We have studied genetic variation in salt sensitivity in our animal models, the spontaneously hypertensive rat (SHR) (1,2), and particularly the salt-sensitive sub-strain, stroke-prone SHR (SHRSP) (3,4).

The importance of nutrition in the pathogenesis and prevention of stroke and other cardiovascular diseases was supported by our epidemiological studies which suggest that there is ethnic or genetic variation in salt sensitivity and, by our observation, that stroke could be prevented by diet manipulation even in the SHRSP, which is genetically strongly predisposed to stroke (5,6).

EPIDEMIOLOGICAL ANALYSIS OF DIET AND HYPERTENSION

Our epidemiological survey, the so-called WHO-CARDIAC Study (WHO-Coordinated Cardiovascular Diseases and Alimentary Comparison Study) (7,8), is a multicenter epidemiological study that was designed to investigate the relationship of biological markers of diet to hypertension in a core study and to cardiovascular disease mortality in the complete study. The study has lasted for more than 12 years and is now engaged in the final analyses.

Fifty-seven centers in 25 countries have, so far, participated in the CARDIAC study. Among them, over 20 populations were examined by a Japanese study team in cooperation with local centers. The target population examined for the core CARDIAC study were 100 men and 100 women ranging in age 48 to 56 years, randomly selected from each population. Over 10,000 participants in total were enrolled in the study.

A characteristic of the CARDIAC study was the standardized measurement of blood pressure by an automated blood pressure measurement system (9). This system was used worldwide in the CARDIAC study for objective blood pressure measurement among different populations. These included the Masai who use no salt at all.
and had a low urinary sodium excretion of around 2.5 g calculated as NaCl, and practically no hypertension in the age range of the CARDIAC survey.

Another characteristic of the study was the use of biological markers for 24-hr urine and blood samples for objective nutritional estimations. To make it less difficult to collect 24-hr urine, we developed a new device—the so-called aliquot cup (10)—which enabled us to collect 24-hr urine samples successfully even in developing countries like Tibet where the population drinks salted tea and has an excessive salt intake of about 18 g daily with frequent severe hypertension.

DIFFERENT SALT SENSITIVITY SUGGESTED BY EPIDEMIOLOGICAL SURVEY

The results of CARDIAC study suggest the importance of salt sensitivity. This worldwide cross-sectional analysis showed for the first time that, in different populations, the means of both systolic and diastolic blood pressure (SBP, DBP) were positively related to 24-hr urinary sodium excretion, indicating that excess salt intake is a risk factor for hypertension (5,6,8).

Another risk factor for hypertension is obesity: body mass index (BMI) was positively correlated with both SBP and DBP. In contrast, urinary magnesium excretion was inversely related to SBP and DBP (8), indicating a beneficial effect of magnesium intake on blood pressure, as shown by our experiments in SHRSP (5,6,11).

The CARDIAC study also showed that stroke mortality was positively correlated with urinary sodium excretion and also with the sodium to potassium ratio, indicating an adverse effect of sodium and a beneficial effect of potassium on stroke (12).

In contrast, stroke mortality was inversely correlated with serum cholesterol concentrations (12); this might be a reflection of the dietary intake of animal protein, a beneficial nutrient for stroke prevention as proven experimentally in SHRSP (13). Other epidemiological studies in Japan and the U.S. have also confirmed the similar inverse relationship between serum cholesterol and stroke, especially hemorrhagic stroke mortality.

The CARDIAC study, thus, showed that excess salt intake is a major risk factor for hypertension and stroke. However, detailed observation of the association between blood pressure and urinary sodium excretion shows that the relationship between salt and hypertension is not straightforward. Masai people and the Chinese in Guanzhou, who excreted less than 6 g of salt per day, had low blood pressure due to their low sodium intake. However, in black people in Dar Es Salaam, Tanzania, blood pressure was higher than would have been expected from their relatively low urinary sodium excretion of 5 g per day and their blood pressure was similar to that of Japanese and Brazilians who appeared to be consuming twice as much salt a day as judged by their high urinary sodium excretion around 12 g. These data indicate there may be genetic differences in salt sensitivity in hypertension (14).
CLINICAL EXPERIMENT ON ETHNIC DIFFERENCES IN SALT SENSITIVITY

