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GENETIC DISSECTION OF HUMAN BLOOD PRESSURE VARIATION: COMMON PATHWAYS FROM RARE PHENOTYPES

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I. Introduction

Heart disease and stroke are the number one and number three leading causes of death in the United States, accounting for more than a third of all deaths annually. Epidemiologic studies have established a number of risk factors for these diseases, including hypertension, high cholesterol, diabetes mellitus, and smoking. Prospective randomized trials of blood pressure lowering, cholesterol reduction, and smoking cessation have established the causal relationship of these risk factors to disease, because modifying these parameters prevents adverse clinical outcomes, including death.

In the case of cholesterol, initial therapeutic agents had modest cholesterol-lowering effects that reduced risk of heart attack but did not reduce overall mortality. Understanding the cholesterol biosynthetic pathway ultimately led to identification of rate-limiting steps in the pathway and development of highly potent cholesterol-lowering agents, the HMG-CoA reductase inhibitors. These agents lower cholesterol far more than their predecessors and markedly reduce both morbidity and overall mortality. These studies underscore the importance of identifying the right targets in the right pathways in order to achieve optimal clinical results.

Hypertension is the most common disease of the industrialized world, affecting one billion people world-wide and more than 70% of the elderly population. Treatment of hypertension has clear benefit to reduce the incidence of stroke and heart attack (Multiple Risk Factor Intervention Trial Research Group, 1982; Medical Research Council Working Party,

The Harvey Lectures, Series 100, pages 71–101

1985). Nonetheless, the blood pressure reduction achieved with current single medications is relatively modest in most treated subjects; only a small minority of hypertensive individuals achieve the goals of blood pressure reduction, leaving considerable room for improvement in therapy. Efforts to improve therapy, however, have been complicated by a lack of understanding of the main pathways that determine long-term blood pressure homeostasis and the key points in these pathways that would represent the best therapeutic targets. This uncertainty arises from the complex physiologic regulation of blood pressure, with inputs from the brain, heart, adrenal, kidney, vasculature, and endocrine systems (Fig. 3.1). Consequently, the field has been plagued with many proposals as to where primary abnormalities might lie with little substantiating data.

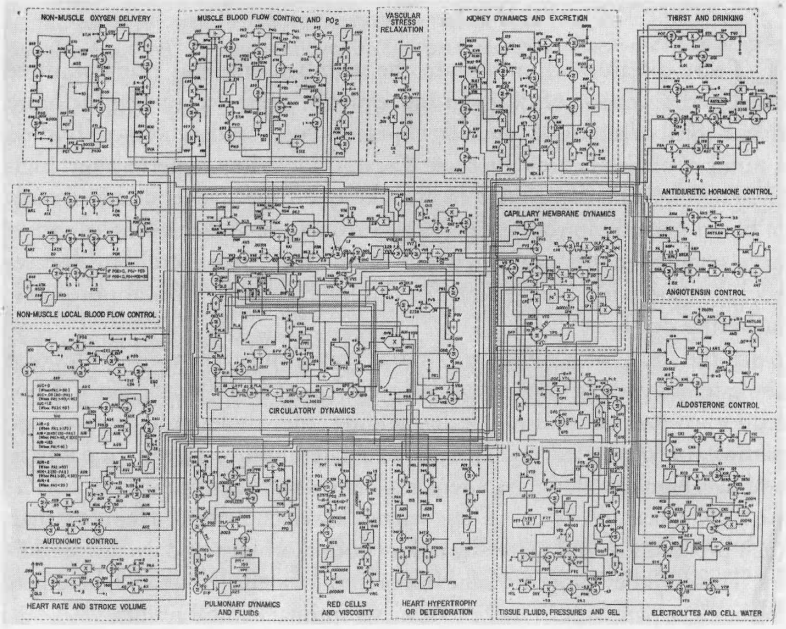

Fig. 3.1. Model of blood pressure homeostasis. The wiring diagram of a computer model of blood pressure homeostasis devised by Arthur Guyton. The model illustrates the many organs and physiologic systems that have input into blood pressure homeostasis. Reproduced with permission from the *Annual Review of Physiology*, Volume 34, copyright 1972 by Annual Reviews, www.annualreviews.org.

II. Genetic Approaches to Understand Complex Traits

In the setting of such complex systems biology, genetic approaches have considerable merit. Most importantly, they can definitively settle the question of causality that commonly plagues analysis of complex systems.

There are multiple approaches one might take to this problem. We were initially inspired by genetic approaches in model organisms, in which complex systems like development have been effectively dissected by large-scale mutagenic screens, looking for mutations that impart large effects in order to identify the key genes and pathways that affect the trait in question (Nusslein-Volhard and Wieschaus, 1980). This suggested a search not for common alleles in the general population that might impart incremental effects on the trait, but for rare alleles with very large effects that should define the important underlying pathways that control the trait. While one cannot perform mutagenesis in humans, one can take advantage of the presence of the more than 12 billion copies of the human genome in existence among the world's inhabitants. With a 3 billion base pair genome and reasonable estimates of the mutation frequency, mutations altering virtually every base in the genome that are compatible with survival exist somewhere on the planet, and they come to medical attention. Owing to high rates of consanguinity in a number of cultures, even rare recessive alleles can be found in the homozygous state.

These considerations motivated our efforts to systematically scour the globe for extreme outliers with either extremely high or extremely low blood pressure, followed by clinical investigation of the index case and the extended kindred to identify distinct physiologic features and to determine whether blood pressure or related endophenotypes show evidence of Mendelian segregation. Among such families, mapping studies comparing the transmission of each chromosome segment to the inheritance of altered blood pressure can pinpoint the location of the underlying disease gene, and screening of genes in the linked interval can identify underlying functional mutations. Importantly, the typical occurrence of independent mutations among unrelated kindreds that segregate with the trait and which show specificity for the trait settle the issue of causality, an important advantage. The demonstration of single gene mutations that impart large effects on blood pressure have the capacity to identify key rate-determining steps and pathways for the behavior of the overall system.

These sites represent logical points at which therapeutic intervention might have the greatest impact. Importantly, as for cholesterol homeostasis, intervention at these rate-determining steps may have the greatest therapeutic impact in the general population, regardless of the presence or absence of mutations in this pathway.

To date, we have used this approach to identify mutations in eight genes that raise blood pressure and another eight genes in which mutation lowers blood pressure. The most striking finding from these studies is that these genes define a final common pathway that regulates renal salt handling: Mutations that increase net renal salt reabsorption raise blood pressure, and mutations that reduce salt reabsorption lower blood pressure.

III. Renal Salt Homeostasis

In order to understand the relationship between salt and blood pressure, a basic knowledge of renal salt homeostasis is required. Every day the kidneys of a normal adult filter 170 liters of plasma containing 1.5 kg of salt. Consequently, on a typical Western 5-g salt diet, the kidneys must reabsorb all but about 0.5% of the filtered load of salt to maintain homeostasis. The amount of water reabsorbed is precisely regulated to maintain a serum sodium concentration very close to 140 mM. As a consequence, increased salt reabsorption initially leads to higher plasma volume; conversely, reduced salt reabsorption results in lower plasma volume.

