In Vitro Study of the Juxtaglomerular Apparatus and Its Implications in the Chronic Kidney Disease

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Online Data Supplement

The renin–angiotensin system (RAS) plays important roles in the regulation of renal functions and blood pressure (BP) and in the maintenance of homeostasis of electrolyte balance and body fluid composition under physiological conditions. For example, during dehydration and sodium depletion, specific actions of the RAS in the specific sites within the kidney are indispensable for maintenance of systemic circulation and glomerular filtration rate (GFR); thus, the RAS and complex renal structures and functions are essential components for survival. On the other hand, under pathological conditions, such as hypertension, diabetes mellitus, and chronic kidney disease, the RAS now becomes a villain that causes damages in the brain, heart, kidney, and vasculatures. Why is the RAS, a single system, beneficial for survival under one condition but harmful in other conditions? We may be able to find an answer in the process of the evolution of life.1–3

I have been engaged in the study of the RAS and renal hemodynamics from the basic and clinical aspects of hypertension and nephropathy. In the basic study, I developed unique in vitro methods to directly study the function of the juxtaglomerular apparatus (JGA) that is composed of glomerular afferent and efferent arterioles and a plaque of specialized tubular epithelial cells called the macula densa.4–7 In the clinical research, together with many colleagues, I have performed large prospective cohort studies in chronic kidney disease and also large clinical trials in diabetic and nondiabetic nephropathy or hypertension.8–11

This review summarizes findings of our research on the RAS and renal hemodynamics together with other relevant literature, focusing on the structure–function correlates of the kidney in relation to renal sodium handling and maintenance of GFR. In addition, I will describe a unique circulatory system commonly existing in vital organs, such as brain, heart, and kidney. This circulatory system, which I refer as strain commonly existing in vital organs, such as brain, heart, and kidney. This circulatory system, which I refer as strain vessel, may provide a reasonable explanation for an important clinical observation of chronic kidney disease; that is, even if the GFR is normal, microalbuminuria is closely associated with both a high incidence of cardiovascular events (CVEs) and salt sensitivity of BP in such diseases as hypertension and diabetes mellitus.

In Vitro Study of Renin Release From Isolated JGA

It is now clear that the JGA has ≥2 functions, namely the control of renin release and glomerular hemodynamics. Renin was first discovered by Tigerstedt and Bergman12 in 1898, and subsequent studies clarified close relationships between the sodium balance and renin release. In 1945, based on observations that the macula densa is in close contact with the renin-containing juxtaglomerular cells of afferent arterioles (Figure S1 in the online-only Data Supplement) and that both the juxtaglomerular cells and macula densa exhibit characteristic morphological changes in response to dietary sodium manipulations, Goormaghtigh13 suggested that the macula densa may somehow sense tubular signal and control renin release and GFR. However, this hypothesis had remained unproven for a long time, and there were controversies on whether a low or a high NaCl concentration at the macula densa activates RAS in JGA.14,15 Moreover, the macula densa is not the only structure that has an intimate anatomic relationship with the afferent arteriole. It was pointed out that the connecting tubular segment is also in direct contact with the afferent arteriole, and some investigators suggested that the connecting tubule may also control renin release and glomerular hemodynamics.16,17 After many years of microdissection, I have confirmed that in the superficial nephron, the distal tubular segment after the macula densa runs together with an initial segment of efferent arteriole toward superficial direction and then forms a hairpin curve at the surface and goes back to the end of the parent afferent arteriole18 (Figure S1). The tubular segment now becomes the connecting tubule and runs side-by-side along the entire length of the afferent arteriole in the retrograde direction.

To study the role of the macula densa in renin release directly, I developed unique preparations of microdissected afferent arterioles with or without attached macula densa and incubated them in an isotonic solution.4,5 We found that the basal rate of renin release was lower in the afferent arterioles with attached macula densa than in those without, whereas furosemide stimulated renin release only in the presence of the macula densa. These findings provided the direct evidence...
that the macula densa indeed participates in the regulation of renin release. We speculated that the inhibition of basal renin release by the macula densa may be because of a high NaCl concentration of our incubation medium (Na+ 133 mEq/L; Cl− 126 mEq/L) when compared with that of the tubular fluid at the macula densa in vivo (=60 mEq/L). We also found that adenosine antagonist theophylline abolished the differences in basal renin release between the preparations with and without macula densa, suggesting that an enhanced adenosine production induced by high NaCl at the macula densa inhibited renin release. The direct effect of NaCl concentration at the macula densa on renin release was subsequently studied by Skøtt and Briggs, who used isolated JGA preparation in which the macula densa segment was perfused. They showed that decreasing NaCl concentration at the macula densa increases renin release. The studies using the isolated JGA preparation contributed significantly to our understanding of the mechanism of macula-mediated renin release, such as important roles of adenosine, prostaglandins, and nitric oxide.

