII treatment, which was similar in both IL-10KO and WT mice.

Renal Excretory Responses

The values for excretory parameters in urine collected on the last day of the experimental period are given in Table S1 in the online-only Data Supplement. The mean values of $U_{NOx}V$ and $U_{ISO}V$ are also given in Table S1 and are illustrated in Figure 3. IL-10KO groups showed higher $U_{NOx}V$ (Figure 3A) and $U_{ISO}V$ (Figure 3B) values than WT groups. HS groups generally showed higher values of these parameters in both strains except that $U_{ISO}V$ was not increased in the HS-IL-10KO group, which could be the effect of marked increase in NO production in that group. Because identical NS or HS diets were given and the quantity of their food intake was similar both in WT and in IL-10KO, it is reasonable to conclude that dietary factor(s) other than salt content did not contribute to the differences in $U_{NOx}V$ between these strains of mice.[10,18]

Renal eNOS Expression

The images of Western blot analysis for total eNOS protein in the renal tissue are shown in Figure 4A, and their densitometer values are illustrated in Figure 4B. Protein expression

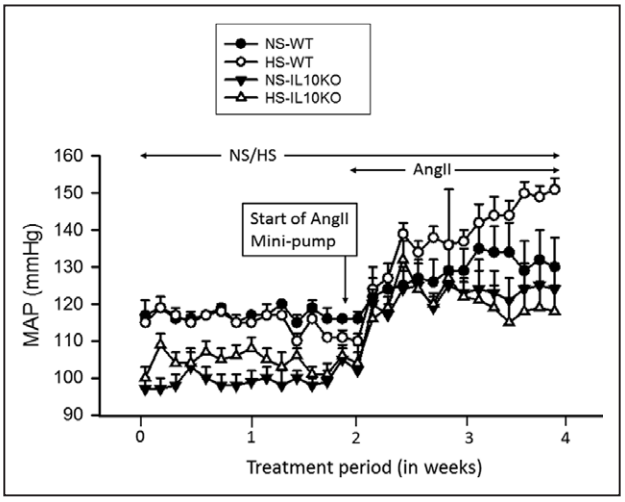

Figure 1. Mean arterial pressure (MAP) responses in interleukin-10 gene knockout (IL-10KO) and wild-type (WT) mice fed with either normal salt (NS) or high salt (HS) diets before and during AngII (angiotensin II; 400 ng/min per kilogram of body weight) treatment. The values at 0 day represent basal values at the start of the experiment. n=6 in NS-WT; n=5 in HS-WT; n=5 in NS-IL-10KO; n=6 in HS-IL-10KO.

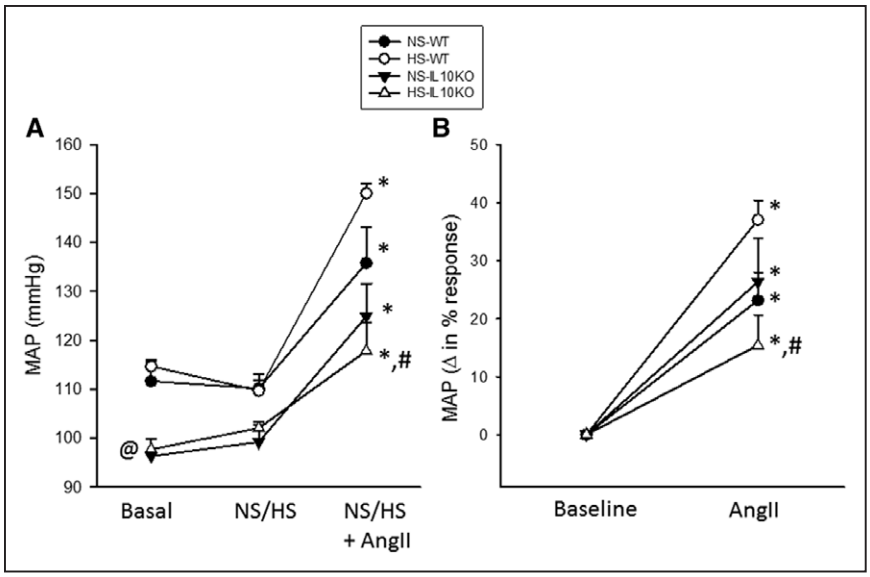

Figure 2. A, Absolute changes in mean arterial pressure (MAP) in response to normal salt (NS) or high salt (HS) diets before and during AngII (angiotensin II; 400 ng/min per kilogram of body weight) treatment. Basal represents the values at the start of the experimental protocol; NS/HS represents the values just before the day of AngII minipump implantation; NS/HS+AngII represents the values on the last day of the experimental period. **B**, Percent changes in MAP due to AngII treatment; The percent responses are calculated considering the values on the day just before AngII infusion as baseline values. AngII represents values on the last day of AngII treatment. **P*<0.05 vs NS/HS or baseline value, #*P*<0.01 vs HS-wild-type (WT) group; @*P*<0.001 vs WT group.

of eNOS was higher both in NS-IL-10KO (≈2-folds) and HS-IL-10KO (≈3-folds) compared with NS-WT and HS-WT, respectively. The density of the β-actin band was not different between samples, indicating equal loading in all lanes.