The kidneys achieve salt homeostasis by a complex, integrated set of exchangers, cotransporters, and channels that act in distinct nephron segments (Fig. 3.2). Bulk reabsorption occurs proximally, with progressively smaller fractions reabsorbed in each subsequent nephron segment. About 60% of the filtered sodium load of salt is reabsorbed by Na^+/H^+ exchange in the proximal tubule, 30% by the action of a Na^+–K^+–$2Cl^-$ cotransporter in the thick ascending limb of Henle, 7% by the action of a Na–Cl cotransporter in the distal convoluted tubule, and the last 2% by the action of the renal epithelial sodium channel (ENaC). Reabsorption of Na^+ via ENaC is electrogenic, providing the electrical driving force to support either paracellular Cl^- reabsorption or K^+ and H^+ secretion.

The activity of this final channel, ENaC, is regulated by the renin–angiotensin system. The renin–angiotensin system is activated when the

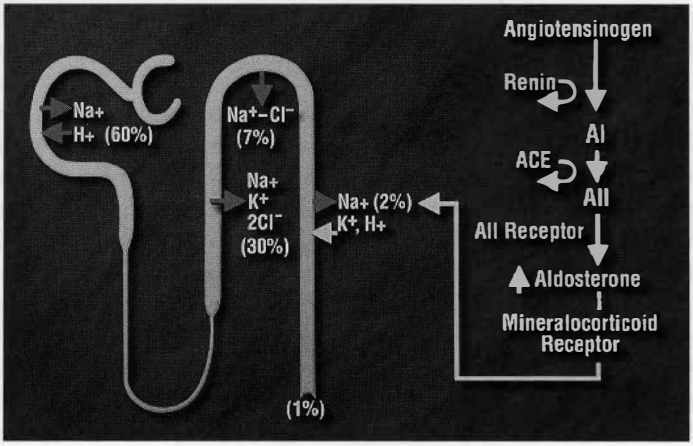

Fig. 3.2. Elements contributing to renal salt homeostasis. A diagram of a nephron is shown. Sodium filtered at the glomerulus is reabsorbed by four major mediators distributed along the nephron as described in the text. Reabsorption of Na^+ by the epithelial sodium channel in the distal nephron is regulated by the activity of the renin–angiotensin system via regulated production of the steroid hormone aldosterone.

kidney senses reduced chloride delivery to the thick ascending limb of Henle, resulting in secretion of the aspartyl protease renin by cells of the juxtaglomerular appratus. Renin cleaves the circulating protein angiotensinogen to angiotensin I, which is further cleaved by angiotensin converting enzyme to the active peptide hormone angiotensin II. Angiotensin II binds to specific receptors in the adrenal glomerulosa, which leads to increased secretion of the steroid hormone aldosterone; aldosterone binds to specific receptors in principal cells of the distal nephron, which ultimately leads to increased activity of the epithelial sodium channel.

The mutations our group has identified impart large effects on blood pressure and/or related traits. In each case, families from around the world have been identified and clinically characterized, and disease-causing mutations have been identified. In nearly every case, biochemical and clinical studies have established the specific mechanisms by which mutations increase or decrease renal salt reabsorption.

IV. Mutations that Raise Blood Pressure

A. Glucocorticoid-Remediable Aldosteronism (GRA)

GRA is an autosomal dominant trait featuring early onset of severe hypertension (Sutherland et al., 1966). Physiologically, affected patients have elevated levels of aldosterone despite suppression of renin levels. Interestingly, aldosterone secretion in these patients is driven by adrenocorticotropic hormone (ACTH), the normal secretagogue for cortisol, rather than angiotensin II. By investigation of families with GRA, we demonstrated that this disease is caused by a gene duplication produced by unequal crossing over between two genes involved in adrenal steroid biosynthesis (Fig. 3.3; Lifton et al., 1992a,b). One of these genes encodes aldosterone synthase, which is the rate-limiting step in aldosterone biosynthesis in the adrenal glomerulosa and whose expression is normally regulated by angiotensin II signaling. The other gene encodes steroid 11-beta hydroxylase, which is employed in cortisol biosynthesis in adrenal fasciculata and whose expression is regulated by adrenocorticotropic

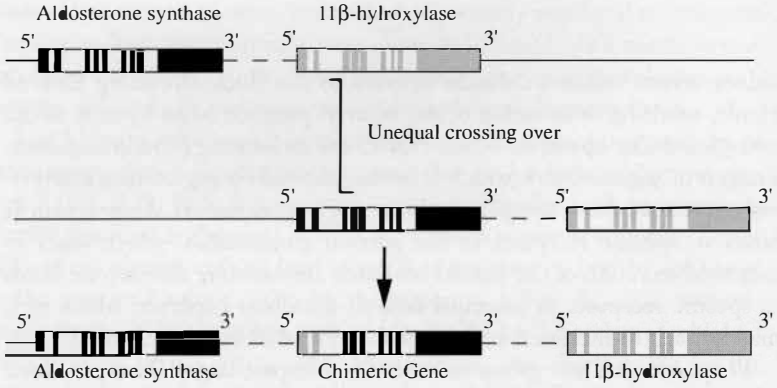

Fig. 3.3. The genetic basis of the hypertensive disease glucocorticoid-remediable aldosteronism. Unequal crossing over between genes encoding aldosterone synthase and steroid 11-beta hydroxylase produces a chimeric gene duplication fusing 5′ regulatory sequences of 11-beta hydroxylase to coding sequences of aldosterone synthase. This leads to ectopic expression of aldosterone synthase enzymatic activity in adrenal fasciculata under control of ACTH.

hormone (ACTH). These two genes have recently evolved from a common ancestor; they are tightly linked on chromosome 8 in head-to-tail configuration and are 95% identical in DNA sequence. The chimeric gene duplications that cause GRA fuse ACTH-responsive regulatory elements from 11-hydroxylase onto coding sequences that result in aldosterone synthase enzymatic activity (Fig. 3.3). This results in expression of the rate-limiting enzyme for aldosterone synthesis in the wrong tissue, the adrenal fasciculata, under control of the wrong hormone, ACTH. At the expense of maintaining ACTH levels to support normal cortisol secretion, affected subjects consequently have sustained aldosterone secretion.

This mutation results in hypertension by the following sequence: Chronic aldosterone secretion leads to increased sodium reabsorption via ENaC; water follows to maintain isotonicity of plasma, leading to increased intravascular volume; this increases venous blood return to the heart, and the cardiac stroke volume and cardiac output consequently increases; by Ohm's law, this increase in cardiac output results in elevated blood pressure. This elevation in blood pressure is sufficient to suppress the activity of the renin angiotensin system; however, this fails to reduce aldosterone secretion, since aldosterone secretion is now under control of ACTH.

B. Mendelian Hypertension Exacerbated by Pregnancy

Aldosterone signals via the mineralocorticoid receptor, a member of the nuclear hormone receptor family. By investigation of a unique family with unexplained severe hypertension with suppressed renin and aldosterone secretion, we defined a new disease, demonstrated that it was transmitted as a simple autosomal dominant trait, and demonstrated that it is caused by gain of function mutation in the mineralocorticoid receptor (Geller et al., 2000). The disease-causing mutation is missense, and it lies in the ligand binding domain of the mineralocorticoid receptor, substituting leucine for a native serine in helix 5 (Fig. 3.4). Biochemical studies in mammalian cells demonstrated partial activation of the mutant receptor in the absence of added steroid; more impressively, steroids such as progesterone that lack a 21-hydroxyl group, which normally bind but fail to activate the receptor, become very potent agonists for the mutant receptor.