To investigate this question, we conducted clinical experiments in male volunteers—13 Tanzanians, 11 Brazilians, and 6 Japanese—after approval of the protocol by the ethics committees. Volunteers, who were healthy young men averaging around 24 years of age, were given a basal diet containing 3 g of NaCl for the first 7-day low-salt period, and then over the following 7 days, they were given high-salt diets containing 21 g NaCl for Tanzanians and Brazilians and 25 g NaCl for Japanese (we had noted that the Japanese were rather salt-resistant in our previous experiment).

Blood pressure measured objectively by the automated measurement system (9), stabilized on the fifth day of the low-salt period and was compared with the values obtained on the fifth day of the high-salt period. A significant rise in SBP was observed only in Tanzanian blacks 5 days after the salt loading, and not in Brazilians and Japanese, indicating that there are ethnic differences in salt sensitivity in hypertension (15).

EPIDEMIOLOGICAL ANALYSIS OF ETHNICITY IN SALT SENSITIVITY

To study a possible ethnic difference in salt sensitivity, we applied multiple regression analysis to within-center analysis of various factors related to blood pressure such as BMI and urinary electrolytes, after adjustment for other confounding variables such as sex and age in 47 centers (16). BMI showed a positive correlation to SBP in most centers (43 out of 47; 91.5%) and in 36%, the correlations were significant. However, urinary sodium excretion showed both positive and negative correlations to SBP (in 27 and 20 centers, respectively) and significant positive and negative associations were noted only in 1 center for each. This relatively slight association may be due to the fact that one 24-hr urine sample cannot be regarded as a representative sample for each individual because of the large daily variation of urinary sodium excretion.

To show the strength of the association between sodium and blood pressure, blood pressure slopes on 24-hr urinary sodium excretion were obtained as partial regression coefficients in a multiple linear regression model after adjusting for other confounding factors. Pooled regression coefficients of the 53 populations that were obtained by weighting the inverse of their partial regression coefficient in the multiple linear regression model, showed a significant independent association of sodium with blood pressure. The slopes of blood pressure on 24-hr sodium excretion (mm Hg/mmol) were calculated for SBP and DBP, respectively: SBP = -0.2460 to +0.1430; DBP = -0.2820 to +0.6100. The slopes of DBP with Na distributed more widely than those with SBP (17).

Fifty-three populations could be classified into six groups with respect to ethnicity...
and geographic location. Ten Japanese populations including three migrant populations in Hawaii and Brazil showed relatively clear negative associations. The Chinese populations and six Mediterranean groups had no strong tendency. Seventeen European, North American, and Oceanic Caucasian groups had an almost symmetrical distribution ranging from $-0.1$ to $+0.1$, excluding one exceptionally strong positive slope in Sofia, Bulgaria ($+0.310$). Central and South American groups and blacks in Africa and North America had a weak negative tendency except for a weak positive association for Dar Es Salaam, Tanzania ($+0.088$).

We extended our analysis by using the simple linear regression model to study relationships between dietary factors and blood pressure slope on sodium excretion among seven Japanese populations that had a wide distribution of blood pressure slope on sodium. Among various biological markers of dietary factors, urinary Na/K ratio had clear negative association with blood pressure slope on sodium (SBP: $B = -0.07$, $r = -0.655$, $p = 0.110$; DBP: $B = -0.08$, $r = -0.893$, $p = 0.007$). This may imply that those who have hypertension should be advised to reduce their salt intake and such public health education is more common in the areas where people's intake of salt is high.

Beyond this possible bias, these data may indicate that the association of blood pressure with sodium could be influenced by sodium intake level, especially the Na/K ratio, as well as by other nutritional factors and related physiological adaptations, as some of our previous experiments in animal models suggested.

