Published in final edited form as:

*Hypertension*. 2020 February ; 75(2): 449–457. doi:10.1161/HYPERTENSIONAHA.119.13822.

# A New Mechanism for the Sex Differences in Salt-Sensitive Hypertension - the Role of Macula Densa NOS1β-Mediated Tubuloglomerular Feedback

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## Abstract

Females are relatively resistant to salt-sensitive hypertension than males, but the mechanisms are not completely elucidated. We recently demonstrated a decisive role of macula densa neuronal nitric oxide synthase β (NOS1β)-mediated tubuloglomerular feedback (TGF) in the long-term control of glomerular filtration rate (GFR), sodium excretion and blood pressure. In the present study, we hypothesized that the macula densa NOS1β-mediated TGF mechanism is different between male and female, thereby contributing to the sexual dimorphism of salt-sensitive hypertension. We used microperfusion, micropuncture, clearance of FITC–inulin and radio telemetry to examine the sex differences in the changes of macula densa NOS1β expression and activity, TGF response, natriuresis and blood pressure after salt loading in wild type and macula densa–specific NOS1 knockout (KO) mice. In wild type mice, a high salt diet induced greater increases in macula densa NOS1β expression and phosphorylation at Ser 1417, greater nitric oxide (NO) generation by the macula densa, and more inhibition in TGF response *in vitro* and *in vivo* in females than males. Additionally, the increases of GFR, urine flow rate and sodium excretion in response to an acute volume expansion were significantly greater in females than males. The blood pressure responses to Angiotensin II plus a high salt diet were significantly less in females than males. In contrast, these sex differences in TGF, natriuretic response and blood pressure were

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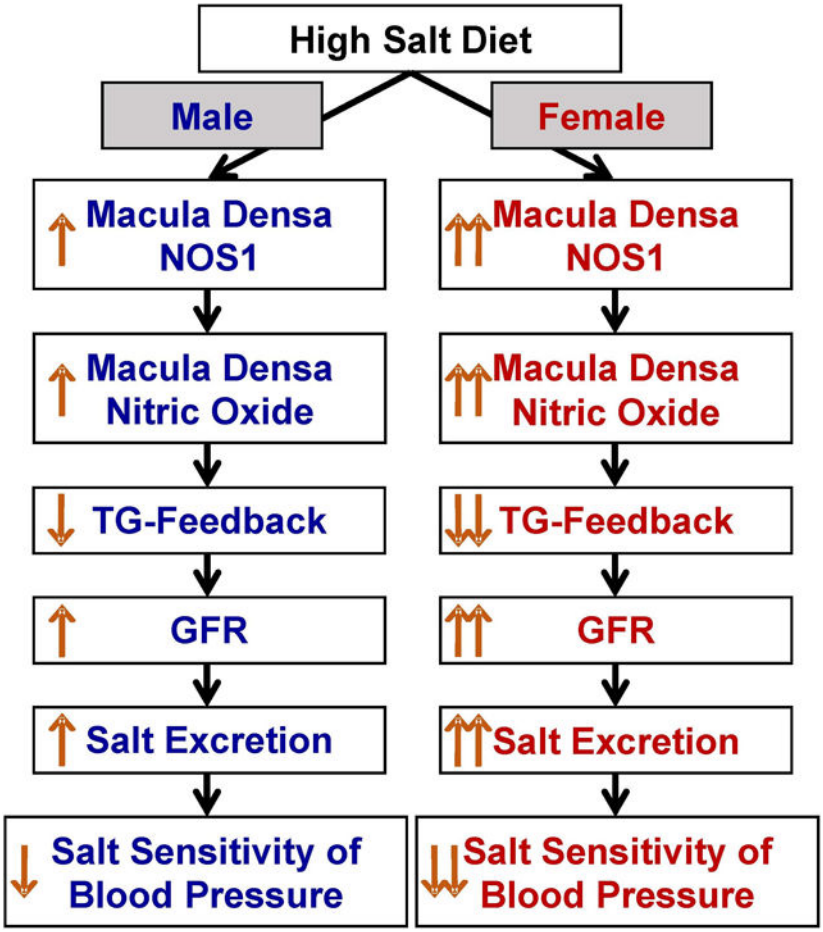
DISCLOSURES

None.

largely diminished in KO mice. In conclusion, macula densa NOS1β-mediated TGF is a novel and important mechanism for the sex differences in salt-sensitive hypertension.

## Graphical Abstract




### Keywords

salt sensitivity hypertension; gender differences; macula densa; neuronal nitric oxide synthase; tubuloglomerular feedback

## INTRODUCTION

Hypertension affects about 30% of American adults and more than half of hypertensive patients are salt-sensitive.[1-4] Before menopause, women have lower risk for most cardiovascular events, including salt-sensitive hypertension, than men.[5-8] Moreover, various animal models of salt-sensitive hypertension also exhibit sex-related differences in blood pressure.[9-13] However, the underlying mechanisms for the sexual dimorphism of salt-sensitive hypertension have not been fully clarified.[5,14]

Increases in glomerular filtration rate (GFR) in response to salt loading play a vital role in the rapid elimination of sodium to maintain salt-water balance and normal blood pressure. Impaired responses of GFR to salt loading have been observed in both humans[15,16] and animal models[17,18] with salt-sensitive hypertension. Tubuloglomerular feedback (TGF) is an important mechanism in control of GFR. It describes a negative feedback between tubule and afferent arteriole (Af-Art), in which an increase in NaCl delivery to the macula densa promotes the release of adenosine and/or ATP that constricts the Af-Art and thereby induces a tonic inhibition of single nephron GFR.[19-21] Nitric oxide (NO) generated by neuronal nitric oxide synthase (NOS1) in the macula densa is a major modulator of TGF response, which buffers or attenuates the TGF response via a cGMP-dependent pathway.[22-25] Recently, several studies from our laboratory have demonstrated that NOS1β is the primary and salt-sensitive splice variant of NOS1 in the macula densa, which contributes to most of the NO generation by the macula densa in response to salt loading;[26-28] mice with the deletion of NOS1 from the macula densa exhibit augmented TGF response, impaired natriuresis and salt-sensitive hypertension.[27] However, all of the above studies were performed in male mice.[26-28] It is unknown whether the macula densa NOS1 β-mediated TGF in response to salt loading is different in females and if this difference in TGF responsiveness contributes to the sexual dimorphism of salt-sensitive hypertension.

