Salt-Sensitive Hypertension and Inducible Nitric Oxide Synthase

Form-Function Dichotomy of a Coding Region Mutation, *Mutatis Mutandis*

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The earliest recorded association of dietary salt with blood pressure was reported by Huang Ti Nei Ching Su Wein (ca. 1500 B.C.), who noted that "if large amounts of salt are taken, the pulse will stiffen or harden" (page 82). In 1904, Ambard and Beauchard first demonstrated rigorously that dietary salt can increase blood pressure in normal volunteers. Throughout the 20th century, the relationship between dietary salt and essential hypertension has been debated; however, evolving epidemiological data and recent evidence in nonhuman primates, coupled with a growing understanding of the molecular determinants of sodium handling, clearly support the view that certain individuals defined as salt-sensitive have a propensity to hypertension when exposed to dietary salt. It is estimated that 50% to 75% of hypertensives are salt-sensitive.

In the 1960s, Dahl first showed that the hypertensive response to salt was genetically determined. He developed two strains of inbred rat, a salt-sensitive (Dahl S) strain in which blood pressure increased in response to dietary salt and a salt-resistant (Dahl R) strain in which blood pressure did not increase in response to dietary salt. These rats have served as the basis for many genetic studies of salt-sensitive hypertension and its end-organ sequelae for many years; yet, until recently, the molecular basis for this genetic sensitivity remained unknown.

In this issue of *Circulation Research*, Ying et al. provide functional genetic evidence for the molecular basis of salt sensitivity in this animal model. They identified a mutation in the coding sequence of the inducible nitric oxide synthase gene (*NOS2*) that leads to a gene product that is more rapidly cleaved by the proteasome than the wild-type gene product. Functional studies of the expressed protein in conjunction with studies of protein stability were used to support the authors’ conclusion that a lower level of normally functioning inducible nitric oxide synthase accounts for salt-sensitive hypertension in this model.

To understand the basis for this study and the interpretation of the results, we must first review the role of nitric oxide (NO) in the renal handling of salt. The normal mammalian response to dietary salt in normotensive individuals is to increase renal NO production, leading to a nitrite-uresis and a natriuresis; these changes are accompanied by an increase in urinary cGMP and renal vasodilation, indicating that the increased NO is bioactive. In salt-sensitive individuals, this response is blunted or absent, can be mimicked by selective inhibitors of inducible nitric oxide synthase, and can be reversed by L-arginine. Moreover, Deng and Rapp originally reported in a genetic linkage study that the *NOS2* gene cosegregates with blood pressure in the Dahl S rat, a finding that was not subsequently confirmed in rats or humans. These conflicting results led Deng to conclude that *NOS2* was not itself the primary genetic determinant of salt-sensitive hypertension but a gene that modified the response to dietary salt.

All three isoforms of nitric oxide synthase are found in the kidney. Neuronal nitric oxide synthase is found principally in the macula densa and the inner medullary collecting duct; endothelial nitric oxide synthase is expressed primarily in endothelial cells of the renal vasculature; and inducible nitric oxide synthase is constitutively expressed as two structurally distinct forms, a macrophage form and a vascular smooth muscle form, with the former found most abundantly in the medullary thick ascending limb and the latter in renal vascular smooth muscle cells. In light of its location in the macula densa, neuronal nitric oxide synthase is likely to be involved in tubuloglomerular feedback; however, dietary salt has also been shown to increase neuronal nitric oxide synthase expression acutely in the inner medullary collecting duct of the rat, suggesting that NO derived from this enzymatic source may also be responsible, at least in part, for the adaptive renal response to salt loading. In support of this hypothesis, selective infusion of antisense phosphorothioate oligodeoxynucleotides against *NOS1* mRNA into the renal medulla or the intramedullary administration of a selective neuronal nitric oxide synthase inhibitor has been shown to produce salt-dependent hypertension. In contrast to these acute responses of the neuronal isoform to salt loading, chronic administration of salt leads to reduced expression of neuronal nitric oxide synthase, bringing into question its role in the normal, chronic renal homeostatic response to dietary salt.

Owing to its location in vascular endothelial cells, endothelial nitric oxide synthase is likely to modulate glomerular resistance, renal blood flow, and glomerular filtration. The expression of this isoform normally increases in response to...
acutely and chronically acutely and chronic salt loading, principally in the renal cortex. The regional renal concentration of NO is highest in the medulla, which is best explained by this site being the principal site of inducible nitric oxide synthase expression. This isoform is a high-capacity enzyme for the synthesis of NO, which can, in turn, inhibit the Na\(^+\)-K\(^+\) ATPase\(^{22}\) and regulate sodium reabsorption in the medullary thick ascending limb.\(^ {24}\) Importantly, medullary expression and activity of inducible nitric oxide synthase normally increase in response to acute and chronic salt loading\(^ {25}\); however, its expression is decreased in response to dietary salt in Dahl S rats,\(^ {26}\) and targeted disruption of the \(\text{NOS}2\) gene in mice leads to salt-sensitive hypertension,\(^ {27}\) suggesting that this isoform is of central importance in the homeostatic renovascular response to dietary salt.