Thus, there are several factors that might influence the association between sodium and blood pressure. These include genetics, ethnicity, salt sensitivity (which may be affected by sodium intake), potassium, and other nutritional factors such as calcium, magnesium, dietary fiber, protein, and alcohol. There are obviously limitations to the analysis of genetic variation in salt sensitivity if the analysis is only based on limited epidemiological data.

**SALT SENSITIVITY IN SHRSP**

Despite such limitations in epidemiological studies, and those on salt sensitivity in particular, investigation into the genetic variation has made rapid progress thanks to various hypertensive rat models. SHRSP rats, selectively bred from SHR, are unique models that not only develop severe hypertension with blood pressures of over 210 mm Hg, but also without exception develop hypertensive complications such as hemorrhagic and ischemic stroke (3,4).

SHRSP were selectively established by keeping the offspring of the A substrains of SHR which died of stroke, while stroke-resistant SHR from the B and C substrains develop hypertension of around 200 mm Hg but rarely develop stroke (3).

While we were attempting to establish SHRSP, we noted that they developed more severe hypertension than stroke-resistant SHR (SHRSP) when they were given 1% salt in their drinking water (18). Because normotensive control Wistar-Kyoto
(WKY) rats do not develop hypertension in response to salt-loading, salt sensitivity—that is, a blood pressure rise induced by salt loading—is characteristic of stroke-prone SHR. The SHRSP rat is therefore one of the best models available for analyzing genes related to salt sensitivity. For this purpose, F2 progeny were bred by cross-breeding between stroke-prone SHR and WKY strains, and we studied linkage between salt-loaded blood pressure and gene locus in the F2 segregate generation according to the procedure for linkage analysis (19).

Two sets of F2 progenies—198 rats in total—were produced independently. They were given 1% salt in drinking water from the age of 5 months. Blood pressure was measured in the conscious animal before salt loading and 12 days, 1, 2, 3, and 7 months after salt loading using the photoelectric oscillometric tail cuff method (UR1000, UEDA Tokyo, Japan). Diastolic pressure was calculated from systolic pressure and mean pressure.

All F2 rats were killed at the age of 1 year under anesthesia. Genomic DNA was extracted from the liver by a standard method using phenol-chloroform. Over 100 microsatellite regions were amplified by polymerase chain reaction (PCR), then electrophoresed on acrylamide or agarose gel and stained with ethidium bromide. Primers were synthesized according to published sequences with a DNA synthesizer (PCR-Mate 391, ABI) or purchased from Research Genetics (Huntsville, AL). Statistical analysis was performed by analysis of variance (ANOVA). Linkage map and QTL localization were done with MAPMAKER/QTL program obtained from Dr. Eric Lander (Whitehead Institute, Cambridge, MA).

GENE LOCI RELATED TO BASAL AND SALT-LOADED HYPERTENSION

From the blood pressure distribution at 5 to 13 weeks of age in stroke-prone SHRSP, WKY, and F1 and F2 progenies from the cross between them, we could estimate that the degree of genetic determination of blood pressure is about 60% and that more than three major genes are involved in the heredity of genetic hypertension (20). These F2 progeny are useful for detecting genes related to basal blood pressure rise by linkage analysis.

We were able to identify the gene locus strongly related to basal blood pressure by our linkage analysis. The LSN gene—that is, leucosyalin—coding leukocyte glycoprotein on rat chromosome 1 was closely linked with SBP and DBP in both male and female F2 rats before salt loading (21). This means a gene in the vicinity of LSN causes spontaneous hypertension at the early stage of hypertension without much influence from environmental factors such as salt intake. Further studies are needed to clarify the gene in this locus and its pathophysiologic role in hypertension.

The gene related to salt-sensitive hypertension was first investigated by linkage analysis of the DNA sets from F2 supplied by Dr. Ganten, by both Hilbert’s (22) and Lander’s groups (23) who noted linkage between the angiotensin I converting enzyme (ACE) gene from SHRSP with blood pressure rise in response to excess
salt intake. We also noted the similar linkage by using our own F2DNA set and observed that only homozygotes from SHRSP but neither heterozygotes nor homozygotes from normotensive Wistar rat genes showed any significant salt-induced increase in pressure, indicating that the ACE gene or the chromosome region near the ACE gene is related to the heredity of salt-sensitive hypertension, and that its mode of the inheritance is recessive (19).