In the present study, we hypothesized that a salt loading induces greater increases in macula densa NOS1β expression and activity in females than males; the higher expression and activity levels of macula densa NOS1β in females promote lower TGF response and higher GFR, thereby facilitating sodium excretion and protecting against salt-sensitive hypertension. Microperfusion, micropuncture, renal clearance of FITC–inulin and radio telemetry methods were utilized to examine the sex differences in the changes of macula densa NOS1β expression and phosphorylation, NO generation in the macula densa, TGF responsiveness, natriuretic response and mean arterial pressure (MAP) following a high salt diet in both wild type and macula densa–specific NOS1 knockout (KO) mice.

## METHODS

Data, analytical methods, and study materials are available from the corresponding author on reasonable request. The detail methods are available in the online supplement.

### Animals

C57BL/6 mice (male and female, 12 weeks old) were purchased from Jackson Laboratory. The KO mice (male and female, 12 weeks old) were generated by crossing $NKCC2^{cre}$ mice with $NOS1^{flox/flox}$ mice as we described previously.[27] The number of animals used was indicated in the figure legends. Mice were fed with either high salt diet (4.0% NaCl; Envigo,) or normal salt diet (0.4% NaCl; Envigo) for 2 weeks. All protocols were approved by the Institutional Animal Care and Use Committee at the University of South Florida College of Medicine.

### Statistical analysis

Statistical analysis was performed using Prism 8 (GraphPad Software; San Diego, CA). The effects of interest were tested using t-test, or two-way analysis of variance (ANOVA) followed by Sidak multiple comparisons test when appropriate. Data were presented as a mean ± SEM, and a p-value <0.05 was considered statistically significant.

## RESULTS

### A high salt diet induces greater increases of macula densa NOS1β expression and phosphorylation at Ser 1417 in female than male mice

To determine the sex differences in the effect of salt loading on the expression and phosphorylation levels of NOS1 splice variants in the macula densa, we measured the protein levels of NOS1 and P-NOS1 in the renal cortex,[27,29,30] where most of the NOS1 comes from macula densa cells, in male and female C57BL/6 mice fed either a normal salt diet or a high salt diet (Figure 1A). The NOS1β and P-NOS1β/NOS1β levels in the renal cortex were not significantly different between male and female mice on a normal salt diet. A 2 weeks of high salt diet increased the NOS1β and P-NOS1β/NOS1β levels by 68.7±10.9% and 49.3±12.8% respectively in male mice, and by 246.1±21.2% and 129.1±26.9% respectively in female mice (Figure 1B and 1C). These results demonstrated that the salt loading induced greater increases of macula densa NOS1β expression and phosphorylation at Ser 1417 in female than male mice.

### A high salt diet induces greater NO generation at the macula densa in female than male mice

To determine the sex differences in the effect of salt loading on the NO generation in the macula densa, we measured the TGF-induced NO generation in male and female C57BL/6 mice fed either a normal salt diet or a high salt diet.[27,29] The TGF-induced NO generation by the macula densa was not significantly different between male and female mice on a normal salt diet. A 2 weeks of high salt diet increased the production of NO by 58.1±7.1% (from 117.1±10.7 to 184.8±15.2 units/min) in male mice and by 109.9±8.3% (from 115.1±7.1 to 241.3±9.7 units/min) in female mice when NaCl concentration in tubular perfusate was increased from 10 to 80 mM (Figure 1D). These results demonstrated that the salt loading induced greater NO generation by the macula densa in female than male mice.

### A high salt diet induces more inhibitions of TGF response *in vitro* and *in vivo* in female than male mice

To determine the sex differences in the effect of salt loading on TGF responsiveness, we measured TGF response *in vivo* with micropuncture in male and female C57BL/6 mice fed either a normal salt diet or a high salt diet.[27,29] The TGF response *in vivo* was not significantly different between male and female mice on a normal salt diet. Following a 2 weeks of high salt diet, the TGF response *in vivo*, indicated by $\Delta P_{sf}$, was reduced by 27.4±12.8% (from 5.26±0.69 mmHg to 3.81±0.67 mmHg) in male mice and by 54.1±13.1% (from 5.49±0.78 mmHg to 2.52±0.72 mmHg) in female mice (Figure 2A and 2B).

To eliminate systemic confounding factors such as hormones and sympathetic activity, we also measured TGF response *in vitro* in isolated and double perfused JGAs. The TGF response *in vitro* was not significantly different between male and female mice on a normal salt diet. Following a 2 weeks of high salt diet, the TGF response *in vitro*, indicated by the change in luminal diameter of Af-Art, was decreased by 32.3±12.6% (from 4.3±0.7 μm to 2.9±0.5 μm) in male mice and by 61.1±15.9% (from 4.1±0.8 μm to 1.6±0.7 μm) in female mice (Figure 2C and 2D).

These results demonstrated that the salt loading induced more inhibition of TGF response in female than male mice.

## The sex differences in the effect of salt loading on TGF response are dependent on macula densa NOS1β

To determine the significance of macula densa NOS1β in the sex differences in the effect of salt loading on TGF responsiveness, we measured the changes of TGF response *in vivo* and *in vitro* following a 2 weeks of high salt diet in male and female $NOS1^{flox/flox}$, as well as KO mice.

