Letters to the Editor

Concordant Genetic and Physiological Data Are Required for Candidate Genes

To the Editor:

In a recent article, Chen et al.1 present additional data on differences in the biochemical and physiological properties of the inducible form of nitric oxide synthase (NOS2) between Dahl salt-sensitive (S) and Dahl salt-resistant (R) rats. The data establish that the enzyme responds differently to increasing concentrations of L-arginine with regard to nitrite production, such that the S enzyme is less active at a given substrate concentration than the R enzyme. This makes sense as a potential mechanism for hypertension in S rats, which would be expected to produce less NO. Also, there was a single base change in the gene for NOS2 that results in an amino acid substitution and a DNA restriction fragment length polymorphism (RFLP); this RFLP appeared to be unique to S rats among the several strains tested.

The question arises as to the causative relationship between the above interesting changes and blood pressure differences between S and R rats. In the last paragraph of their article, Chen et al provide a rather misguided attempt to reconcile their biochemical/physiological data with our genetic data.

Chen et al quote our early genetic linkage data2 using F2 populations derived by crossing S with Wistar-Kyoto rats (WKY) or crossing S with the Milan normotensive strain (MNS) that localizes a blood pressure quantitative trait locus (BP QTL) to a region of rat chromosome 10. This region does include NOS2 and did initially support NOS2 as a locus causing inherited differences in blood pressure. Subsequent data using congenic strains, however, definitively ruled out NOS2 as a locus causing inherited differences in blood pressure between S and MNS.3 Congenic strains substituting a relatively large region of chromosome including NOS2 from MNS into the S background showed a marked reduction in blood pressure compared with S. However, when a congenic substrain was constructed to include only the MNS-derived region containing NOS2 and small flanking chromosomal segments on either side of NOS2, the blood pressure effect was lost, effectively eliminating NOS2 as a candidate for the QTL between S and MNS and localizing the QTL to an adjacent chromosomal segment. This work was published in April 1997 but was not quoted by Chen et al.

Chen et al correctly quoted our result that NOS2 in an F2 population derived from S and R rats does not cosegregate with blood pressure. The most logical reason that NOS2 cosegregates with blood pressure in an F2 population (S×MNS) and not in an F2 population (S×R) is that the different normotensive strains introduce different alleles at the BP QTL and/or different genetic backgrounds.4 If S and R are not polymorphic for the QTL on chromosome 10, it follows that the physiological/biochemical differences observed by Chen et al in NOS2 between S and R have nothing to do with inherited differences in blood pressure. Chen et al reject this obvious and logical explanation in favor of an explanation based on spurious logic. They state that “the unique presence of the NOS2 RFLP in the S strain, but not in other strains of rat including WKY, suggests that the normotensive strain that was used to produce the F2 cross with S rats was not the factor that produced different results with respect to cosegregation of S NOS2 with blood pressure.” Basically, they are stating that strain associations (in this case, the NOS2 RFLP association with blood pressure across several strains) are more powerful than linkage analysis on hundreds of rats. Such strain comparisons for drawing cause and effect conclusions between traits and blood pressure have plagued the hypertension community for decades and were shown to be spurious 15 years ago.5

Unfortunately, enzymatic differences and an RFLP in NOS2 that make biological sense as regulators of gene expression at blood pressure by themselves prove absolutely nothing about NOS2 as a BP QTL without genetic evidence to corroborate the idea. So far, the genetic evidence is solidly against that proposition. It may be useful to realize that 1 in every 1500 bases is likely to be different between the inbred S and R strains.6 Because the mammalian genome is 3×109 bases in size, there will be ~2×106 such differences, some of which are bound to cause interesting biological differences that have nothing to do with blood pressure. Systematic genetic analysis is the only way to sort the wheat from the chaff, and the genetic data cannot be dismissed as was done by Chen et al.

In summary, there are nice physiological/biochemical differences between S and R in the NOS2 enzyme, but these are not supported as a causative factor in inherited hypertension by genetic linkage analysis in S and R rats. In a comparison of S and MNS, there is a major BP QTL near NOS2, but NOS2 has been eliminated as the QTL in that strain comparison by use of congenic strains.

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Response

We are gratified that Dr Rapp has taken an interest in our recent work1 and welcome the opportunity to further discuss the issue of the potential relationship between NOS2 and salt-sensitive hypertension. Dr Rapp has truly been instrumental in development of a fascinating and important model of salt-sensitive hypertension and hypertensive nephrosclerosis: the Dahl/Rapp salt-sensitive (S) rat.2 He is now in the process of using genetic tools to define the genes that are involved in hypertension in this model. Unfortunately, at present no gene has been shown to play a definitive role in salt-sensitive hypertension.