GENDER DIFFERENCE IN SALT SENSITIVITY

This ACE locus, however, was not linked to the blood pressure levels 12 days after salt loading in female F2 rats (24). In a search for gene loci linked to salt-loaded blood pressure in females, we examined nine markers on chromosome 3: KR81, sodium channel 2, catalase, M1TR719, M1TR244, prion protein, D3KY03, M1TR593, and endothelin 3 loci. We found two markers, M1TR593 and M1TR719, located between the KR81 and ET3 locus on chromosome 3. M1TR593 was located about 13.2 cM away from the D3KY03 locus and M1TR719 was about 6.7 cM away from the catalase locus (24).

In female F2 progenies, we found the M1TR719 locus was closely linked to salt-loaded blood pressure but not to blood pressure before salt-loading. Both SBP and DBP in the homozygote of the gene locus from SHRSP were significantly higher than in the heterozygote and in the WKY homozygote (24). However, such a link with blood pressure was not noted in the male F2 progeny, indicating that this locus on chromosome 3 is linked to blood pressure after 12 days of salt-loading only in female F2 progeny.

The M1TR244 locus situated 17.6 cM away from M1TR719 was also closely linked to salt-loaded blood pressure. Both SBP and DBP in SHRSP homozygotes for this locus were significantly higher than in heterozygotes and WKY homozygotes 12 days after salt-loading, but not before salt-loading, at the age of 5 months.

Again, this link with blood pressure was not noted in the male F2 progeny, indicating that gene loci involved in early blood pressure changes after salt loading were different in males and females.

In the female F2 progeny, several loci were significantly linked to salt-sensitive high blood pressure and the candidate region was widely distributed over about 121 cM on chromosome 3. The highest LOD scores for the trait were 6.3 and 3.8 for SBP and DBP. The M1TR244 locus was most strongly linked to SBP 12 days after salt loading. However, this locus was not linked to salt-loaded high blood pressure in the male F2 progeny.

Hilbert et al. (22) and Deng et al. (25) reported that the loci on chromosome 3 did not co-segregate with salt-sensitive hypertension in F2 progeny. Discrepancy of the data may be due to the fact that, in the former report, the authors used the combined data from both males and females of F2 progenies from a cross between SHRSP and WKY, and that in the latter, the authors studied male F2 progeny from crosses between Dahl-salt-sensitive rats and WKY.
GENES RELATED TO LONG-TERM SALT SENSITIVITY

Because the blood pressure changes induced by short-term salt loading for 12 days were not large enough for statistical analysis, due to individual differences in the responses, we observed longer-term blood pressure changes at 1, 2, 3, and 7 months after salt loading. Blood pressures after salt loading were, on average, significantly higher than the pressures before salt loading at the age of 5 months in both males and females. Although average SBP in male F2 progeny was 15 mm Hg higher than in females before salt loading, the blood pressure rise due to salt loading was more marked in the females than in the males after long-term sodium loading.

We examined nine markers (D10Mgh11, D10Mgh6, NGFR, GHGP, ACE, D10Mgh4, RR1023, D10Mgh2, and D10Mgh1) on chromosome 10 by the PCR method. Several loci situated around the ACE locus were significantly linked with salt-sensitive high blood pressure with longer periods of salt-loading (3 and 7 months). Homozygotes for both ACE and RR1023 from SHRSP showed significantly higher blood pressure than heterozygotes and WKY homozygotes. The RR1023 locus was most strongly linked with SBP after 7 months of salt loading.

We constructed a map around the ACE locus on chromosome 10. Analysis by MAPMAKER/QTL showed that one of the salt-sensitive hypertensive genes in male SHRSP rats existed in 33 cM region on chromosome 10 (26). We speculate that it is situated around the RR1023 locus rather than the ACE locus as first reported.