In $NOS1^{flox/flox}$ strain, TGF responses *in vitro* and *in vivo* were not significantly different between male and female mice on a normal salt diet. In male $NOS1^{flox/flox}$ mice on high salt diet, the TGF response *in vivo* and *in vitro* were reduced by 23.1±9.6% (from 5.22±0.72 to 4.01±0.50 mmHg) and 25.1±13.4% (from 3.75±0.53 to 2.81±0.50 μm) respectively compared with normal salt diet. In female $NOS1^{flox/flox}$ mice on high salt diet, the TGF response *in vivo* and *in vitro* were reduced by 49.3±17.3% (from 5.20±0.76 to 2.63±0.91 mmHg) and 62.4±5.3% (from 4.06±0.72 to 1.53±0.21 μm) respectively compared with normal salt diet. The high salt diet-induced inhibitions of TGF *in vivo* (Figure 3A and 3C) and *in vitro* (Figure 3D and 3F) were greater in female than male $NOS1^{flox/flox}$ mice by 1.6±0.4 μm and 1.36±0.62 mmHg respectively.

In KO strain, TGF response *in vivo* and *in vitro* were not significantly different between male and female mice on a normal salt diet, but significantly enhanced compared with $NOS1^{flox/flox}$ mice. Following a 2 week of high salt diet, neither TGF response *in vivo* (Figure 3B and 3C) nor *in vitro* (Figure 3E and 3F) were significantly changed in KO mice or significantly different between males and females.

These results demonstrated that the sex differences in the effect of salt loading on TGF responsiveness were mediated by macula densa NOS1β in mice.

## Macula densa NOS1β contributes to the sex differences in natriuretic responses to acute volume expansion

To determine the significance of macula densa NOS1β in the sex differences of natriuresis, we measured the changes in GFR, urine flow rate and sodium excretion after an acute volume expansion in male and female $NOS1^{flox/flox}$, as well as KO mice.[27]

In $NOS1^{flox/flox}$ strain, the basal levels of GFR, urine flow rate and sodium excretion were not significantly different between male and female mice. In male $NOS1^{flox/flox}$ mice, an

acute volume expansion increased GFR by 43.4±9.3% (from 637.8±33.3 to 913.2±47.8 μl/min/g KW), urine flow rate by 107.7±28.4% (from 4.9±0.6 to 10.1±1.4 μl/min/g KW), and sodium excretion by 324.7±32.9% (from 0.90±0.08 to 3.81±0.18 μEq/min/g KW) compared with baselines. In female NOS1$^{flox/flox}$ mice, an acute volume expansion increased GFR by 60.3±14.1% (from 635.4±44.6 to 1014.6±56.7 μl/min/g KW), urine flow rate by 128.3±18.7% (from 5.1±0.5 to 11.7±1.5 μl/min/g KW), and sodium excretion by 380.2±59.5% (from 0.98±0.09 to 4.69±0.35 μEq/min/g KW) compared with baselines. The acute volume expansion-induced increases in GFR (Figure 4A), urine flow rate (Figure 4B) and sodium excretion (Figure 4C) were greater in female than male NOS1$^{flox/flox}$ mice by 103.8±22.2 μl/min/g KW, 1.4±0.3 μl/min/g KW and 0.80±0.27 μEq/min/g KW, respectively.

In KO strain, the basal levels of GFR, urine flow rate and sodium excretion were not significantly different between male and female mice and similar to NOS1$^{flox/flox}$ strain. Following an acute volume expansion, the changes in GFR, urinary flow rate and sodium excretion were not significantly different between males and females. The acute volume expansion increased GFR by 24.4±1.9% (from 601.4±28.3 to 748.3±37.1 μl/min/g KW) and 20.5±7.8% (from 627.9±45.7 to 755.1±46.5 μl/min/g KW) (Figure 4A), urine flow rate by 55.4±20.3% (from 4.5±0.4 to 7.1±1.2 μl/min/g KW) and 60.2±27.7% (from 4.5±0.6 to 7.3±1.4 μl/min/g KW) (Figure 4B), and sodium excretion by 162.9±63.3% (from 0.85±0.14 to 2.22±0.53 μEq/min/g KW) and 189.3±80.4% (from 0.89±0.12 μm to 2.37±0.50 μEq/min/g KW) (Figure 4C) compared with baselines in males and females, respectively.

These results demonstrated that the sex differences in natriuretic responses to the acute volume expansion were dependent on macula densa NOS1β in mice.

### Macula densa NOS1β contributes to the sex differences in salt-sensitive hypertension

To determine the significance of macula densa NOS1β in the sex differences of salt-sensitive hypertension, we measured blood pressure responses to a subpressor dose of Ang II plus salt loading in male and female NOS1$^{flox/flox}$, as well as KO mice.[27,31,32]

In NOS1$^{flox/flox}$ strain, the basal levels of MAP were not significantly different between male and female mice on a normal salt diet. Following a 2 weeks of high salt diet along with Ang II infusion, MAP raised by 46.2±5.3% (from 96.9±3.9 mmHg to 141.3±4.7 mmHg) in males and by 17.6±4.8% (from 97.6±3.8 mmHg to 114.7±5.3 mmHg) in females (Figure 5A and 5B) compared with baselines. The increases of blood pressure were significantly less in female than male NOS1$^{flox/flox}$ mice by 27.5±4.4 mmHg.

In KO strain, the basal levels of MAP were not significantly different between male and female mice on a normal salt diet and similar to NOS1$^{flox/flox}$ strain. Following a 2 weeks of high salt diet along with Ang II infusion, MAP raised by 60.9±8.9% (from 98.5±4.2 mmHg to 158.2±4.9 mmHg) in males and by 50.2±7.3% (from 97.6±3.1 mmHg to 146.5±3.2 mmHg) in females (Figure 5A and 5B) compared with baselines. The sex differences in the increases of blood pressure were 10.8±5.5 mmHg in KO mice, which was significantly diminished compared with that in NOS1$^{flox/flox}$ mice (27.5±4.4 mmHg).

These results demonstrated that macula densa NOS1β played a significant role in the sex differences of salt-sensitive hypertension in mice.