Molecular modeling followed by site-directed mutagenesis has shown that the disease-causing mutation creates a key van der Waals interaction

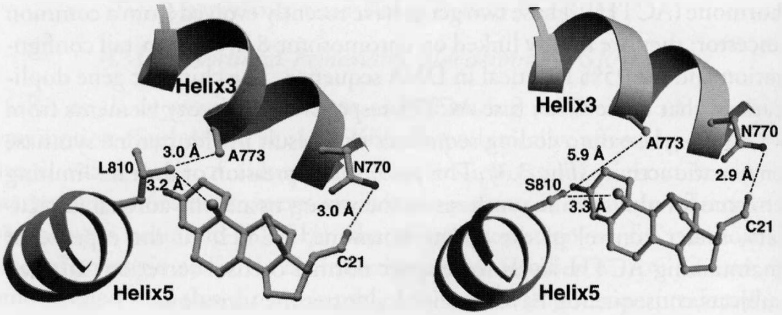

Fig. 3.4. Hypertension caused by mineralocorticoid receptor mutation. Molecular model of aldosterone binding to the mineralocorticoid receptor is shown. The wild-type receptor with Serine at position 810 is shown at right, and the mutant receptor with leucine at position 810 is shown at left. L810 is capable of a new van der Waals interaction with alanine 773 in helix 3, near the site where the 21-hydroxyl group of aldosterone interacts with asparagine 770. This new interaction eliminates the requirement for the steroid 21-hydroxyl group, resulting in receptor activation by progesterone and other steroids.

between helix 5 and helix 3 of the ligand binding domain. This new interaction replaces the requirement for an interaction between the steroid 21-hydroxyl group and helix 3 of the receptor, allowing steroids lacking this moiety to become potent receptor agonists. These observations make a clinical prediction: in normal pregnancy, progesterone levels rise 100-fold; if progesterone is indeed an agonist for this mutant receptor, women harboring this mutation should develop severe hypertension. This is indeed the case. The five pregnancies among women harboring this mutation have been accompanied by the development of extreme hypertension, necessitating early delivery or termination of pregnancy. This is the first demonstration of a molecular mechanism underlying this common complication of pregnancy and demonstrate that it can arise from the abnormal action of a normal hormone.

C. Liddle Syndrome

Downstream of the mineralocorticoid receptor lies its major target, the epithelial sodium channel (ENaC). ENaC is composed of homologous subunits encoded by three different genes, alpha, beta, and gamma. Mutations in ENaC subunits cause another Mendelian form of hypertension

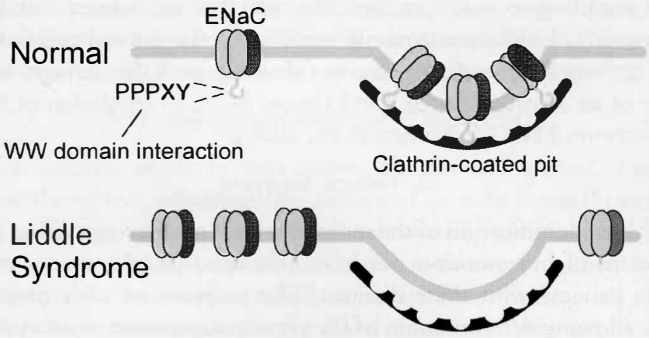

Fig. 3.5. Mutations in the epithelial sodium channel (ENaC) cause the hypertensive disease Liddle syndrome. Mutations that delete or modify the PPPXY motif in the cytoplasmic tail of the beta or gamma subunits of ENaC cause Liddle syndrome by impairing the clearance of ENaC from the cell surface via endocytosis through clathrin-coated pits.

known as Liddle syndrome. This disease features early onset hypertension transmitted as an autosomal dominant trait; affected patients have hypertension despite suppression of the renin–angiotensin system and reduced levels of aldosterone. By investigation of families with this disease, we have shown that Liddle syndrome is caused by mutations in the beta or gamma ENaC subunits (Fig. 3.5; Shimkets et al., 1994, Hansson et al., 1995); disease-causing mutations eliminate or alter single amino acids in the cytoplasmic C-termini of these proteins. The target sequence for these mutations is a PPPXY segment shared by these two subunits (Schild et al., 1996). This shared sequence motif is required for the normal removal of ENaC from the cell surface by endocytosis via clathrin-coated pits (Shimkets et al., 1997). This sequence is recognized by WW domains of Nedd-4, a ubiquitin ligase, implicating Nedd-4 in this clearance mechanism (Staub et al., 1996). These mutations thus result in a prolonged half-life and increased levels of ENaC at the cell surface (Shimkets et al., 1997). These channels are active and mediate increased sodium reabsorption, thus feeding into the same final common pathway for hypertension as patients with GRA and mineralocorticoid receptor mutations. These findings establish that increased renal sodium reabsorption alone is sufficient to produce hypertension in humans, independent of mineralocorticoid receptor action.

It is tempting to speculate that the clearance mechanism for ENaC discovered via Liddle syndrome is involved in the normal regulation of ENaC activity by aldosterone. Recent data supports this notion, linking activity of an aldosterone-regulated kinase, SGK, to regulation of Nedd-4's activity on ENaC (Ichimura et al., 2005).

D. Genetic Screening

With the identification of the molecular basis of these autosomal dominant forms of hypertension, we have developed simple genetic tests to identify patients with these diseases. The purposes of such testing are several, allowing determination of the prevalence of these diseases among the hypertensive population, permitting studies of the impact of these mutations on blood pressure and clinical outcomes in an unbiased fashion by the study of extended kindreds, and permitting specific therapy tailored to the mutation among affected subjects. Over the last decade, we have screened over 1000 samples sent by physicians from around the world for evaluation of unexplained hypertension. Among these, we have identified 42 new kindreds with GRA and 12 new kindreds with Liddle syndrome, as well as over 200 additional affected subjects identified from their relationship to an affected index case. The strongest predictors of a positive test are onset of hypertension before the age of 20 and a first-degree relative with the same finding; among such subjects, 25% have mutations in one of the above genes.

From these studies, we have also shown that these genes on average impart a very large quantitative effect on blood pressure, approximating 30 mm Hg in the case of GRA. This marked effect on blood pressure has important clinical consequences for affected patients: Patients with GRA have a dramatic increase in the risk of intracranial hemorrhage before the age of 45, most often due to rupture of aneurysms of the circle of Willis. Prospective screening of affected subjects has identified asymptomatic aneurysms that have resulted in premorbid surgical intervention. Identification of these patients has also permitted therapeutic intervention with agents tailored to the underlying primary abnormality.

V. Mutations that Lower Blood Pressure

We are equally interested in mutations that lower blood pressure, because loss of function mutations that reduce blood pressure may define

therapeutic targets at which pharmacologic inhibitors would have beneficial blood-pressure-lowering effects. We have approached this problem by investigating newborns with life-threatening hypotension.

A. Pseudohypoaldosteronism Type I (PHAI)

PHAI features severe hypotension in the neonatal period. There are autosomal recessive, autosomal dominant and sporadic forms (Hanukoglu, 1991). Affected subjects have severe salt wasting despite marked elevations in renin and aldosterone. In addition to salt wasting, they also have striking hyperkalemia and metabolic acidosis. These findings all point to defective Na^+ reabsorption in the distal nephron, since impaired Na^+ reabsorption at this site would be expected to impair secretion of K^+ and protons.