TNF-α and IL-6 Levels

Figure 5 depicts plasma and renal tissue levels of TNF-α (Figure 5A and 5B) and IL-6 (Figure 5C and 5D) at the last day of the experimental period. TNF-α level was higher in plasma (69±6 versus 34±4 pg/mL) and in renal tissue (208±15 versus 95±11 pg/mg protein) in HS-WT compared with NS-WT mice. However, TNF-α level was lower in plasma (30±3 versus 180±44 pg/mL) and in renal tissue (206±23 versus 277±62 pg/mg protein) in HS-IL-10KO compared with that in NS-IL-10KO mice. However, IL-6 level was lower in plasma (13±1 versus 26±6 pg/mL) and higher in renal tissue (148±29 versus 71±12 pg/mg protein) in HS-WT compared with that in NS-WT mice. IL-6 level was higher in plasma (16±2 versus 8±2 pg/mL) but lower in renal tissue (108±18 versus 198±34 pg/mg protein) in HS-IL-10KO compared with that in NS-IL-10KO mice.

Glomerular Sclerosis

The representative images of the periodic acid–Schiff–stained kidney sections providing the extent of glomerular sclerosis are given in Figure 6A, and the percent of the sclerotic areas is illustrated in Figure 6B. The percent sclerotic area in NS-IL-10KO was higher than that in NS-WT mice (25±1% versus 14±2%). However, the percent sclerotic area was not statistically different between HS-WT and HS-IL-10KO mice (16±3% and 22±3%).

Renal Interstitial Fibrosis

The representative images of Gomori trichrome-stained sections providing the extent of renal interstitial fibrosis are given in Figure 6C, and the percent of the fibrotic areas is illustrated in Figure 6B. The fibrotic area in the renal interstitium is higher in NS-IL-10KO (11±1% versus 8±1%) compared with NS-WT mice. There was no significant difference in the fibrotic area between HS-WT (10±1%) and HS-IL-10KO (11±0.4%) mice.

Discussion

It is generally known that chronic HS intake alone induces no or minimal changes in blood pressure in normal conditions, but it exaggerates the hypertensive and renal injury responses to elevated AngII level in many rodent models.[8–10] In the present study, it has been demonstrated that such HS-induced exaggerated hypertensive response (commonly termed as SSH) to chronic AngII administration (400 ng/min per kilogram body weight) is minimal rather absent in IL-10KO mice compared with the corresponding blood pressure response in WT mice (Figures 1 and 2). Although AngII-induced increase in MAP during NS intake was similar in both strains, the observed exaggerated MAP response to AngII administration with HS intake in WT mice was virtually absent in IL-10KO mice (Figure 2A and 2B). This finding indicates that the endogenous level of

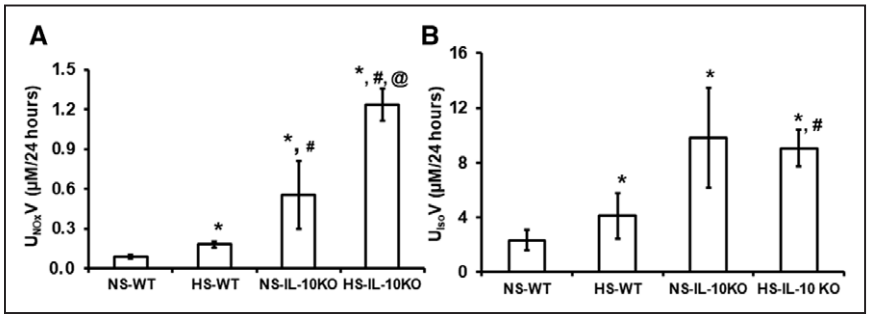

Figure 3. Mean values of urinary excretion rates of nitrate/nitrite ($U_{Nox}V$; **A**) and 8-isoprostane ($U_{ISO}V$; **B**) on the last day of the experimental period with angiotensin II (400 ng/min per kilogram body weight) treatment. **P*<0.05 vs normal salt (NS)-wild type (WT); #*P*<0.05 vs high salt (HS)-WT; @*P*<0.05 vs NS-interleukin-10 gene knockout (IL-10KO).