## DISCUSSION

The present study demonstrated the significance of macula densa NOS1β-mediated TGF mechanism in the sexual dimorphism of salt-sensitive hypertension. We found that a high salt diet induced higher levels of macula densa NOS1β expression and phosphorylation at Ser 1417, greater NO generation at the macula densa and lower TGF response in female than male wild type mice. Moreover, female mice exhibited a greater natriuretic response to acute volume expansion and lower MAP in response to Ang II infusion plus a high salt diet. In contrast, these sex differences in TGF response, natriuresis and blood pressure were largely diminished in KO mice.

It is well-known that the sexual dimorphism of salt-sensitive hypertension exists in both humans[5-8] and experimental animals.[9-13] However, the underlying mechanisms for the sex differences in salt-sensitive hypertension have not been fully clarified.[5,14] Our recent studies have demonstrated the significance of macula densa NOS1β-mediated TGF mechanism in the long-term control of sodium excretion and blood pressure.[26-28] However, all of these previous studies were undertaken on male mice only without a comparison to females. The role of macula densa NOS1β-mediated TGF mechanism in the sexual dimorphism of salt-sensitive hypertension remains to be determined.

In the present study, we found that the expression level of NOS1β in the renal cortex was similar between male and female C57BL/6 mice on a normal salt diet, while females had a greater increase in NOS1β expression than males on a high salt diet. In addition, it has been reported that the phosphorylation of NOS1 at $Ser^{1417}$ by cAMP-dependent protein kinase (PKA) increased the NOS activity,[33-35] and the phosphorylation at Ser847 by calmodulin-dependent protein kinase (CaM-K) decreased its activity.[36,37] In this study, we found that the phosphorylation level of NOS1β at $Ser^{1417}$ in the renal cortex was not significantly different between male and female C57BL/6 mice on a normal salt diet, while females had a greater upregulation in NOS1β phosphorylation at $Ser^{1417}$ than males on a high salt diet. Furthermore, the TGF-induced NO production in the macula densa was similar between male and female C57BL/6 mice on a normal salt diet, but significantly higher in females than males on a high salt diet. These results demonstrated that the salt loading induced greater increases in both expression and activity of macula densa NOS1 β in female than male mice. Consistent with our findings in C57BL/6 mice, the renal cortical NOS1 expression was shown to be similar between male and female Sprague Dawley rats,[38,39] as well as spontaneously hypertensive rats[40] on a normal salt diet. However, the sex differences in NOS1 expression on a high salt diet were not examined in these studies. It was also reported that manipulation of sex hormones did not significantly alter the renal cortical NOS1 expression or activity in spontaneously hypertensive rats[41] and Fischer-344 rats,[42] suggesting that the sex hormones may not play an essential role in regulating the expression or phosphorylation of NOS1 in the macula densa. Since the PI3K/Akt[43-46] and cAMP/PKA[35,47,48] pathways have been recognized to participate in the regulation of NOS1 expression and phosphorylation, we speculate that the sex differences existing in these

signaling pathways[49-54] might be associated with the sexual dimorphism of the salt loading-induced changes in macula densa NOS1β, which will be examined in the future studies.

NO generated by NOS1β at the macula densa is a key modulator of TGF response, which buffers or attenuates the TGF response via a cGMP-dependent pathway.[22,23,54] The TGF mechanism describes a negative feedback between tubule and Af-Art, where an increase in NaCl delivery to the macula densa promotes the release and formation of ATP and/or adenosine, which then constricts the Af-Art, thereby resulting in a tonic inhibition of single nephron GFR.[55-58] It was reported that TGF response was similar between the age-matched male and female FVB/N mice with a sodium-controlled diet (0.25% NaCl).[59] Nevertheless, the sex differences in the effect of salt loading on TGF response remain unknown. In the present study, we found that the TGF response was not significantly different between male and female C57BL/6 mice on a normal salt diet, which is consistent with the previous finding in FVB/N mice. However, female mice had a greater inhibition in TGF response than male mice on a high salt diet. Recently, our laboratory generated a macula densa-specific NOS1 knockout mouse strain by crossing an $NKCC2^{cre}$ line with a $NOS1^{flox/flox}$ line. This floxed mouse line targets the exon 6 of the NOS1 gene and excision of this exon by Cre recombinase inactivates all splice variants of NOS1.[27,60] Furthermore, because the expression of NOS1 is negligible in the thick ascending limb compared with that in the macula densa,[61,62] this $NKCC2^{cre}/NOS1^{flox/flox}$ line is considered as a macula densa–selective NOS1 knockout model. Thus, in the present study, this KO model was utilized to determine the significance of macula densa NOS1 in the sexual dimorphism of TGF response on a high salt diet. We found that the salt loading-induced inhibition of TGF was significantly greater in female than male $NOS1^{flox/flox}$ mice, but the sex differences were almost eliminated in the KO mice, indicating that the sex differences in the effect of salt loading on TGF responsiveness are mediated by macula densa NOS1β.

The increase of GFR plays a critical role in sodium excretion following salt loading to restore salt-water balance and protect against salt-sensitivity of blood pressure. An acute salt loading via saline infusion or chronic salt loading via high salt diet was reported to increase GFR in healthy normotensive subjects without significant changes in blood pressure.[63-67] In contrast, the salt loading-induced rises of GFR were attenuated or absent in salt-sensitive subjects with significant elevations in blood pressure.[15,16] Similar phenotypes were also observed in experimental animal models. A saline infusion or high salt diet increased GFR in Dahl salt-resistant rats without significant changes in blood pressure. On the contrary, the salt loading-induced rises of GFR were largely attenuated in Dahl salt-sensitive rats with significant increases in blood pressure.[17,18,68,69] Moreover, sexual dimorphism of pressure-natriuresis has been shown in Sprague-Dawley rats, wherein females exhibited a greater sodium excretion than males at a defined renal perfusion pressure.[70,71] However, the mechanisms underlying these sex differences in natriuresis remain unclear. In the present study, to determine the significance of macula densa NOS1β in the sex differences of natriuretic responses to salt loading, we measured the changes in GFR, urine flow rate and sodium excretion in response to an acute volume expansion in male and female KO mice and compared with the $NOS1^{flox/flox}$ mice. We found that the increases of GFR, urine flow rate and sodium excretion in response to the acute volume expansion were significantly greater in female than male $NOS1^{flox/flox}$ mice, whereas these sex differences were almost

eliminated in the KO mice, indicating that the sex differences in natriuretic responses to salt loading are dependent on macula densa NOS1β.