**In Vitro Study of Glomerular Hemodynamics**

The GFR and renal blood flow are maintained at a constant level for a wide range of renal perfusion pressures (autoregulation). It is thought that the myogenic response and macula densa–mediated tubuloglomerular feedback (TGF) are the 2 intrinsic mechanisms for renal autoregulation. Using microperfusion techniques, Thurau and Schnermann first reported that changes in the composition of the tubular fluid in the distal nephron affect the GFR at the level of the single nephron, a phenomenon known as TGF. When the distal Cl− concentration and the proximal tubular pressure (an index of single-nephron GFR) were measured simultaneously, they were found to oscillate synchronously, with an increase in the Cl− concentration being associated with a decrease in proximal tubular pressure. Thus, it seems that the TGF is an exquisitely intricate mechanism for maintaining the GFR constant at the level of the single nephron. Because the JGA displays an intimate anatomic relationship between the macula densa and glomerular afferent and efferent arterioles, it was assumed to be the anatomic site of TGF. However, attempts to obtain direct evidence to support this had been hindered by the anatomic complexity of the JGA. Because the JGA is located beneath the tubular layer at some distance from the surface of the kidney, the macula densa is neither accessible to direct micropuncture in vivo nor direct observation of the vascular pole possible. In addition, as described earlier, the connecting tubule may participate in the so-called TGF.

To circumvent these limitations of the in vivo approaches and to directly study the macula densa–mediated glomerular hemodynamics, we developed a novel in vitro preparation in which both the afferent arteriole and the macula densa of a single microdissected JGA is microperfused simultaneously (Figure S2). In this preparation, a fine pressure-measuring pipette (outer diameter, 2–3 μm) was inserted through the perfusion pipette into the afferent arteriole, allowing us to control perfusion pressure in the presence of luminal flow. Using this preparation, we observed that increasing the NaCl concentration of the macula densa perfusate constricts the afferent arteriole in the terminal segment close to the glomerulus. This observation provided direct evidence that the macula densa participates in the control of glomerular hemodynamics. We also studied the myogenic response by perfusing the afferent arteriole with various intraluminal pressures and found that the site of myogenic response was more proximal than that of the macula densa–mediated response. Thus, the myogenic and macula densa–mediated responses are located in series along the afferent arteriole. Thus, the myogenic response is the first to respond to changes in renal perfusion pressure to prevent changes in glomerular capillary pressure, whereas any changes in single-nephron GFR that are not prevented by the myogenic response are reflected as changes in NaCl concentrations at the macula densa, with subsequent tuning of vascular resistance of the terminal segment of the afferent arteriole. Such interactions of the myogenic and macula densa–mediated afferent arteriolar responses may enable the kidney to achieve extremely efficient autoregulation.

We subsequently performed a series of experiments and reported that afferent arteriolar constriction induced by a high NaCl concentration at the macula densa was attenuated by an adenosine A1 antagonist, while it was augmented by inhibition of nitric oxide synthase in the macula densa. The roles of nitric oxide and adenosine in macula densa control of glomerular hemodynamics were confirmed by later experiments performed in the target gene–manipulated animal models. In addition, we invented a method of removing the endothelium from microperfused afferent arterioles with the use of antibody against factor VIII and complements. By perfusing 2 afferent arterioles simultaneously, one with free flow and the other without (Figure S3), we found that both myogenic and angiotensin II (Ang II)–induced constrictions were weaker in the presence of luminal flow, whereas either removing the endothelium or inhibiting nitric oxide synthase augmented the vasoconstriction, abolishing these differences. We also perfused efferent arterioles either in the orthograde direction through the glomerulus or in the retrograde direction. By comparing the vascular responses between the 2 preparations, we were able to examine whether vasoactive substances produced by the glomerulus can influence the vascular resistance of the downstream efferent arterioles. We found that Ang II–induced efferent arteriolar constriction was weaker in the orthograde than retrograde perfusion, and the difference became much less by the pretreatment with indomethacin, suggesting that prostaglandins produced by the glomerulus control the vascular resistance of the downstream efferent arteriole. This may be one of the mechanisms that the glomerulus controls its own capillary pressure.