We have shown that the recessive form of this disease is caused by diverse loss of function mutations in subunits of ENaC, the same channel in which gain of function mutations cause Liddle syndrome (Fig. 3.6; Chang et al., 1996). We have also shown that the dominant form of

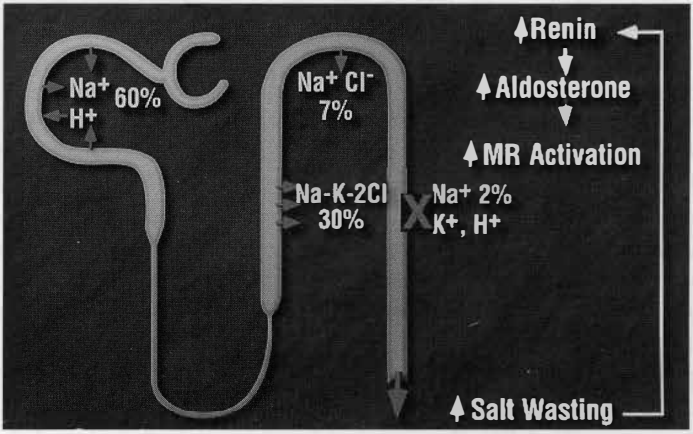

Fig. 3.6. Mutations in ENaC cause the hypotensive disease pseudohypoaldosteronism type I. Loss of function mutations in ENaC result in massive salt wasting leading to activation of the renin–angiotensin system. The loss of ENaC function prevents the increased aldosterone level from increasing Na^+ reabsorption. Due to the loss of electrogenic Na^+ reabsorption via ENaC, K^+ and H^+ secretion are also impaired, resulting in hyperkalemia and metabolic acidosis.

PHAI is caused by heterozygous loss of function mutations in the mineralocorticoid receptor (Geller et al., 1998). Most interestingly, while neonates with either form of PHAI can be gravely ill in the neonatal period, there are striking differences later in life. Patients with ENaC mutations remain extremely dependent upon massive salt supplementation and perpetual treatment to lower serum K^+ levels. Mild intercurrent illness can be life-threatening due to impaired dietary salt intake. In contrast, patients with mineralocorticoid receptor mutations become asymptomatic, usually after age 2, and as adults are entirely asymptomatic, with only a striking elevation in resting aldosterone levels as a biochemical mark of their underlying defect (Geller et al., in preparation).

These findings have several important clinical implications. Most significantly, they require a reconsideration of the long-held view that ENaC is wholly dependent upon aldosterone, and that on a typical 5-g salt diet, with suppression of the renin-angiotensin system, ENaC activity is dispensable. The fact that patients missing ENaC require 20–30 g of salt per day to maintain a semblance of normal intravascular volume indicates the essential role of ENaC for normal homeostasis. These observations underscore the possibility of developing improved antihypertensive agents that target ENaC (see below).

B. Gitelman Syndrome

The recognition that gain or loss of function of ENaC lead to increased or reduced blood pressure prompted consideration of the phenotypes that might result from altered function of other mediators of renal salt reabsorption. These led to the consideration of Gitelman syndrome and Bartter syndrome, two diseases featuring abnormalities in salt, potassium, pH, magnesium, and calcium homeostasis (Bettinelli et al., 1992). We showed that Gitelman syndrome is caused by recessive loss of function mutations in the thiazide-sensitive Na–Cl cotransporter of the renal distal convoluted tubule (NCC) (Simon et al., 1996a). This results in primary salt wasting, along with activation of the renin–angiotensin system, leading to increased ENaC activity, which, in turn, leads to hypokalemia and metabolic alkalosis (Fig. 3.7). In addition, these patients uniformly have both hypomagnesemia due to renal Mg^{2+} loss and hypocalciuria. These latter features are not readily explained from known physiology but indicate an essential requirement for NCC activity in the normal homeostasis of Mg^{2+} and Ca^{2+}.

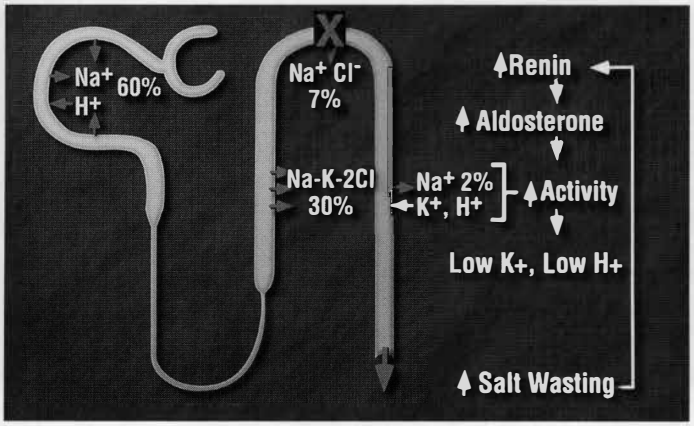

Fig. 3.7. Mutations in the Na–Cl cotransporter (NCC) cause Gitelman syndrome. Loss of function mutations in NCC result in salt wasting and induction of the renin–angiotensin system. This leads to a compensatory increase in Na^+ reabsorption via ENaC, resulting in only modest reduction in blood pressure; the increased lumen-negative potential results in increased electrogenic drive for K^+ and H^+ secretion, leading to hypokalemia and metabolic alkalosis.

We hypothesized that the relatively mild salt wasting seen in this disease should reduce blood pressure. This was tested by clinical investigation of 200 members of an extended kindred segregating two identified NCC mutations. By genotyping kindred members, we were able to compare the blood pressures of family members harboring zero, one, or two mutant copies of the gene. The results demonstrated an average 8-mmHg reduction in systolic blood pressure among homozygous mutant subjects, with no reduction in blood pressure of heterozygotes. This reduction was highly significant but nonetheless was somewhat less than might have been anticipated. This led us to consider whether affected subjects might in some way be compensating for their inherited defect. One obvious means to achieve this would be to simply eat more salt. This was tested by measuring 24-hour urinary Na^+ excretion, which in steady state is the best indicator of dietary salt intake. Interestingly, both the heterozygous and homozygous mutant subjects were found to consume markedly more salt than their wild-type relatives, indicating dietary compensation for the inherited renal defect (Cruz et al., 2001).

These findings have several interesting implications. First, they reveal the complexity of the relationship between salt intake and blood pressure. Epidemiologic studies over the last half-century have examined this relationship, but have struggled to provide generalizable conclusions (Taubes, 1998). Kindreds such as this one provide suggestions as to why this relationship is so confounded. In this family, the individuals with the lowest blood pressure are those that are eating the most, not the least, salt. The recognition that primary renal salt wasting induces increased dietary salt consumption reveals the difficulty in demonstrating a simple relationship between salt intake and blood pressure. Second, these findings also make the interesting point that dietary taste for salt, which might have been considered a complex behavioral trait, can be determined by the activity of a protein in the kidney.