Chronic administration of subpressor dose of Ang II is a well-established and widely-used animal model that mimics many characteristics of salt-sensitive hypertension in humans.[72-75] Many previous studies have demonstrated that females are relatively resistant to subpressor Ang II-induced hypertension compared with males and the sex differences in blood pressure responses are further exaggerated with salt loading.[10,11,59,76] However, the mechanisms for these sex differences in subpressor Ang II-induced salt-sensitive hypertension have not been fully clarified. In the present study, to determine the significance of macula densa NOS1β in the sexual dimorphism of salt-sensitive hypertension, we measured the blood pressure responses to subpressor Ang II along with a high salt diet in male and female $NOS1^{flox/flox}$ mice, as well as KO mice. We found that the increases of blood pressure were nearly 30 mmHg greater in male than female $NOS1^{flox/flox}$ mice, whereas the sex differences in the increases of blood pressure were less than 10 mmHg in the KO mice, which indicates that macula densa NOS1β plays an essential role in the sexual dimorphism of subpressor Ang II-induced salt-sensitive hypertension. In addition, our recent study[27] has demonstrated that high salt diet significantly increased the MAP by about 10 mmHg in the KO mice, but not in the $NOS1^{flox/flox}$ mice. Therefore, it is the true salt-sensitivity of blood pressure. The reason why we added Ang II is to exaggerate the blood pressure response to high salt diet.

Although the macula densa NOS1β-mediated TGF is an important mechanism for the sex differences in salt-sensitive hypertension, we are aware that the knockout of macula densa NOS1 does not completely eliminate the differences in blood pressure responses to subpressor Ang II plus salt loading between male and female mice, and the other factors also contribute to the sexual dimorphism of salt-sensitive hypertension. For example, macula densa angiotensin type 2 receptor ($AT_2R$)-mediated TGF response was reported to be critical in the sex differences in Ang II-induced hypertension[59]. Whereas several other studies demonstrated that Ang II enhanced TGF response and stimulated cytosolic calcium increases in the macula densa cells through only $AT_1$ receptors but not $AT_2$ receptors.[77,78] Many previous studies have also indicated the involvement of sex hormones in the sexual dimorphism of salt-sensitive hypertension. It was reported that the sex differences in the DOC-salt hypertension were completely abolished by gonadectomy.[79,80] Ovariectomy was shown to exacerbate the development of hypertension in females to a comparable level of males in Dahl salt-sensitive rats.[81-83] The pressor responses to Ang II were found to be attenuated in males with castration and augmented in females with ovariectomy.[10] Moreover, a previous study from our laboratory demonstrated that testosterone enhanced TGF response by stimulating superoxide production in macula densa cells via androgen receptors.[84] However, whether the effects of these factors are dependent on macula densa NOS1-mediated TGF response is still elusive and needs to be determined in future studies.

## PERSPECTIVES

This study demonstrated a novel mechanism for the sex differences in salt-sensitive hypertension, wherein a high salt diet induces higher levels of macula densa NOS1β

expression and phosphorylation at Ser 1417 in females than males, which results in a greater inhibition in TGF response, thereby facilitating sodium excretion and protecting against salt-sensitivity in blood pressure. These findings establish a critical role of macula densa NOS1β-mediated TGF mechanism in the sexual dimorphism of salt-sensitive hypertension.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

SOURCES OF FUNDING

This work was supported by American Heart Association Predoctoral Fellowship and American Society of Nephrology Ben J. Lipps Research Fellowship Awards (to J. Zhang.), American Society of Nephrology Ben J. Lipps Research Fellowship Award (to J. Wei.), American Physiological Society STRIDE Summer Research Fellowship Award (to L. Qu.), American Heart Association Career Development Award 18CDA34110441 (to L. Wang.), the National Institutes of Health grants DK099276, HL142814, and HL137987 (to R. Liu.), and National Natural Science Foundation of China 81900347 (to J. Zhu.).

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## NOVELTY AND SIGNIFICANCE

### What Is New?

Using a variety of sophisticated techniques and a novel macula densa-specific NOS1 knockout model, we identified a new mechanism for the sex differences in salt-sensitive hypertension, wherein salt loading induces more upregulation in the expression and activity of macula densa NOS1β in females than males, which results in greater inhibition in tubuloglomerular feedback response, thereby facilitating sodium excretion and protecting against salt-sensitivity in blood pressure.

### What Is Relevant?

Females are relatively resistant to salt-sensitive hypertension compared with males. However, the underlying mechanism for the sexual dimorphism of salt-sensitive hypertension has not been fully elucidated.

## Summary

Macula densa NOS1β-mediated tubuloglomerular feedback is a novel mechanism for the sex differences in salt-sensitive hypertension.