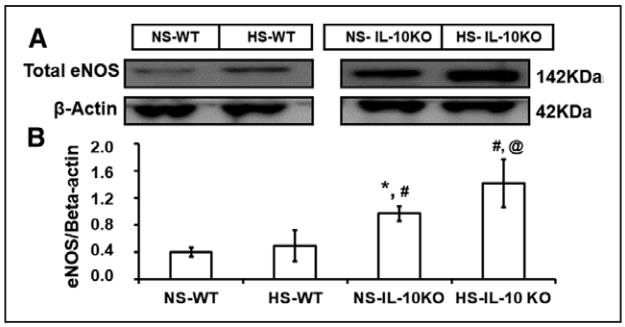

Figure 4. eNOS (endothelial nitric oxide synthase) enzyme protein expression in the renal tissue. **A**, Representative images of total eNOS expression. **B**, Mean densitometer value (arbitrary units) of eNOS expression. **P*<0.05 vs normal salt (NS)-wild type (WT); #*P*<0.05 vs high salt (HS)-WT; @*P*<0.05 vs NS-interleukin-10 gene knockout (IL-10KO). β-actin was used as loading control.

IL-10 provides a prohypertensive role in the condition of elevated AngII level during HS intake. Although AngII-induced exaggerated hypertension during HS intake is mechanistically linked with the development of oxidative stress condition,[8–10] a proinflammatory process is also suggested recently in the pathophysiology of SSH and associated renal injury.[9] The striking absence of AngII-induced salt sensitivity in IL-10KO mice in the present study indicates that endogenous IL-10 may also contribute to such proinflammatory process in the development of SSH. Although widely regarded as one of the anti-inflammatory cytokines, IL-10 has also been recognized for inducing proinflammatory reactions in many cellular functions.[19] In the present study, it is also observed that combined AngII treatment with chronic HS intake generally downregulated the formation of proinflammatory cytokines (TNF-α and IL-6) in the renal tissues of IL-10KO mice as opposed to their increases during similar treatment in WT mice (Figure 5). Thus, these findings indicate that the endogenous formation of IL-10 is involved in a proinflammatory response during chronic HS intake in the condition of elevated AngII level.

In the present study, it is noted that the basal MAP is lower in IL-10KO mice than that in WT mice. Such lower MAP in IL-10KO compared with WT mice was also reported earlier both in conscious[20] (recoded by radio-telemetry) and in anesthetized mice[21] (recorded from carotid artery catheter). However, a previous study[7] in conscious mice reported no differences in the basal MAP or the increases in MAP (recorded by tail-cuff plethysmography) in response to a high dose of AngII (≈2.5× higher than that used in the present study) treatment between IL-10KO and WT mice. Such difference in the findings in that study[7] compared with the present and earlier studies[20,21] could be related to the differences in the blood pressure measuring techniques and the differences in the doses of AngII. Although the reason for a low MAP in IL-10KO was not clearly identified in earlier studies,[20,21] it was suggested to be a nonspecific effect of IL-10 gene ablation or could be because of lower blood volume associated with the lower erythrocyte count observed in IL-10KO than that in WT.[21] In the present study, however, it is observed that $U_{NOx}V$, as well as the renal expression of eNOS, was higher in IL-10KO, indicating an enhanced NO production in this strain compared with WT. Such increase in NO production seems to have contributed to low MAP in IL-10KO mice. As total eNOS protein expression is high in IL-10KO mice, it is conceivable that an increase in $U_{NOx}V$ (a marker for increased NOS activity) in this strain of mice is, at least in part, because of the increase in eNOS activity. We have considered to examine eNOS protein expression over other NOS isoforms because it was shown earlier that the NO derived from eNOS mainly regulates baseline vascular resistance, renal blood flow, and modulates AngII-induced hypertensive responses in mice.[22,23] Although both eNOS and nNOS (neuronal NOS) isoforms are present in the renal tissue,[24] a contributing role of nNOS in the regulation of blood pressure is not supportive because it has been observed that blood pressure in nNOS knockout mice is similar to that in WT mice.[23] It is also reported[22] that the inducible NO synthase isoform is mostly absent or minimally present in the mice kidney. Thus, it is conceivable that an increase in NO production mostly via enhanced eNOS activity results in low MAP and the attenuated AngII-induced hypertensive response in HS-IL-10KO mice. However, further investigation may be needed to determine the specific roles of other NOS isoforms in inducing NO production in the condition of IL-10 deficiency.