C. Bartter Syndrome

The related disease Bartter syndrome features a typically far more severe form of salt wasting that has high morbidity and mortality in the neonatal period. Affected subjects are now recognized to have, in addition to hypokalemia and metabolic alkalosis, mild to severe hypercalciuria, with a substantial fraction developing nephrocalcinosis resulting in renal failure. This disease proves to result from a failure of salt reabsorption in the renal thick ascending limb of Henle (TAL) and define the pathway by which salt traverses the TAL (Fig. 3.8). To date, loss of function mutations in four genes have been identified that result in related phenotypes. One of these, the Na–K–2Cl cotransporter encoded by *NKCC2*, mediates the entry of salt from the lumen into the epithelium of this nephron segment; homozygous loss of function mutations in this gene are one cause of Bartter syndrome (Simon et al., 1996b).

Potassium entering epithelial cells of the TAL via NKCC2 is recycled back into the lumen by the K^+ channel ROMK, and recessive loss of function mutation in this channel results in a related phenotype (Simon et al., 1996c). The explanation for this phenocopy is that fluid in the TAL is high in Na and Cl but low in K. Consequently, without the "recycling" of K^+ entering the cell back into the lumen via ROMK (Fig. 3.8), there is not sufficient K^+ in the lumen to permit normal extraction of Na^+ and Cl^- by NKCC2. Interestingly, these patients have severe salt wasting, but their K^+ loss is much less than seen among patients with NKCC2 mutations. The reason for this is likely that ROMK is also used

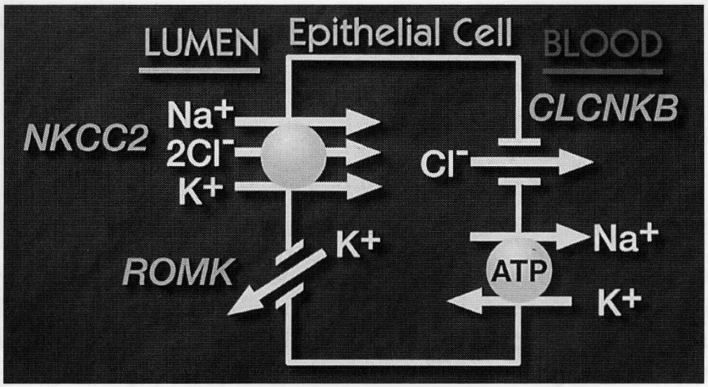

Fig. 3.8. Mutations that impair salt reabsorption in the thick ascending limb of Henle (TAL) cause the hypotensive disease Bartter syndrome. Mutations in genes that mediate apical entry of Na–K–2Cl, the recycling of K^+ back into the lumen, or exit of Cl^- across the basolateral membrane in the TAL typically cause severe salt wasting and Bartter syndrome.

for K^+ secretion in the distal nephron. This observation has interesting therapeutic implications (see below).

NaCl entering the epithelial cell of the TAL must return to the bloodstream by traversing the basolateral membrane. Recessive loss of function mutations in the Cl^- channel encoded by *CLCNKB* results in another subset of patients with Bartter syndrome and identifies this channel as a required component of this exit step (Simon et al., 1997). Interestingly, this channel, when expressed alone in *Xenopus* oocytes, is inactive, suggesting the requirement for an accessory subunit. Recently, Birkenhager and colleagues (2002) have identified such an accessory subunit of this channel by idenification of mutations in the protein Barttin in another group of Bartter patients.

Genotype–phenotype correlations have demonstrated that much of the clinical variability seen among Bartter patients is explained by the different genes that are mutated to produce this phenotype. For example, patients with mutations in CLCNKB or Barttin have more modest hypercalciuria and less often have nephrocalcinosis, a feature that is nearly always found among patients with mutations in NKCC2 and ROMK. Patients with mutations in ROMK typically present in the neonatal

period with paradoxical hyperkalemia and evolve over time to hypokalemia that rarely requires K^+ supplementation to the extent that other Bartter patients do.

VI. New Pathways from Recent Genetic Studies

A. A Complex Metabolic Syndrome Due to Mitochondrial Mutation

In the general population, hypertension is significantly associated with a number of other traits, including insulin resistance, obesity, hyperlipidemia, and hypomagmesemia. Together, these traits are often referred to as the metabolic syndrome. The mechanisms underlying the clustering of these traits has been unknown; however, the prevalence of this syndrome is recognized to affect about 25 million Americans.

We ascertained and performed detailed clinical investigation of 142 blood relatives of a kindred with an unusually high prevalence of hypertension, hypercholesterolemia, and hypomagnesemia (Fig. 3.9; Wilson et al., 2004). We showed that each of these traits is strongly linked to the maternal lineage in this kindred; for example, all 32 kindred members with clinically defined hypomagnesemia were on the same maternal lineage, a finding extremely unlikely to occur by chance ($p = 10^{-9}$). In addition, the fraction of offspring of affected mothers who had hypomagnesemia significantly exceeded the number expected under autosomal dominant models of transmission. Elevated blood pressure and elevated LDL and VLDL cholesterol followed were similarly linked to the maternal lineage.

Since mitochondria are virtually exclusively maternally transmitted in thousands of copies, these findings strongly implicate a mutation in the 16-kb mitochondrial genome as the cause of this syndrome. The mitochondrial genome sequence revealed a novel mutation in the base immediately 5′ to the anticodon of the mitochondrial isoleucine tRNA. The normal uridine at this position is perhaps the most conserved base in all of biology, owing to the essential role of its amino group in stabilizing the anticodon loop, permitting presentation of the anticodon to the codon of the cognate mRNA. Uridine is found at this position in every ile tRNA from archaebacteria to humans and is also conserved in virtually all other tRNAs, with notable exceptions of some initiator methionine tRNAs in eukaryotes. This mutation is homoplasmic among all members of the maternal lineage, regardless of phenotype.

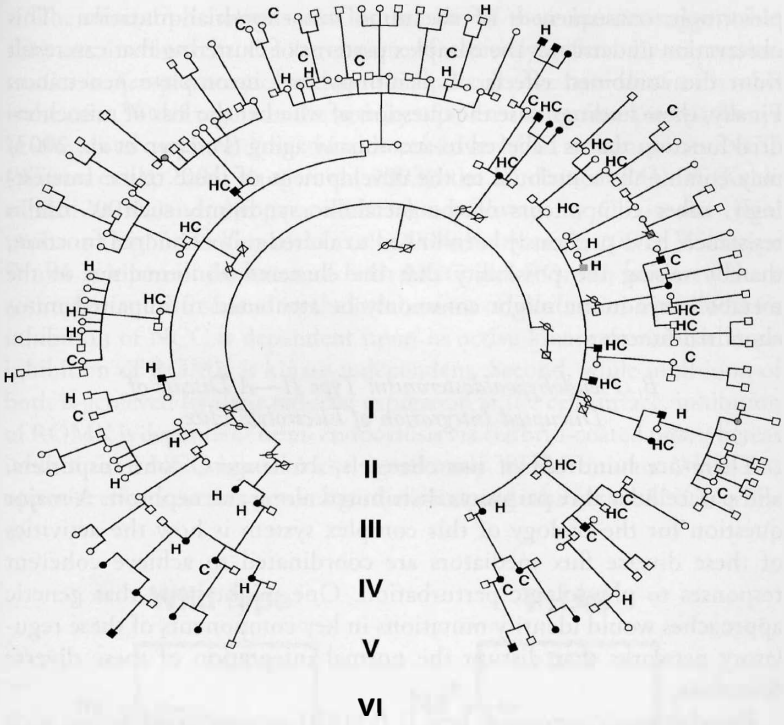

Fig. 3.9. Kindred with mitochondrial hypomagnesemia, hypertension, and hypercholesterolemia. Kindred members with hypomagnesemia, hypertension, and hypercholesterolemia are denoted by filled symbols, the letter H and the letter C, respectively. Each of these traits shows a marked increase on the maternal lineage, with strong support for mitochondrial transmission.