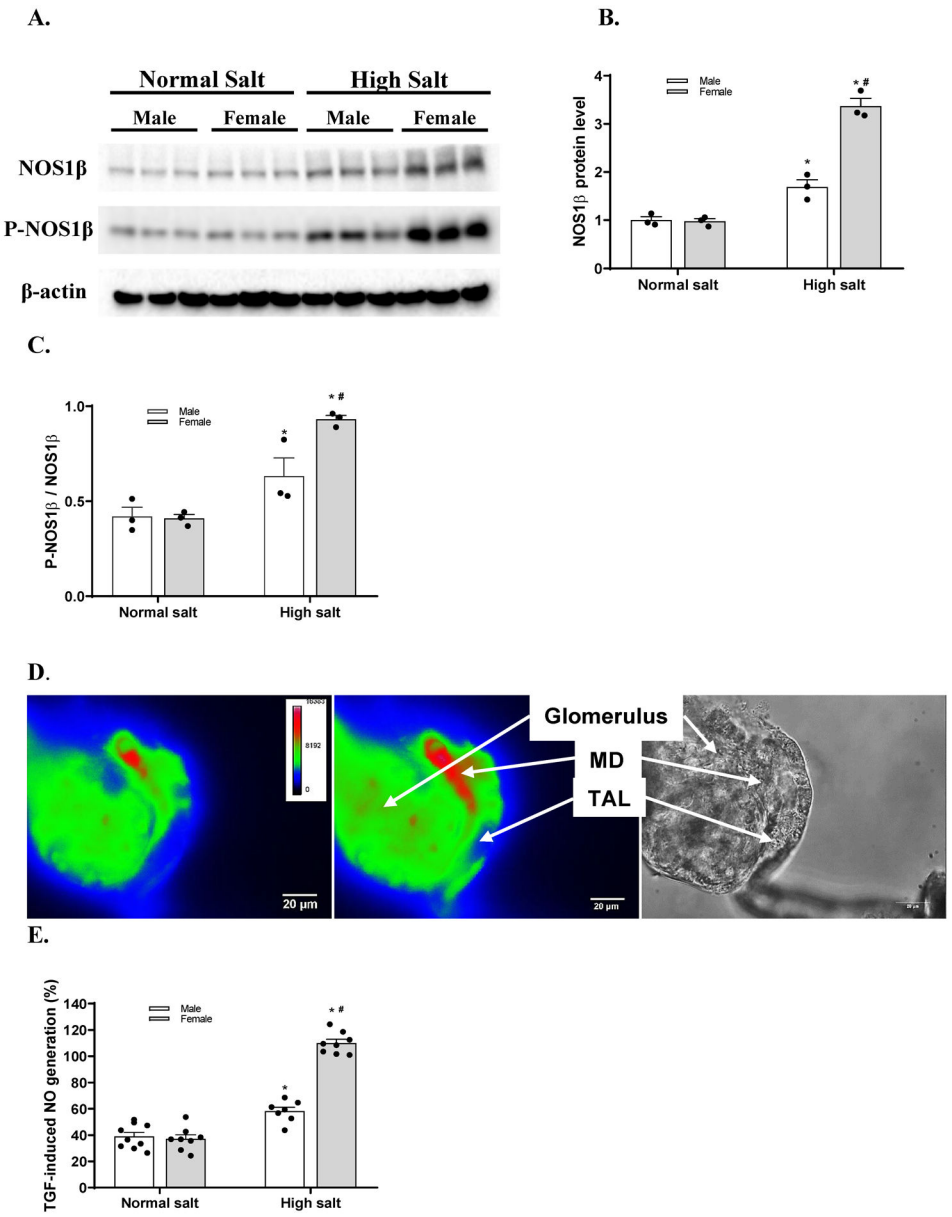


**Figure 1. High salt diet induces greater increases of macula densa NOS1β expression and activity in female mice than male mice.**

(A) The immunoblots of NOS1, P-NOS1 and the loading control of β-actin. The renal cortical levels of NOS1β (B) and P-NOS1β/NOS1β (C) in male and female C57BL/6 mice with normal salt diet or high salt diet. n=3; **P*<0.01 versus normal salt; #*P*<0.01 versus male mice. (D) The NO generation in the macula densa was measured in isolated perfused JGA with DAF-2 DA. The bright field image exhibited the anatomic structure of the perfused JGA. The florescent image of DAF-2 DA loaded JGA showed the NO generation in the macula densa. (E) The TGF-induced NO generation by the macula densa in male and female C57BL/6 mice with normal salt diet or high salt diet. n=7-9; **P*<0.01 versus normal salt; #*P*<0.05 versus male mice. Statistical difference was calculated by two-way ANOVA followed by Sidak multiple comparisons test.

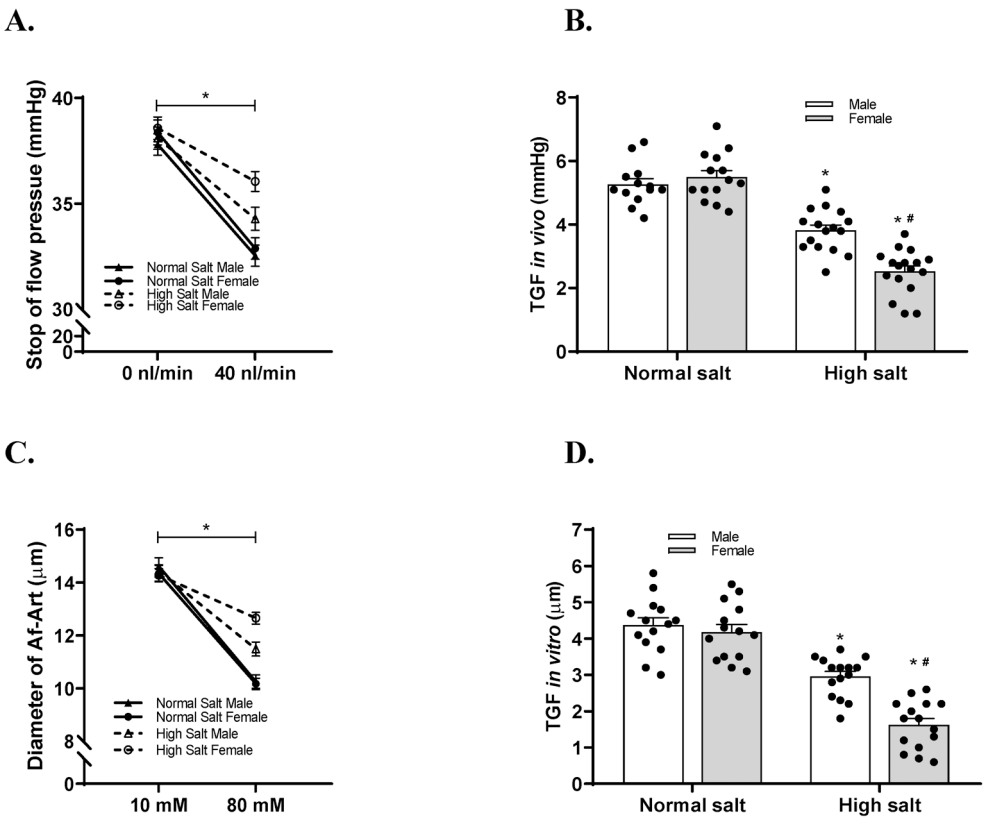


**Figure 2. High salt diet induces more inhibitions in TGF response *in vitro* and *in vivo* in female mice than male mice.**