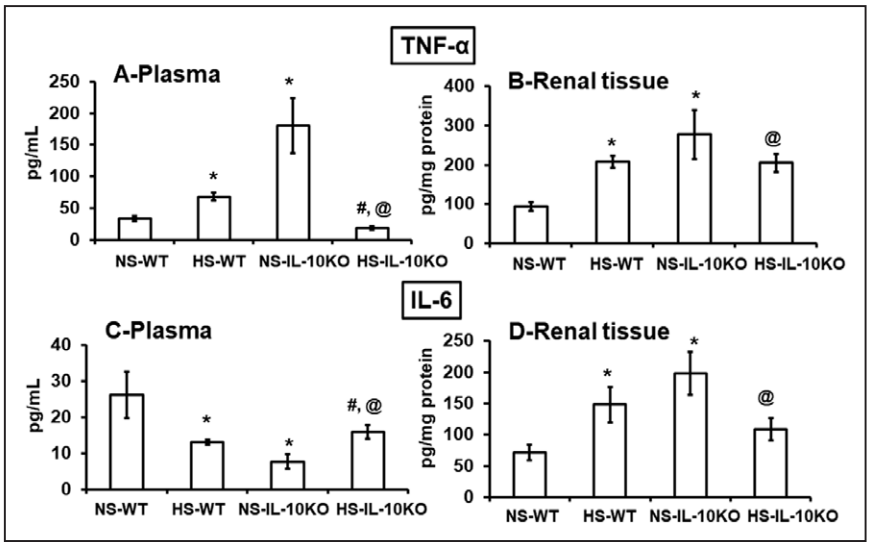

Figure 5. TNF-α (tumor necrosis factor-α) levels (**A**, in plasma; **B**, in renal tissue) and IL-6 (interleukin-6) levels (**C**, in plasma; **D**, in renal tissue) on the last day of the experimental period with angiotensin II treatment (400 ng/min per kilogram body weight). **P*<0.05 vs normal salt (NS)-wild type (WT); #*P*<0.05 vs high salt (HS)-WT; @*P*<0.05 vs NS-interleukin-10 gene knockout (IL-10KO).

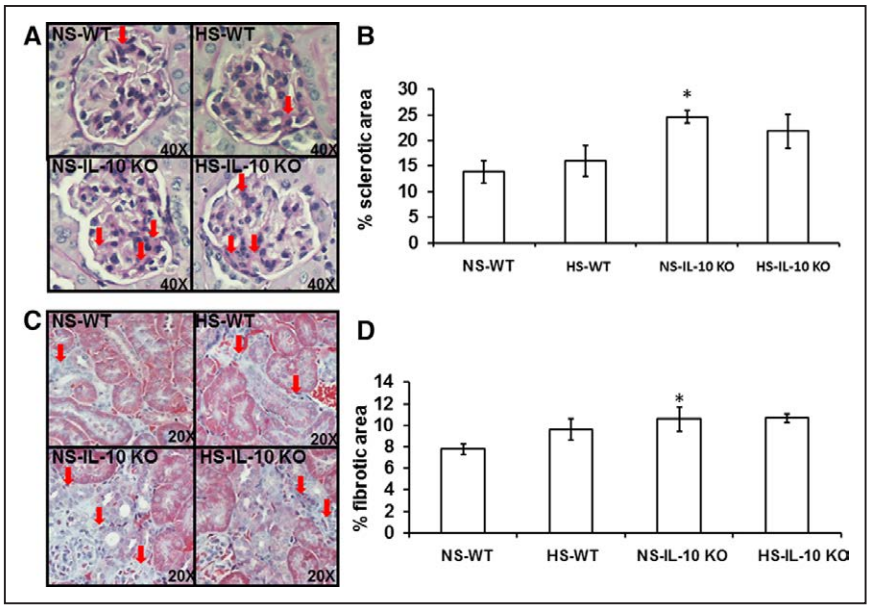

Figure 6. The extent of renal injury (glomerular sclerosis and interstitial fibrosis) at the end of the experimental period with angiotensin II (400 ng/min per kilogram body weight) treatment. **A** and **C**, Representative photomicrographs, **B** and **D** illustrate the mean values of the percent areas of sclerotic area and fibrotic area in the renal tissues, respectively. *P<0.05 vs normal salt (NS)-wild type (WT). HS indicates high salt; and IL-10KO, interleukin-10 gene knockout.