In vitro microperfusion approaches have proven useful to study specific mechanisms of JGA functions. These methods have been successfully applied to various imaging studies and electrophysiological studies of JGA, and readers are referred elsewhere. In addition, Ren et al perfused both afferent arteriole and attached connecting tubule simultaneously and demonstrated that the connecting tubule does indeed control afferent arteriolar tone, and they named this phenomenon connecting TGF. Interestingly, in the connecting TGF, an increase in the tubular NaCl concentration causes dilation of the afferent arteriole, a completely opposite response to that induced by the macula densa. This third intrinsic mechanism...
may help explain renal hemodynamic phenomena that are not completely explained as yet, for example, renal vasodilatation induced by acute sodium loading. However, physiological and pathophysiological significances of the connecting TGF remained to be fully investigated.

Structure and Function of the Mammalian Kidney and Their Teleological Significance

The mammalian kidney is complex with structural and functional heterogeneities, and all of these complexities are needed to maintain homeostasis of body fluid composition. For more details, readers are referred to previous reviews.1,3

The kidney receives abundant blood supply, >90% of which is distributed to the renal cortex. Renal medulla receives much less blood, and therefore, much less oxygen delivery. Because of the characteristic vascular structures (vasa recta bundle) and high oxygen consumption, outer medulla is an anatomic site most susceptible to ischemic injuries. Particularly, medullary thick ascending limbs (mTALs) of the superficial nephron are the tubular segments most vulnerable to ischemic injuries because their location is far from the vascular bundle, and therefore, less oxygen is delivered (Figure S4). Indeed, it has been reported that the characteristic renal pathology of acute kidney injuries is the necrosis of mTALs close to the collecting duct.35 Thus, the task imposed on the kidney is to maintain a high GFR (150 L/day) and a high reabsorption rate, while protecting the mTAL from ischemic injuries by reducing its workload (oxygen consumption) even in the face of limited salt intake and low systemic BP.

This task is sophisticatedly accomplished because of the well-organized renal structure and the site-specific action of Ang II. When sodium intake is low and systemic BP is reduced, renin release from the JGA increases and thereby raising circulating and intrarenal Ang II. It is important to note the heterogeneity of the vasoconstrictor actions of Ang II in the superficial and juxtamedullary afferent arterioles. By microperfusing the afferent arteriole in vitro, we have shown that vasoconstriction induced by intraluminal Ang II is much stronger in the superficial than in the juxtamedullary afferent arterioles, with maximal constriction being 70% and 30%, respectively.36 Because of the strong constriction of the distal branches (superficial afferent arterioles), intraluminal pressure in the proximal segment of the interlobular artery would be increased. Thus, the heterogeneity of Ang II action would decrease superficial nephron GFR and increase juxtamedullary nephron GFR, maintaining the whole kidney GFR at a relatively constant level. In fact, it has been shown that a low-salt diet or administration of Ang II causes the redistribution of renal blood flow and GFR from the superficial cortex to the juxtamedullary cortex.37,38

The redistribution of GFR has an important physiological meaning for efficient recovery of filtered sodium and for avoiding ischemic renal injuries. When juxtamedullary GFR is increased, oxygen demand of the juxtamedullary nephrons would increase. However, mTALs of these nephrons are located in the vicinity of the vasa recta (Figure S4), and therefore, oxygen supply would be enough to avoid ischemic injuries of this nephron segment. On the other hand, the decreases in the single-nephron GFR combined with increased proximal tubular absorption (by increased filtration fraction and Ang II’s direct action) would greatly reduce the amount of sodium delivered to mTALs of superficial nephrons, the most vulnerable tubular segment to ischemic injuries. This would reduce oxygen demand and prevent ischemic injuries of mTALs of the superficial nephrons. Thus, the kidney is sophisticatedly designed structurally and functionally, so that a high GFR and electrolyte balances are maintained even under a low sodium intake and low systemic BP, while protecting the kidney itself from ischemic injuries. When we look into the kidney from the teleological point of view, we can understand the necessity of its complex and well-organized structures and functions.

Microalbuminuria, CVE, and Salt Sensitivity of BP

It has been clearly established that in lifestyle-related diseases, microalbuminuria is a predictor for adverse CVE independently of the level of GFR.39 Studies have now shown that albuminuria even within the reference range (=10 mg/day) is associated with higher incidence and prevalence of cardiovascular disease, and this risk increases in a continuous fashion with the degree of albuminuria. The significance of albuminuria can be recognized when we think of the fact that the amount of albumin in 24 hour-GFR is as large as 6 kg (4.0 g/dLx150 L/day) in normal: why such a miniscule amount of albumin in urine (as little as 10 mg of 6 kg) can be so closely related to CVE? Microalbuminuria is also closely associated with salt sensitivity of BP, and the salt sensitivity is an independent risk factor for CVE even in normotensive subjects.40 The mechanism of the association among albuminuria, CVE, and salt sensitivity of BP is still largely unknown, and it is a focus of intensive research. In contrast to lifestyle-related diseases, a substantial amount of albuminuria does not seem to be a significant risk factor for CVE in primary glomerular diseases. This apparent discrepancy would suggest that what is really important as cardiovascular risk is not the severity of albuminuria but the mechanism by which albumin appears in the urine. To explain reasonably the close inter-relationship among albuminuria, salt-sensitive BP, and CVE specifically in lifestyle-related diseases (but not in primary glomerular diseases), we have proposed strain vessel theory as described below.1,3