Several aspects of these findings are of interest. First, they implicate altered mitochondrial function in the pathogenesis of three new traits: hypomagnesemia, elevated blood pressure, and elevated cholesterol. This adds to the remarkably diverse list of traits that can result from mitochondrial mutation (Wallace, 1999). Second, the clustering of these phenotypes is noteworthy: While virtually all of the clustering occurs on the maternal lineage, within the maternal lineage the distribution of these traits is nearly stochastic; this suggests that these traits are independent,

pleiotropic consequences of the same mitochondrial mutation. This observation underscores the complex patterns of clustering that can result from the combined effects of pleiotropy and incomplete penetrance. Finally, these findings raise the question of whether the loss of mitochondrial function that is believed to accompany aging (Petersen et al., 2003) may commonly contribute to the development of these traits. Interestingly, other components of the metabolic syndrome, such as insulin resistance, have previously been linked to altered mitochondrial function, thereby raising the possibility that the clustered abnormalities of the metabolic syndrome might commonly be attributed to impaired mitochondrial function.

B. Pseudohypoaldosteronism Type II—A Disease of Disrupted Integration of Electrolyte Flux

There are hundreds of ion channels, exchangers, contransporters, and paracellular flux pathways distributed along the nephron. A major question for the biology of this complex system is how the activities of these diverse flux mediators are coordinated to achieve coherent responses to physiologic perturbation. One might hope that genetic approaches would identify mutations in key components of these regulatory networks that disrupt the normal integration of these diverse functions.

Pseudohypoaldosteronism type II (PHAII) appears to define just such a higher-order regulator of function. PHAII features hypertension with hyperkalemia and metabolic acidosis despite otherwise normal renal function; it is transmitted as an autosomal dominant trait (Gordon et al., 1995). Interestingly, the hypertension and hyperkalemia can be corrected by low doses of thiazide diuretics or the removal of chloride from the diet.

We have identified mutations in two novel serine threonine kinases, Wnk1 and Wnk4, as causes of PHAII (Wilson et al., 2001). Mutations in Wnk1 are large deletions in the first intron that increase expression of Wnk1 mRNA. Mutations in Wnk4 are missense and cluster within a 10-amino-acid acidic segment distal to the kinase domain. The expression of these kinases in the kidney is confined to the distal convoluted tubule and collecting duct of the nephron. Outside the kidney, these kinases are expressed in diverse Cl^- transporting epithelia, including pancreatic ducts, hepatic bile ducts, colonic crypts, and sweat ducts.

The clinical phenotypes and localization of these kinases suggest that they are involved in the regulation of electrolyte flux. By expression studies in *Xenopus* oocytes and mammalian cells, we have shown that this is the case. Wnk4 regulates the activity of at least three transport pathways in the distal nephron: the thiazide-sensitive NaCl cotransporter NCC (Wilson et al., 2004; Yang et al., 2003), the K^+ channel ROMK (Kahle et al., 2003), and the paracellular flux pathway for Cl^- in the distal nephron (Fig. 3.10; Yamauchi et al., 2004; Kahle et al., 2004). Wild-type Wnk4 is a potent inhibitor of both NCC and ROMK; however, there are important differences in the mechanism of inhibition. First, Wnk4's inhibition of NCC is dependent upon its active kinase domain, whereas inhibition of ROMK is kinase-independent. Second, while inhibition of both is achieved by their reduced expression at the cell surface, inhibition of ROMK is dependent upon endocytosis via clathrin-coated pits, whereas inhibition of NCC is not. Most significantly, PHAII mutant Wnk4 has opposite effects on these two targets, eliminating inhibition of NCC while

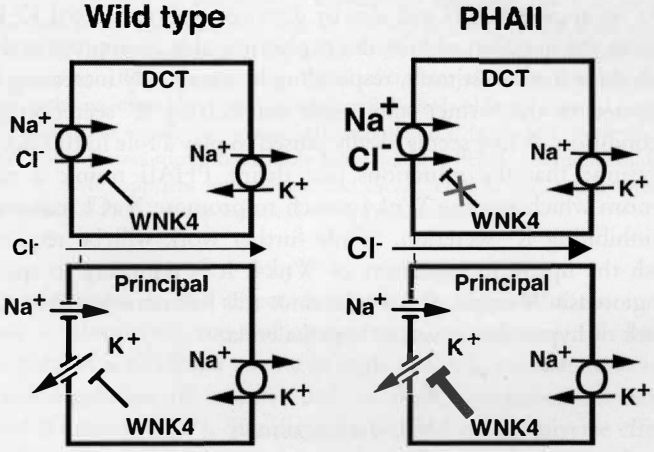

Fig. 3.10. Wnk4 regulates the balance between Cl^- reabsorption and K^+ secretion. Wnk4 inhibits the activity of the NaCl cotransporter in the distal convoluted tubule (DCT) and the K^+ channel ROMK in intercalated cells of the collecting duct. Mutations in WNK4 in patients with pseudohypoaldosteronism type II (PHAII) alleviate inhibition of NCC, augment inhibition of ROMK, and increase paracellular Cl^- permeability. This results in a net increase in NaCl reabsorption and reduced K^+ secretion.

increasing inhibition of ROMK. In addition, Wnk4 has effects on the paracellular flux pathway. When expressed under control of doxycycline in MDCK monolayers, expression of wild-type Wnk4 induces a selective increase in the absolute paracellular permeability for Cl^-. PHAII-mutant Wnk4 imparts a much larger effect to increase paracellular Cl^- permeability.

These effects indicate that Wnk4 has properties of a molecular switch, which enable it to have independent and divergent effects on highly diverse flux pathways. PHAII-mutant Wnk4 is inferred to increase renal NaCl reabsorption by increasing the activity of NCC and by increasing permeability of the paracellular pathway for Cl^-, allowing increased reabsorption of Na^+ with Cl^- in the distal nephron and concomitantly decreasing the electrical driving force to support K^+ secretion. In parallel, PHAII-mutant Wnk4 also inhibits K^+ secretion directly, by inducing clearance of ROMK from the cell surface. These effects of mutant Wnk4 can therefore explain the physiology observed in patients with PHAII.

These observations suggest that Wnk4's actions can explain a classic paradox in renal physiology: Aldosterone is secreted in response to hypovolemia via angiotensin II and also by direct effects of elevated K^+ levels. This raises the question of how the nephron is able to appropriately distinguish these distinct stimuli, responding by maximally increasing NaCl reabsorption in the former state while maximizing K^+ secretion in the latter condition. Wnk4 seems ideally poised to play a role in this decision. We presume that the mutations that define PHAII mimic a normal mechanism which sets the Wnk4 switch to promote NaCl reabsorption while inhibiting K^+ secretion. While further work will be required to establish the upstream regulators of Wnk4, it is tempting to speculate that angiotensin II might play a role, since this hormone is a distinguishing mark of hypovolemia versus hyperkalemia.