Our laboratory has had an active interest in hypertension and the attendant renal failure that develops in the Dahl/Rapp rat. These rats have a defect in NO production that manifests as salt-sensitive hypertension. This defect in NO production is overcome by administration of l-arginine, the amino acid precursor of NO. L-Arginine simultaneously increases NO produc-
tion and lowers blood pressure. This sensitivity to L-arginine is a unique feature of this genetic model of hypertension. Interestingly, there does not appear to be a defect in L-arginine production and metabolism in S rats. We focused on NOS2 because of early studies that suggested involvement of this enzyme in salt-sensitive hypertension. Indeed, studies in normotensive rats also supported the concept that NOS2 is upregulated by dietary salt. Our most recent publication demonstrated a defect in NO production by NOS2 in vascular smooth muscle cells (VSMCs) from S rats compared with VSMCs from salt-resistant (R) as well as Sprague-Dawley rats. This defect in NO production was repaired by addition of L-arginine to the medium in increasing doses. Using a standard approach, we confirmed a point mutation in S NOS2. This created an RFLP that was unique to this strain. We consider these data to be consistent with the possibility that NOS2 is involved in the pathogenesis of salt-sensitive hypertension in S rats, although as detailed by Rapp’s letter, we have not proven a cause and effect relationship. Our findings do not negate the possibility that other genes, such as the β2 subunit of soluble guanylyl cyclase, may also be important in this model of hypertension.

The problem pointed out by Rapp is that these abundant physiological and biochemical observations are in apparent disagreement with the genetic approach used by his laboratory. Reconciling these data may be difficult. In our view, the genetic approach is a very powerful tool that provides definitive results when used properly. However, the “Achilles heel” of this technique is phenotyping. If the phenotype is simple—green eyes versus blue or brown eyes—phenotyping is reliable. If the phenotype is a complex trait such as hypertension, the observations form a gaussian distribution, and distinctions may not be as clear-cut. An accurate tool to determine blood pressure is essential. In the study quoted by Rapp, the phenotype was determined using tail-cuff blood pressures in awake, restrained rats. In the initial studies that validate this technique, the authors appropriately pointed out most of the pitfalls of this approach. They demonstrated that this method did not reliably measure diastolic and mean blood pressure in awake rats. Furthermore, blood pressure determination was made inaccurate by conditions that frightened the animals, such as placing them in restraints during the first week of study or sounds in the environment. The rats were not warmed in that study, but the room was kept at >27°C. In Rapp’s study, the rats apparently were not trained and were restrained and warmed to 28°C; more than 1 operator performed the studies. Presumably, only the systolic blood pressure was used in the analyses, given the known problem with tail-cuff blood pressures, but almost certainly these animals were stressed during determination of blood pressure. Rapp’s observation that NOS2 did not correlate with stress-induced systolic hypertension is not surprising.

In addition to these technical considerations, the condition of the animal can affect blood pressure. Our laboratory demonstrated an exquisite sensitivity of S rat kidneys to dietary salt; renal failure from hypertensive nephrosclerosis was a consistent finding by 3 weeks on a high salt diet and contributed to hypertension in this model. The rats in Rapp’s study were maintained on 2% NaCl chow for 24 days. The role of altered renal function in determination of blood pressures in their animals is unknown but may be an important variable. Finally, response of blood pressure to L-arginine was not demonstrated in any of their strains. In our view, phenotyping, which is the most important step in identifying the gene causing salt-sensitive hypertension in S rats, was performed inadequately. Attempts to collaborate formally with Rapp to understand the differences in results from our laboratories have been unsuccessful to date.

Another potential concern is the lack of demonstration of functional NOS2 in the congenic strains used by Rapp’s laboratory. The genetic context in which a gene is expressed may also vary the functional expression of that gene. A collection of genes, not NOS2 alone, was introgressed into the congenic strains in Rapp’s study. Interaction among these genes is another potential variable that should be considered in these experiments.

More genetic-based approaches that profess to show the gene or genes that control blood pressure in hypertensive strains or “rule out” their involvement will appear in the literature. It is vital that we not overlook the need to carefully document the phenotype of the animals under study. An accurate and reproducible means by which blood pressure is determined cannot be overemphasized.

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