The findings in the present study may indicate an inhibitory role of IL-10 on the formation of NO because eNOS expression is upregulated in IL-10KO mice. However, such characterization of IL-10–induced effect on NO formation may not be as simple because it is also observed that NOS inhibition decreases IL-10 levels both in the plasma and in the renal tissue.[13] Although the IL-10 level is not measured in the present study, we have observed earlier[25] that its' level is lower in plasma but higher in renal tissue of eNOS knockout mice compared with that in WT mice. Previous in vitro studies[26] also demonstrated that the NOS activity, as well as NO production, in uterine tissues from pregnant rats was inhibited by pre-incubation with IL-10, indicating a reciprocal relationship between IL-10 and NO production. The present study also demonstrates that endogenous IL-10 level regulates NO production, as well as tissue eNOS expression, particularly in the kidney. Generally, it has been suggested from previous studies[27–29] that proinflammatory cytokine (s) released from activated T-cells contribute to the development of hypertension and the anti-inflammatory cytokines, particularly IL-10, released from T-regulatory cells helps to mitigate this hypertensive response.[6,30,31] However, the findings in our present study do not support a direct role of IL-10 in the development of SSH although an antihypertensive effect of IL-10 has been suggested earlier.[6,7,31] It is to be emphasized here that the activation of T-cells produces a variety of pro- and anti-inflammatory cytokines, depending on the specific nature of the inflammatory insults. The specific impact of the production of these cytokines in the overall regulation of blood pressure is complex, which is yet a subject of various investigations. The novel finding in the present study indicates that a reciprocal relationship that exists between IL-10 and NO production influencing the regulation of blood pressure during chronic HS intake in the elevated renin–angiotensin system.

In the present study, $U_{ISO}V$ (an indirect marker for oxidative stress) was higher in IL-10KO compared with WT. This is in coherence with an earlier report[7] suggesting the role of endogenous IL-10 in attenuating increases in vascular superoxide and endothelial dysfunction in disease conditions associated with diabetes mellitus and hypertension. The beneficial effects of IL-10 in endothelial dysfunction and other microvascular inflammatory complications have also been demonstrated earlier.[2,4,5,32] It was reported that patients with congestive heart failure register a decreased level of IL-10 in serum[33] and that the progression of vascular injury in several cardiovascular disease models has been retarded by exogenous administration of IL-10.[1] AngII-induced inflammation, oxidative stress, and vascular dysfunction in IL-10KO mice were also shown to be reduced by transferring cultured T-regulatory cells isolated from WT mice that produce IL-10.[31]

The percent areas of glomerular sclerosis and interstitial fibrosis (renal injury parameters) are greater in AngII-treated IL-10KO compared with that in WT in the present study. These findings are consistent with earlier reports that endogenous IL-10 deficiency was involved in both sclerosis and fibrosis of renal tissue induced by many factors, such as ureteral obstruction[34] and ischemia–reperfusion injury.[35] A reduction in IL-10 level was observed during AngII administration,[6] and AngII-induced vascular effects were prevented by exogenously applied IL-10.[30] In the present study, it has been observed that the renal tissue levels of TNF-α and IL-6 are higher in NS-IL-10KO compared with those in NS-WT, supporting the notion that IL-10 exerts an inhibitory role in the generation of proinflammatory cytokines.[1,2,13] However, TNF-α level was lower in HS-IL-10KO compared with NS-IL-10KO. Because the eNOS protein expression, as well as the $U_{NOx}V$, is markedly increased in HS-IL-10KO, the increase in NO production could be a contributing factor in such low level of TNF-α in this strain. It seems that NO production reciprocally regulates proinflammatory cytokines because it is reported that NOS inhibition increase TNF-α[13,15] in mice and NO production enhances anti-inflammatory activity in some disease conditions.[36] It is also noted that the level of IL-6 is lower in plasma but higher in the renal tissue of NS-IL-10KO compared with that in NS-WT, indicating that the changes in the plasma level of IL-6 may not always reflect tissue production of this cytokine.[33,37] The reason for such

variability in the regional IL-6 level in these AngII-treated mice is not yet clearly understood. However, it is also reported that many conditions, including AngII treatment, enhance soluble IL-6 receptors in the serum which, on binding to IL-6, exerts biological actions in different organs or cell types.[38,39] Although no direct data are yet available, it is possible that an increase in plasma soluble IL-6 receptors may occur during HS/AngII treatment, which may bind with IL-6 to reduce its concentration in the plasma. Further experiments may be needed to examine this possibility. It should also be emphasized here that such variability in the levels of pro- and anti-inflammatory cytokines in different tissues may not be unexpected because the production of these cytokines depends on the local or global inflammatory responses in different conditions.