VII. Implications

A. Pathophysiology of Hypertension

These studies have unequivocally established a causal relationship between alteration of net renal salt reabsorption and altered blood pressure in humans; mutations that increase salt balance raise blood pressure and mutations that reduce salt balance lower blood pressure (Fig. 3.11).

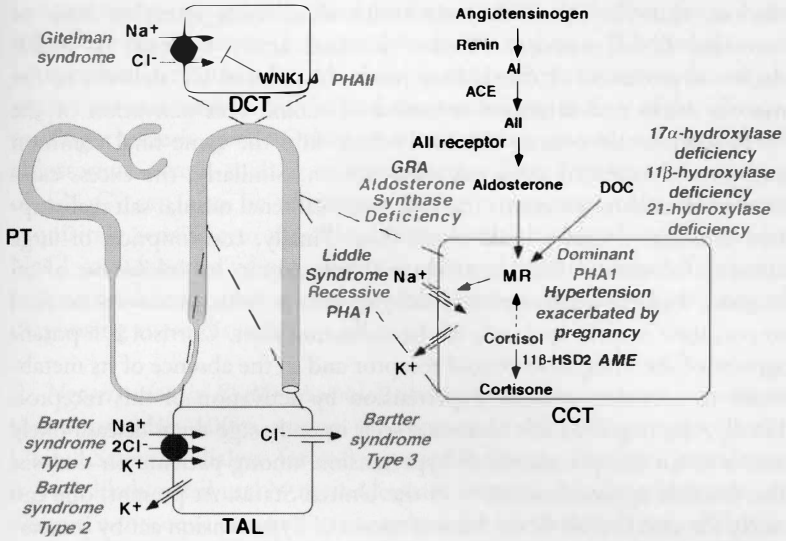

Fig. 3.11. Diagram of a nephron. The pathways mediating NaCl reabsorption in epithelial cells of the thick ascending limb of the loop of Henle (TAL), distal convoluted tubule (DCT), and the cortical collecting tubule (CCT) are indicated, along with the renin–angiotensin system, the major regulator of renal salt reabsorption. Inherited diseases affecting these pathways affect blood pressure and are indicated. Abbreviations: AI, angiotensin I; ACE, angiotensin converting enzyme; AII, angiotensin II (AII); MR, mineralocorticoid receptor; GRA, glucocorticoid-remediable aldosteronism; PHA1, pseudohypoaldosteronism, type 1; AME, apparent mineralocorticoid excess; 11 β-HSD2, 11β-hydroxysteroid dehydrogenase-2; DOC, deoxycorticosterone; PT, proximal tubule.

The mechanisms of this effect can readily be explained via an initial increase in plasma volume and cardiac output; over time, this hemodynamic pattern is modified to one of high systemic vascular resistance by the local regulation of vascular beds to match perfusion to metabolic demand (Guyton, 1991). Significantly, while there are diverse effects on K^+, Ca^{2+}, and Mg^{2+} homeostasis, in these diseases the vector for NaCl consistently predicts the resulting effect on blood pressure.

These observations raise the question of whether other forms of hypertension also arise via increased renal salt reabsorption. The answer is unequivocally yes, because virtually all of the acquired forms of hypertension arise via this mechanism. Aldosterone-producing adenoma is one

obvious example, in which constitutive aldosterone secretion leads to increased ENaC activity. Another is renal artery stenosis, in which decreased perfusion of one kidney results in reduced Cl^- delivery to the macula densa and increased secretion of renin. This activation of the renin–angiotensin system ultimately feeds into the same final common pathway of increased renal salt reabsorption. Similarly, the excess catechols in pheochromocytoma increase proximal renal tubular salt reabsorption and also decrease renal blood flow. Finally, consumption of large amounts of natural licorice produces hypertension by inhibition of an enzyme, 11-beta hydroxysteroid dehydrogenase, which converts cortisol to cortisone in principal cells of the collecting duct. Cortisol is a potent agonist of the mineralocorticoid receptor and in the absence of its metabolism to cortisone induces hypertension by activation of this receptor. Finally, the impaired salt clearance seen in end-stage renal disease likely accounts for the prevalence of hypertension among patients on dialysis; this fraction approaches 100% in the United States. At present, one can make the case that all of the known causes of hypertension act by increasing net salt balance, either by altering renal salt handling directly or as a secondary effect resulting either from impaired blood flow to the kidney or from impaired glomerular filtration.

Given that the genetic and acquired forms of hypertension act via altered renal salt handling, it is tempting to speculate that the common forms of hypertension will also arise by this mechanism with either a direct effect on renal salt handling or an indirect one via reduced glomerular filtration due to primary glomerular or renal vascular effects. Large population studies are underway that will address whether rare or common variants in genes in these pathways play a role in common forms of hypertension. Regardless of the answer to this latter question, however, the recognition that blood pressure can be modulated from very low to very high levels by alteration of renal salt balance has important implications for the treatment of hypertension.

B. Therapeutic Implications

1. Tailored Therapy for Rare Diseases. For the rare Mendelian hypertensive diseases discussed above, knowledge of their pathogenesis provides the opportunity to direct therapy to the primary underlying abnormality rather than an empiric approach. This is of clinical importance

because without diagnosis, these patients are frequently thought to have refractory hypertension. For example, in the case of GRA, patients can be specifically treated with physiologic doses of glucocorticoids, which suppresses secretion of ACTH and shuts down expression of the disease-causing chimeric gene. Other treatments that counter the effects of aldosterone excess, such as mineralocorticoid antagonists or inhibitors of ENaC, can also be effective. Similarly, patients with PHAII are exquisitely sensitive to thiazide diuretics, which reduce activity of the NaCl cotransporter whose activity is elevated in this disease. The value of specific therapy among these patients underscores the importance of diagnosing these diseases.

2. Hypertension in ESRD. As noted above, hypertension has a prevalence that approaches 100% among the more than 300,000 patients in the United States on dialysis for end-stage renal disease. This high prevalence is very likely attributable to inadequate removal of salt and water on dialysis, a consequence of efforts to reduce the number and time of dialysis sessions. Recent studies that employ more frequent or longer dialysis permit more net salt and water removal; these substantiate a dramatic reduction in the prevalence of hypertension (Chan et al., 2002) and strongly motivate modification of dialysis practice.

3. Treatment of Essential Hypertension. These genetic studies reveal variation in renal salt handling as a key site for long-term regulation of blood pressure in humans. Most significantly, mutations in this process impart very large effects on the trait, demonstrating the ability of this system to modulate blood pressure from extremely low to extremely high values. These observations implicate renal salt handling as a key target for therapeutic intervention in the treatment of hypertension. This suggests that drugs that target this pathway might be superior to agents that act on other pathways. Recent randomized controlled trials support this notion. For example, the 33,000-patient ALLHAT trial found that chlorthalidone, an inhibitor of the NaCl cotransporter, was as good as or better than other agents for all major clinical outcomes (ALLHAT Officers et al., 2002). Similarly, the Life study, a large randomized controlled trial of the angiotensin II receptor blocker losartan versus a beta blocker, showed superiority of losartan in prevention of stroke and overall morbid outcomes (Dahlof et al., 2002). These findings strongly support the primary

importance of reducing salt balance in the treatment of hypertension, which is now reflected in national guidelines (Chobanian et al., 2003).