(A-B) TGF response *in vivo* was indicated by the change of stop flow pressure when the tubular perfusion rate was increased from 0 to 40 nl/min. TGF response *in vivo* was measured and compared in male and female C57BL/6 mice with normal salt diet or high salt diet. n=10-13 tubules/4-5 mice; $*P<0.01$ versus normal salt; $^{\#}P<0.05$ versus male mice. (C and D) TGF response *in vitro* was indicated by the change of Af-Art diameter while the macula densa perfusate was switched from 10 to 80 mM NaCl. TGF response *in vitro* was measured and compared in male and female C57BL/6 mice with normal salt diet or high salt diet. n=11-12; $*P<0.01$ versus normal salt; $^{\#}P<0.05$ versus male mice. Statistical difference was calculated by two-way ANOVA followed by Sidak multiple comparisons test.

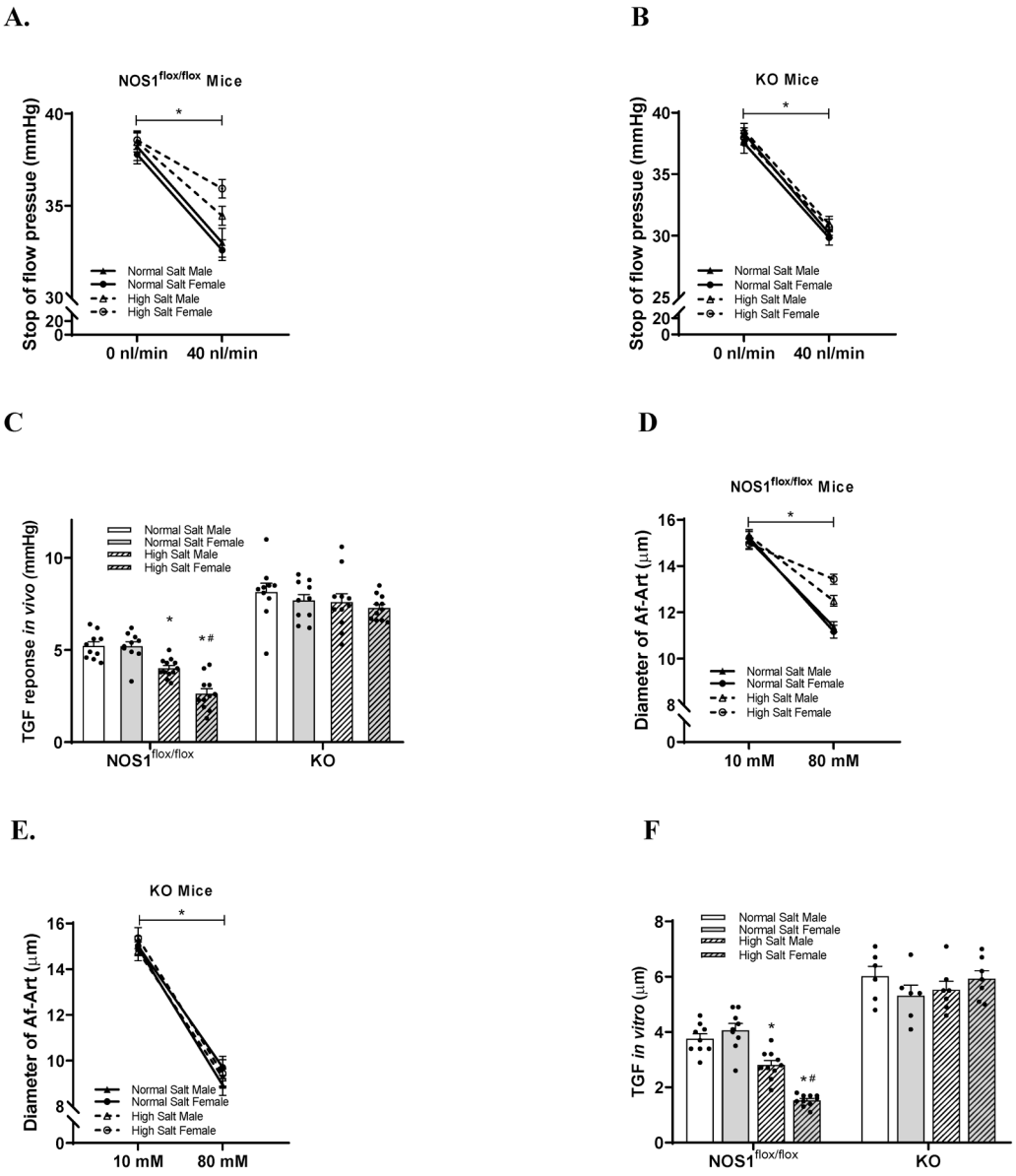


**Figure 3. The sex differences in the effect of salt loading on TGF response are mediated by macula densa NOS1β.**

(A-C) TGF response *in vivo* (n=10-12 tubules/3-5 mice) and (D-F) TGF response *in vitro* (n=6-10) in male and female NOS1$^{flox/flox}$ mice, as well as KO mice with normal salt diet or high salt diet. *$P$<0.01 versus normal salt; $^{\#}P$<0.05 versus male mice. Statistical difference was calculated by two-way ANOVA followed by Sidak multiple comparisons test.