It is also interesting to note that blood pressure was generally higher in WT than that in IL-10KO, yet the renal injury parameters are generally higher in IL-10KO than those in WT, indicating that AngII-induced renal injury may occur independent of an increase in blood pressure as reported[40] previously. It should be emphasized that both blood pressure–dependent and independent factors contribute to the development of AngII-induced renal injury. Thus, it is not unexpected that a similar degree of renal injury can be associated with a variable degree of blood pressure in the different groups of mice as observed in the present study. The findings in the present study indicate a comparatively lesser degree of renal injury in IL-10KO mice than that which would be generally expected in such a condition of deficiency in anti-inflammatory cytokine. This is mostly because of an increase in NO production in this IL-10KO strain that exerts anti-inflammatory action[41] to provide a protective role against AngII-induced renal injury. This is consistent with a report from Walley et al[42] that NO downregulates proinflammatory protein and mRNA expression during acute lung injury by an effect upstream of the activation of nuclear factor-κB, which binds to the promoter region of the proinflammatory cytokine genes.

In conclusion, the results of this investigation demonstrate that there is an increase in NO generation because of enhanced eNOS activity that occurs in the mice lacking the IL-10 gene. Such enhanced production of NO helps to mitigate hypertensive and renal injury responses to chronic AngII and HS intake in the condition of IL-10 deficiency. These novel findings provide the evidence that IL-10 can act as prohypertensive, proinflammatory cytokine during chronic HS intake in the conditions in which AngII level is elevated.

Perspectives

The implied prohypertensive role of IL-10 in SSH induced by AngII would have significant impact on the therapeutic approaches in the management of inflammatory renal injury associated with many hypertensive conditions. Such prohypertensive action of IL-10 achieved by reducing NO formation may also be a vital component in the pathophysiology of many critical conditions, including sepsis. The acute early phase of sepsis is usually characterized by an excess of proinflammatory cytokines, as well as NO production, while IL-10 level is predominantly increased during the secondary phase in which NO production is significantly attenuated.[43] Such an increase in IL-10 production in the late stage of sepsis helps to reduce proinflammatory cytokines, as well as NO formation, which would have further detrimental effects in critically ill patients.

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Disclosures

None.

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Novelty and Significance

What Is New?

- The role of IL-10 (interleukin-10; anti-inflammatory cytokine) in the development of salt sensitivity has not been addressed earlier in a comprehensive manner. This present study specifically investigates the blood pressure responses to chronic high salt (HS) intake and AngII (angiotensin II) treatment in the condition of IL-10 deficiency. The results demonstrate that IL-10 deficiency itself do not induce salt sensitivity rather attenuates hypertensive response induced by chronic AngII treatment with HS intake. This is a novel finding because many previous studies implicated the opposite notion that a decrease in IL-10 may propagate AngII-induced cardiovascular dysfunction.
- The observation that IL-10 deficiency enhances endogenous nitric oxide production, as well as endothelial nitric oxide synthase protein expression induced by chronic AngII treatment and HS intake, is also a novel finding in this study. This reciprocal relationship between endogenous IL-10 and nitric oxide production during AngII treatment or HS intake was not demonstrated in any previous study.

What Is Relevant?

- This finding of a reciprocal compensatory interaction between nitric oxide and IL-10 is significant because it is indicated that an inhibition of IL-10 activity, rather than IL-10 replacement, would be an adequate therapeutic target for the management of salt-sensitive hypertension induced by elevated renin–angiotensin system.

Summary

The findings in the present study document a reciprocal compensatory interaction between nitric oxide and IL-10 in modulating AngII-induced responses during HS intake. These findings also provide the evidence that IL-10 can act as prohypertensive, proinflammatory during chronic HS intake in the conditions in which AngII level is elevated.

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Evidence for Prohypertensive, Proinflammatory Effect of Interleukin-10 During Chronic High Salt Intake in the Condition of Elevated Angiotensin II Level

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EVIDENCE FOR PRO-HYPERTENSIVE, PRO-INFLAMMATORY EFFECT OF INTERLEUKIN-10 DURING CHRONIC HIGH SALT INTAKE IN THE CONDITION OF ELEVATED ANGIOTENSIN II LEVEL

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Short title: AngII treatment in IL-10 deficient mice.

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Materials and Methods

Blood Pressure Monitoring in Conscious Mice: After 10 days of acclimatization, radio transmitters (TA11PA-C10, DSI) were implanted in all the mice from both WT and IL-10KO groups to monitor the arterial blood pressure continuously as described previously.[1, 2] Mice were placed on a 12:12-h light-dark cycle and received food and water ad libitum throughout the study. After 8–10 days of recovery, we began monitoring systemic blood pressure and heart rate continuously using the telemetry data acquisition system (DSI, St. Paul, MN). The mice were monitored for 3 days on standard laboratory diet and resting blood pressure and heart rate were recorded before being switched to a high-salt diet (only for HS-WT and HS- IL-10KO mice) for the remainder of the study.