Recently, Chen et al\(^ {28}\) identified a point mutation in the \(\text{NOS2}\) gene in Dahl S rats that led to the substitution of proline for serine at position 714. In their report, Ying et al\(^ {7}\) attempt to determine the functional relevance of this mutation. Using a transient transfection system, they demonstrate that the mutant enzyme generates less nitrogen oxides than does the wild-type enzyme. This reduced activity was associated with a decrease in the half-life of the protein but no change in the steady-state levels of \(\text{NOS2}\) mRNA compared with the wild-type enzyme. Inhibition of proteasomal activity restored the half-life of the mutant enzyme to that of wild type. Purified mutant enzyme had a catalytic efficiency \((k_m/K_m)\) that was identical to that of purified wild-type enzyme. These results led the investigators to conclude that enhanced posttranslational proteasomal degradation of the mutant enzyme, rather than an intrinsic abnormality in enzyme function, accounts for the comparative decrease in NO production in response to dietary salt in Dahl S animals.

At first blush, these conclusions seem sound and well supported by the primary data. On closer scrutiny, however, there are inconsistencies in the results that weaken this interpretation and raise questions about alternative experiments that might have been performed. First, the protein expression data are inconsistent with the activity data measured in intact cells by nitrite accumulation. The Western blots show \(\approx 50\%\) less mutant protein than wild-type protein in the steady state; yet, at 100 \(\mu\text{mol/L}\) of \(l\)-arginine, the rate of nitrite accumulation is only \(\approx 30\%\) less for the mutant than wild-type enzyme. At the maximal \(l\)-arginine concentration used—200 \(\mu\text{mol/L}\)—the rate of nitrite accumulation does show an approximate 50% reduction for the mutant enzyme compared with the wild-type enzyme. However, at this substrate concentration, the rate has not yet reached a plateau for the wild-type enzyme but has done so for the mutant enzyme. These results suggest that higher concentrations of \(l\)-arginine would likely be associated with a greater difference between mutant and wild-type enzymes. This variability in the ratio of activities of the mutant and wild-type enzymes as a function of substrate concentration cannot be explained solely by the reduction in protein expression.

That \(l\)-arginine restores the normal renal response to dietary salt is inconsistent with the protein half-life data as well as the kinetic data. One explanation for the benefit of \(l\)-arginine is that it may provide more substrate for endothelial nitric oxide synthase; however, the renal expression of this isoform is significantly decreased in Dahl S rats given dietary salt.\(^ {26}\) Moreover, \(l\)-arginine should not restore normal NO production by inducible nitric oxide synthase if the kinetic data show no appreciable increase in the rate of nitrite production at concentrations of \(l\)-arginine above 100 \(\mu\text{mol/L}\) for the mutant enzyme. Alternatively, \(l\)-arginine actions distinct from its serving as a nitric oxide synthase substrate, such as its antioxidant potential, may also explain this benefit.\(^ {29}\)

Studies of the activities of purified mutant and wild-type enzymes were conducted using saturating concentrations of cofactors. In that the \(S714P\) mutation is located between flavin binding sites, Ying et al\(^ {7}\) should have measured enzyme activities over a range of flavin mononucleotide and flavin adenine dinucleotide concentrations at saturating concentrations of substrate, as well as cofactor affinities for the mutant and wild-type enzymes, before concluding that the intrinsic activities of the enzymes are identical. The local concentration of flavins is likely to be significantly lower intracellularly than the saturating concentrations used in the purified enzyme assays,\(^ {30}\) supporting the relevance of this important control.

Enhanced generation of reactive oxygen species plays a critical role in the genesis of hypertension and its end-organ sequelae.\(^ {31}\) These reactive oxygen species, especially superoxide anion and lipid peroxyl radicals, can inactivate nitric oxide by forming peroxynitrites. Recent data from our group suggest that dietary salt increases the production of reactive oxygen species in plasma and urine of Dahl S rats, that this effect is associated with a decrease in renal function and urinary cGMP levels as blood pressure rises, and that these effects can be completely prevented by the administration of \(\alpha\)-tocopherol.\(^ {32,33}\) These interesting results suggest that there is, in fact, nothing wrong whatsoever with the response of inducible nitric oxide synthase to dietary salt or with the intrinsic activity or expression of the enzyme itself; rather, an increase in reactive oxygen species cannot be adequately accommodated in the Dahl S rat, thereby impairing the bioactivity of NO generated by nitric oxide synthase. Of course, in this environment of enhanced oxidant stress, the \(S714P\) mutant enzyme could be more susceptible to oxidative modification than the wild-type enzyme, which could, in turn, lead to increased protein turnover. This possibility should, therefore, also have been examined in an in vitro system designed to assess the susceptibility of the mutant isoform to oxidative inactivation and degradation as a means to reconcile these two seemingly disparate mechanisms.

Other controls that would have provided more support for the authors’ conclusions include quantifying the (presumptive) differences in ubiquitination of the mutant and wild-type enzymes and measuring the rate of nitrite production in cells treated with the proteasome inhibitor. These determinations, if consistent with the data presented in this report, would better substantiate the conclusions.

Of course, the optimal experiment needed to prove the hypothesis definitively is one in which expression of the wild-type enzyme in Dahl S rats eliminates its salt sensitivity.
Short of this crucial experiment, and in light of the inconsistent genetic linkage studies as well as the absence of evidence for a homologous mutation in salt-sensitive humans, one can, at best, conclude that this mutant inducible nitric oxide synthase gene is either a disease-modifying gene that cosegregates with another critical allele(s) or that this mutation is the principal genetic determinant of salt-sensitive hypertension—but only in this rat strain. Clearly, additional studies are required to place the role of inducible nitric oxide synthase in the pathobiology of salt-sensitive hypertension in the proper perspective. The study by Ying et al7 adds one more piece to this most interesting puzzle.

References
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