GENE-NUTRITION INTERACTION IN SALT SENSITIVITY

Although we detected two gene loci linked to blood pressure after short-term salt loading in females and to blood pressure after long-term salt loading in males, we previously reported that the salt-sensitive blood pressure rise was greatly influenced by other nutritional factors. One series of experiments in SHRSP showed that SHRSP developed severe hypertension and all died from stroke before 100 days of age when salt loading was started at the age of 40 days (6).

However, the development of severe hypertension was somewhat attenuated and the average life span was prolonged two-fold under the same salt loading conditions when the animals were fed on soy protein, calcium, and magnesium, which counteract the adverse effects of salt. The combination of these beneficial nutrients clearly delayed the development of stroke and prolonged the lifespan. Therefore, even in salt sensitive SHRSP, blood pressure levels, as well as the development of stroke, were greatly influenced by the intake of other nutrients. Those nutrients that have been proved to affect the salt sensitively in SHRSP are dietary fiber, potassium, calcium, magnesium, and protein.

Blood pressure response to salt intake is, therefore, influenced by two major
factors: genetic and environmental. As demonstrated by the experiments in SHRSP, salt sensitivity is modified by other nutritional factors. Such gene-environmental or gene-nutritional interaction makes the whole relationship between salt intake and blood pressure more complicated, as observed in our epidemiological survey.

CONCLUSION

Because excess salt intake has an adverse effect on hypertension, particularly in salt-sensitive SHRSP rats, and salt sensitivity has been shown to have ethnic variation in our worldwide survey, there is a need for further study of salt sensitivity, and particularly its related genes and genetic mechanisms, to predict the development of hypertension by more reliable detection of genes related to salt sensitivity. It is likely that progress could also be made in preventing hypertension and its complications by improvement in nutrition. Further studies on the genetic prediction and nutritional prevention of hypertension and related cardiovascular diseases are needed with the aim of eradicating these diseases in the 21st century.

REFERENCES


**DISCUSSION**

**Dr. Hossmann:** The extension of the life span of these animals under different dietary regimens is very impressive. Do they eventually die of stroke or do they die of some other disease?

**Dr. Yamori:** They die of stroke, but when they are maintained under specific pathogen-free conditions, they can survive longer. After a stroke event, their condition becomes weaker and they die. Both ischemic and hemorrhagic stroke occur.

**Dr. Ganten:** SHR and SHRSP rats are genetically very close. Is there a difference between SHRSP and SHR with respect to genetic loci in your studies?

**Dr. Yamori:** The ratio of polymorphism between the SHRSP and SHR is 15%. If you compare SHRSP with Wistar-Kyoto control, the polymorphism ratio is about 45%.

**Dr. Ganten:** But what is the difference genetically with respect to sodium sensitivity?
Have you been able to pin down a locus which differentiates SHRSP and SHR with respect to sodium sensitivity?

**Dr. Yamori:** No, it is very difficult to relate the phenotype to a genetic locus.

**Dr. Guesry:** You showed that the calcium diet associated with soya had a negative effect on systolic blood pressure in rats, while you also showed the results of an epidemiological study on humans from 47 centers which showed the opposite—a positive correlation between calcium, and systolic blood pressure. Is there a true difference between rats and humans?

**Dr. Yamori:** These results depended on urinary calcium analysis. We could not directly analyze the dietary contents or absorption of calcium. People with higher blood pressure excrete more calcium. This doesn't mean that calcium is implicated in the pathogenesis of the hypertension. If we examine renal physiology, we find that calcium is excreted with sodium and is also influenced by blood pressure.

**Dr. Grobbee:** There are some good data to suggest that there are hypertensive patients who lose calcium from the kidneys irrespective of their sodium intake (1), and this may produce a need for calcium that would explain some of the discrepancies. One of the problems that has been raised regarding salt is that physical activity may be an important confounding variable. Do you have any data on physical activity in your study, and to what extent do you judge this to be an important confounder, in particular for the sodium associations?

**Dr. Yamori:** It was very difficult to assess physical activity and questionnaires are not reliable in humans. We have collected data on 24-hr heart rates, but so far only in limited numbers and, further, more data are needed to reach a definite conclusion.

**REFERENCE**