Animal Treatment: After the recording of resting blood pressure and heart rate, mice were fed with either a NS (0.03% NaCl) or HS (4% NaCl) diet for 2 weeks to evaluate the responses to HS alone in IL-10KO mice. After 2 weeks, osmotic mini-pumps (Alzet, Durect, Cupertino, CA) were implanted in these mice and a chronic infusion of AngII (dissolved in 0.9% saline) was given for the last 2 weeks at a rate of 400 ng/min/kg bw. The mini-pump was implanted under sterile conditions subcutaneously under the scapula through an incision in the mid-scapular region using isoflurane anesthesia. The antibiotic tetracycline (500 mg/L) was given in the drinking water 24 hour (hr) before the surgery and continued for the duration of the experimental period.[1, 2] During the experimental period, the mice were allowed to drink freely from the water bottles attached to the cages. Food and water intake, along with body weight, of each mouse was measured during the experimental period.

Collection and analysis of urine and blood samples: Twenty-four-hour urine samples were collected from conscious mice using metabolic cages only on the last day to evaluate the excretory parameters during AngII infusion with or without HS intake in these mice. As these mice were subjected to continuous monitoring of blood pressure by telemetry implantation, the collections of 24 hr urine using metabolic cages were only made on the last day of the experimental protocol after the telemetry recordings had been discontinued. All the groups of mice were placed in metabolic cages and urine and the necessary parameters were collected at the same time. Animals were housed individually in metabolic cages, and urine was collected for 24 hr into sterile tubes. Urine volumes were determined from each urine collection, and samples were centrifuged (3,000 rpm/5 min; 4°C) and preserved for determining urinary excretion of sodium and potassium. Urinary excretion of sodium and potassium were assessed by flame photometry.[1, 2] As we had observed an attenuated hypertensive response to AngII+HS treatment in IL-10KO mice compared to that in wild-type (WT) mice during the experiment, we also aimed to determine the changes in the urinary excretion rate of nitric oxide (NO) metabolites, nitrate/nitrite ($U_{NOx}V$) and in renal tissue endothelial NO synthase (eNOS) expression to understand the possible role of NO in mediating this response. Urine samples were preserved with 2-propanol (6.5%) to prevent the growth of bacteria that may degrade the nitrate/nitrite in the stored samples.[2] To determine the urinary excretion rate of oxidative stress marker, 8-isoprostane ($U_{Iso}V$), urine samples were collected into tubes containing butylated hydroxytoluene (10 µl of 5 mg/ml solution in ethanol per 1 ml sample) to prevent further oxidative production of 8-isoprostane.[2, 3] Concentration of nitrate/nitrite and 8-isoprostane in urine samples was

determined colorimetrically (Assay Designs, Ann Arbor, MI) and by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI)[2, 3] respectively.

At the end of the experimental period, all the animals were euthanized and blood was collected. Plasma was immediately separated from the blood and stored at -80ºC for estimation of pro-inflammatory cytokines (IL-6 & TNF-alpha) as conducted earlier.[4] The kidneys were also removed. One kidney was stored at −80°C for cytokine measurement and protein expression of total endothelial nitric oxide synthase (eNOS) and the other kidney was fixed in 10% formalin solution and paraffin□embedded for evaluation of renal injury. For measuring the levels of pro-inflammatory cytokines in the kidney, a portion of renal tissue (about 100 mg in weight) was homogenized in sterile phosphate-buffered saline containing protease inhibitor at 4°C. Kidney homogenates were centrifuged at 9000 g for 10 min at 4°C. Supernatants were transferred to clean micro-centrifuge tubes and stored at −80°C until analyzed.

Enzyme-linked Immunosorbent Assay for IL-6 and TNF-α level: Levels of IL□6 and TNF□α in plasma and supernatants from kidney tissue homogenates were measured by enzyme□linked immunosorbent assay (ELISA) using Ready-SET-go kits (eBioscience, Inc., San Diego, CA).[4] The detection levels of the kits (standard curve range) are as follows: IL-6 Kit (Catalogue no. 88-7064-22), 4–500 pg/mL; TNF-α Kit (Catalogue no. 88-7324-86), 8–1000 pg/mL. The levels of cytokines in renal tissue were normalized by protein concentration (measured by Bio□Rad detergent compatible protein assay method) (Cat. No. # 500□0112; Bio-Rad, Hercules, CA).