Nonetheless, it is apparent that treatment with existing single agents is insufficient to achieve adequate blood pressure lowering in many patients. One explanation for this is the interference of counter-regulatory mechanisms, like the increased dietary salt intake documented among patients genetically deficient for the NaCl cotransporter NCC, the target of thiazide diuretics (Cruz et al., 2001). These observations suggest that initial therapy with inhibitors of the NaCl cotransporter plus an ACE inhibitor or AII antagonist should achieve synergistic effects; this has been substantiated by clinical study. These observations argue that the long-standing practice of starting therapy with a single agent is flawed, and they suggest more rational approaches that start with synergistic combination therapies; recent national consensus recommendations reflect such a change in approach (Chobanian et al., 2003).

C. New (and Old) Therapeutic Targets

These findings also have implications for new therapeutic development for the treatment of hypertension applicable to the general population. The most attractive targets are those that are pharmacologically tractable and that induce the largest blood-pressure-lowering effects with the fewest side effects. The phenotype resulting from loss of a gene product is a strong predictor of both the efficacious and adverse effects of a pharmacologic inhibitor of the gene product. The finding that loss of function mutations in a number of ion channels and cotransporters result in marked salt wasting and reduced blood pressure indicates that these channels are poised at key points in integrated physiology and moreover are not of redundant function; these genetic findings suggest that pharmacologic antagonists of these same channels and transporters would have potent antihypertensive diuretic effects. New targets that are predicted to have large effects on salt balance and blood pressure include the potassium channel ROMK, the chloride channel CLCNKB, and potentially the WNK kinases. Importantly, the paucity of clinical phenotypes in other organs among patients lacking these transporters and channels suggests that specific antagonists would not have limiting adverse effects in other organs.

There are specific features of these targets that might give their antagonists particular utility. For example, limiting side effects of treatment with existing antagonists of the Na–K–2Cl cotransporter are the development

of marked hypokalemia and deafness at higher doses. The potassium channel ROMK is used for K^+ recycling in the TAL and for K^+ secretion in the collecting duct. As a result, individuals deficient for ROMK have massive Na^+ depletion but only mild potassium wasting; their hearing is also normal. These findings suggest that ROMK antagonists would have potent diuretic effects like furosemide without the hypokalemic or loss of hearing side effects. These might be superior agents for treatment of hypertension as well as congestive heart failure.

Finally, some channels previously thought to be poor targets have been resurrected by this work. Existing ENaC antagonists such as amiloride are typically poor antihypertensive agents, which contributed to the false impression that this channel normally contributes little to salt balance. The massive salt wasting seen in patients who are deficient in ENaC reveals that this channel is absolutely essential for normal salt homeostasis. The limited efficacy of amiloride is attributable to the action of this drug as a competitive antagonist for Na^+; on a typical high salt diet, amiloride cannot effectively compete with Na^+ to achieve channel antagonism. These considerations strongly suggest high efficacy of a potent ENaC antagonist; in this case the resulting volume depletion could not be compensated by activation of the renin–angiotensin system, since ENaC is itself the means by which this compensation is achieved. Due to its role as the final arbiter of net Na^+ reabsorption, ENaC could be the ideal target; nonetheless, it is presently unknown whether hyperkalemia or metabolic acidosis would be limiting adverse effects at higher levels of channel inhibition.

D. Evolutionary Considerations

Given the strong evidence of the role of salt in the pathogenesis of hypertension, the role of salt in the evolutionary history of humans is worth considering. Sub-Saharan Africa is one of the most notoriously salt-poor environments on earth. It is consequently highly likely that genes that promoted avid salt retention were highly adaptive and strongly selected in mankind's ancestral environment. After the diaspora of humans to salt-rich environments, however, one can anticipate that those same alleles that were once adaptive lost their selective advantage and might indeed have become deleterious due to their promotion of hypertension and its morbid consequences. If this is correct, one might expect to see ethnic variation in the prevalence of hypertension, with those whose ancestors left Africa 100,000 years ago having a lower incidence of

hypertension than those who only recently have been exposed to a high salt diet. These considerations may help explain the markedly higher prevalence of hypertension among African Americans and other recent descendents of native African populations.

One prediction of this proposal is that alleles that promote salt retention in the African population should be selected against and be reduced in frequency in populations that long ago left Africa. For example, variants in the angiotensinogen gene have been implicated as having modest but significant effects on blood pressure (Jeunemaitre et al., 1992). Intriguingly, the angiotensinogen allele conferring higher blood pressure has a frequency near 100% in native African populations (Lifton et al., 1993) but has been reduced to only 35% in Caucasian and Japanese populations.

E. Understanding the Working of Integrated Systems

An important aspect of these genetic studies is their ability to provide new insights into the integrated systems biology of blood pressure and renal salt homeostasis. For example, the NaCl cotransporter of the DCT normally mediates reabsorption of ~7% of the filtered salt load, whereas the Na^+ channel ENaC is responsible for only 1–2%. It is nonetheless impossible to predict the impact of the loss of either of these due to the unknown ability of other nephron segments to compensate for the loss of one of these systems. Thus it is revealing that patients missing the NaCl cotransporter have only about 8-mmHg reduction in blood pressure while patients deficient in ENaC require lifelong massive salt supplementation; we infer that the loss of NCC can be compensated by increased activity of ENaC and/or other pathways, whereas loss of ENaC activity cannot be reasonably compensated. This underscores the role of ENaC as a key regulated step in the overall salt-retaining pathway.

Second, these studies have identified the Wnk kinases as a previously unknown regulatory system that has divergent effects on multiple components of the salt retaining and K^+ secreting pathway. Prior to the identification of the role of Wnk kinases in the regulation of ion flux, it was commonly held that the integration of function of diverse flux pathways was achieved indirectly, *en passant*, as a consequence of the combined effects of changing electrochemical driving forces, hemodynamics, and tubular flow. Perhaps not surprisingly, the kidney has evolved

additional mechanisms to achieve more direct control over the integrated function of these pathways. In addition to being of interest from a systems biology perspective, the Wnk kinase pathway represents a potentially interesting new target for therapeutic intervention.

Finally, these studies also provide further insight into integrative physiology by identifying secondary effects of mutations in these salt-handling pathways. For example, the marked effects of loss of function Na–Cl cotransporter mutations in producing renal Mg^{2+} loss and Ca^{2+} retention reveals unanticipated interrelationships between handling of salt in the DCT and these divalent cations that would not have been predicted from known physiology. The mechanisms linking genotype to phenotype in this case are not presently understood, but indicate a deeper relationship between homeostasis of salt and divalent cations than previously appreciated.

VIII. Conclusions

Genetic studies of humans with blood pressures at the extremes of the distribution have established that primary alteration of net renal salt reabsorption alters blood pressure in humans. The finding that the same pathway is involved in many different diseases that feature altered blood pressure suggests that altered renal salt handling likely underlies all forms of hypertension. These findings have implications for the improved selection and combination of medications to lower blood pressure. In addition, new potential therapeutic targets have been identified at which antagonists are likely to have improved efficacy and reduced adverse effects compared with current therapies. These findings promise to improve the treatment of hypertension and reduce its morbid outcomes. Finally, the utility of investigating humans with extreme blood pressure phenotypes to define the fundamental pathways that regulate this trait serves as a model for similar approaches to other common complex traits in humans.

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