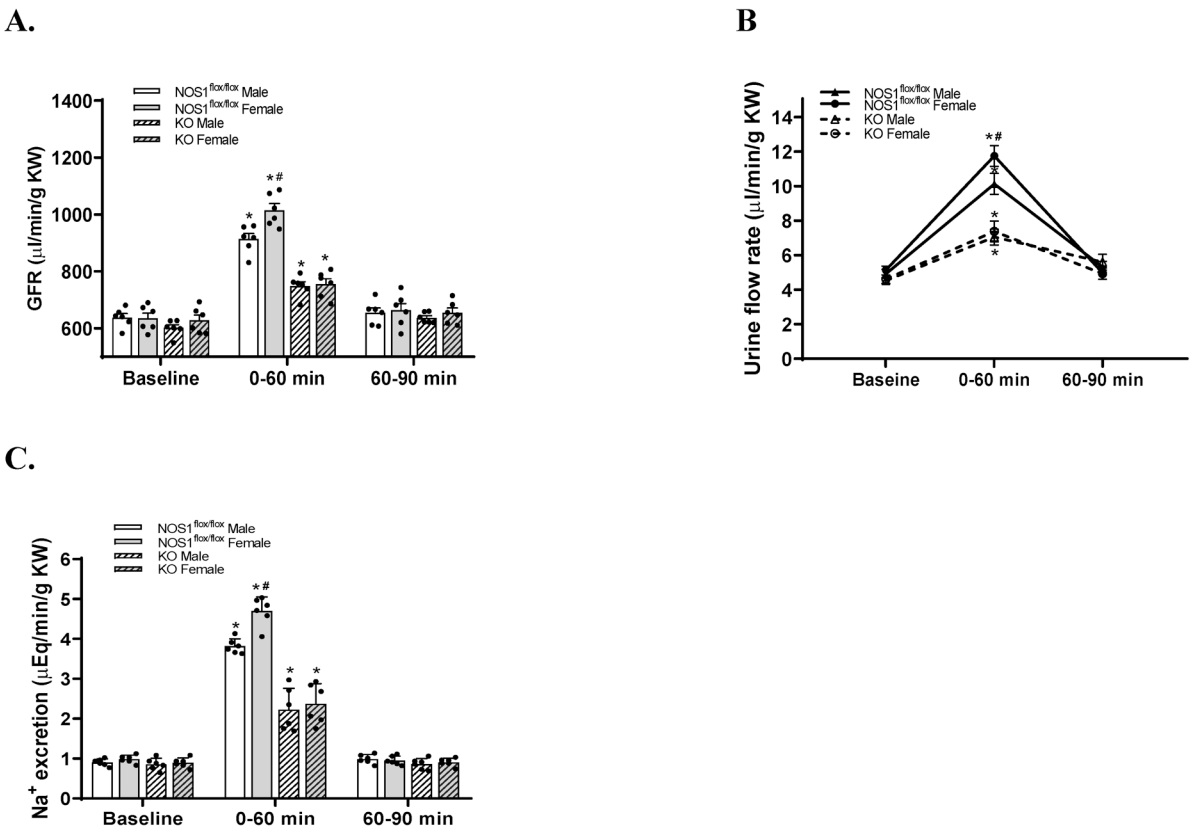


**Figure 4. The sex differences in natriuretic responses to acute volume expansion are dependent on macula densa NOS1β.**

The changes in GFR (A), urine flow rate (B) and sodium excretion (C) during the 0-60 minute period and 60-90 minute period after acute volume expansion in male and female NOS1flox/flox mice, as well as KO mice. n=6; **P*<0.01 versus baseline; #*P*<0.05 versus male mice. Statistical difference was calculated by two-way ANOVA followed by Sidak multiple comparisons test.

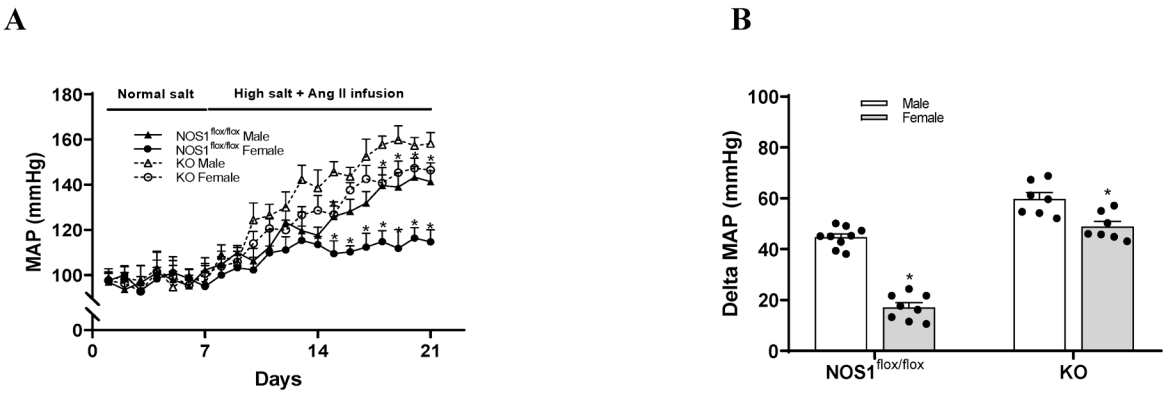


**Figure 5. The sex differences in salt-sensitive hypertension are dependent on macula densa NOS1β.**

(A and B) The MAP responses to sub-pressor Ang II infusion plus high salt diet in male and female NOS1$^{flox/flox}$ mice, as well as NOS1KO mice. n=7-9; *$P$<0.01 versus normal salt; $^{\#}P$<0.05 versus female mice. Statistical difference was calculated by two-way ANOVA followed by Sidak multiple comparisons test.