Western-blot Analysis for Renal eNOS expression: The overall procedure to conduct this western-blot analysis was the same as performed earlier.[5, 6] A portion of renal tissue (involving both cortex and medulla) was lysed by radio-immuno-precipitation assay lysis buffer (RIPA buffer; Sigma-aldrich, St. Louis, MO) in the presence of 1 mmol/l PMSF, 1 mmol/l sodium orthovanadate, 1 mmol/l sodium fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin for 20 min on ice. Whole tissue lysates were centrifuged at 10,000 g for 20 min at 4°C, and the supernatants were collected. The protein concentrations were determined by the Bio-Rad protein assay (Cat. No. #500-0112; Bio□Rad, Hercules, CA). Samples of 50 μg were separated by 10% SDS polyacrylamide gel electrophoresis and transferred by electro-blotting onto a nitrocellulose membrane (Hybond; Amersham Biosciences, NJ).[5, 6] 15 μg of protein per lane was loaded in electrophoresis gel. For the immunoassay, the membranes were blocked in 5% (wt/vol) nonfat dry milk in 1 × PBS-0.2% Tween 20 for 1 hour at 4°C with primary antibodies. Polyclonal rabbit anti-eNOS antibody and monoclonal anti-β-actin (Sigma-Aldrich) were used. Immuno-complexes were detected through horseradish peroxidase-conjugated goat anti-mouse antisera (Amersham Biosciences), followed by enhanced chemiluminescence reaction (ECL, Pierce Biotechnology).

Evaluation of Renal Injury: The formalin-fixed paraffin-embedded kidney sections were used for analyzing glomerulosclerosis using Periodic-acid-Schiff (PAS) staining[1, 7, 8] and interstitial fibrosis using Gomori's trichrome staining.[1, 9]

Estimation of Glomerulosclerosis: The extent of glomerulosclerosis was evaluated quantitatively by automatic image analysis of each glomerulus using PAS-stained renal sections (Mass Histology Service, Worcester, MA).[1, 7, 8] Twenty images from each kidney slide with at

least one glomerulus per field were photographed using a Nikon Eclipse 50i microscope equipped with a Nikon DS Camera Head (DS Fi1) and DS camera control unit (DSU2). A dark purple color in the glomerulus was recognized as sclerosis. Percentage area covered by sclerosis in glomeruli in each field was analyzed using the Nikon NIS-Elements software (version 2.34). The percentage data obtained for each of the 20 images was averaged to obtain the percentage area of sclerosis for the entire slide.

Estimation of Interstitial Fibrosis: The extent of interstitial collagen-positive area (fibrosis) was evaluated quantitatively by an automatic image analysis of renal section occupied by interstitial tissue staining positively for collagen in Gomori's trichrome-stained sections.[1, 9] Formalin-fixed paraffin-embedded renal sections were stained with a plasmin stain (chromotrope 2R) and a connective tissue fiber stain (aniline blue) combined in a solution of phosphotungstic acid to which glacial acetic acid had been added. This stained the collagen blue, which indicates fibrosis. Slides were photographed as described above. Percentage area covered by collagen in each field was analyzed using the Nikon NIS-Elements software (version 2.34). The percentage data obtained for each of the 20 images were averaged to obtain the percentage area of fibrosis for the entire slide.

Results

The values for excretory parameters in urine collected from mice on the last day of the experimental period are given in Table S1 in this supplement section. The mean values of $U_{NOx}V$ and $U_{ISO}V$ are also given in Table S1.

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Table S1: Water intake and urinary excretory parameters in different groups of mice on the last day of the experimental period with chronic treatment with angiotensin II (AngII; 400 ng/min/ kg bw)

Groups of mice Parameters	NS-WT + AngII	HS-WT + AngII	NS-IL-10KO + AngII	HS-IL-10KO + AngII
Water intake (mL/24 hr)	2.8±0.5	3.5±1.2	2.2±0.25	4.9±0.74‡
Urine Volume (mL/24 hr)	1.71±0.38	3.01±0.68*	1.38±0.35	3.00±0.53‡
Urine Volume (mL/24 hr)	1.71±0.38	3.01±0.68*	1.38±0.35	3.00±0.53‡
Sodium Excretion (mM/24 hr)	0.12±0.03	0.52±0.03*	0.11±0.04	1.19±0.20$^{†, ‡}$
Potassium Excretion (mM/24 hr)	0.32±0.08	0.25±0.02	0.36±0.06	0.38±0.06
Nitrate/Nitrite Excretion (mM/24 hr)	0.09±0.01	0.18±0.02*	0.56±0.26$^{*, †}$	1.24±0.12$^{*, †, ‡}$
8-isoprostane Excretion (mM/24 hr)	2.33±0.76	4.14±1.67*	9.79±3.66*	9.07±1.31†

NS-WT= Normal salt fed WT mice, HS-WT= High salt fed WT mice, NS-IL-10KO= Normal salt fed IL-10KO mice, HS-IL-10KO= High salt fed IL-10KO mice. *, P<0.05 vs NS-WT; †, P<0.05 vs HS-WT; ‡, P<0.05 vs NS-IL-10KO.