Microalbuminuria results from glomerular injuries and reduced tubular reabsorption of albumin. Using immunohistochemical staining for albumin, Kralik et al41 reported that the kidney from albuminuric type 2 diabetic subjects showed a heterogeneous pattern of staining, suggesting that albuminuria originates only from a small fraction of nephrons. Using micropuncture techniques, Yoshioka et al42 have shown that histologically intact nephrons are the primary origin of proteinuria in chronic renal disease. Heterogeneity of hypertensive renal tissue injuries has also been observed that renal tissue damages are most obvious in the juxtamedullary region and outer medulla in various hypertensive models,43,44 as well as in humans.45 In addition, we and others have shown that glomerular lesions first appear predominantly in the juxtamedullary nephrons and then extend toward more superficial nephrons in spontaneously hypertensive rats and
Otsuka-Long-Evans-Tokushima-Fatty rats.\textsuperscript{46–48} Such distinct localization of glomerular injuries and mode of progression may be related to anatomic and functional heterogeneities of different nephron populations.

The juxtamedullary glomeruli are located deep in the cortex, and their afferent arterioles arise either from the initial segment of the interlobular artery or directly from the large high-pressure arcuate artery (Figure S5). From the hemodynamic point of view, the juxtamedullary afferent arteriole is a small vessel exposed to a high and pulsatile pressure and is destined to maintain strong vascular tone to provide the large pressure gradient in a short distance between the large arcuate artery and the glomerulus. We refer to these kinds of vessels as strain vessels.\textsuperscript{1–3} In contrast, in the superficial nephrons, a more gradual pressure reduction occurs along the greater length of vasculature, including the entire interlobular artery and afferent arterioles. It is of note that the interlobular artery also participates in renal autoregulation, and, therefore, the feeding pressure of superficial afferent arterioles is substantially lower than that of juxtamedullary afferent arterioles.\textsuperscript{49} These differences in pressure load and vascular tone of arterioles can explain why the anatomic sites that are injured initially or more severely are the juxtamedullary afferent arterioles and glomeruli. Thus, in arteriosclerotic diseases, such as hypertension and diabetes mellitus, renal injury would occur predominantly in the juxtamedullary nephrons, whereas the majority of other nephrons remain relatively intact. This would be expected to result in only minimal increases in urinary albumin excretion.

As discussed above, microalbuminuria may be an early marker of vascular damages of strain vessels in lifestyle-related diseases. Other strain vessels exist most notably in the central nervous system (Figure S5), where many perforating arteries arise directly from large high-pressure arteries, such as anterior, middle, or posterior cerebral arteries, and penetrate into the brain tissues. It is well known that the sites of hemorrhage or infarction in the brain are frequently the areas of blood supply governed by these perforating arteries.\textsuperscript{50} Thus, strain vessel injuries may explain the close link between microalbuminuria and stroke. Indeed, we have shown that in stroke-prone spontaneously hypertensive rats fed a high-salt diet, hypertensive vascular damages occur predominantly in juxtamedullary afferent arterioles, and that the degree of arteriolar damages of the juxtamedullary nephron correlated well with that of perforating arteries of the middle cerebral artery.\textsuperscript{46} Coronary arteries can also be regarded as strain vessels. They arise directly from the aorta, and during the systolic phase, the entire epicardial segments of coronary arteries are exposed to a high pressure. In addition, because the coronary blood flow is intermittent, the endothelium of coronary arteries is exposed to large variations of the shear stress, a condition known to facilitate the formation of atherosclerotic lesions.

From the above discussion, we can also understand why albuminuria is not a risk factor of CVE in primary glomerular disease. Urine abnormality is the first sign of primary glomerular diseases, and its manifestation is often proteinuria (macroalbuminuria) rather than microalbuminuria. The degree of urine abnormalities reflects the degree of glomerular injuries but not strain vessel injuries. Thus, in primary glomerular diseases, albuminuria alone may not be a risk factor of CVE because strain vessels are not the sites of injuries.

The strain vessel theory may also be able to explain a close association of microalbuminuria with salt sensitivity of BP. It is well known that alteration of pressure natriuresis plays an important role in the pathogenesis of salt-sensitive hypertension and that the renal medullary circulation plays a crucial role in the mechanisms of pressure natriuresis.\textsuperscript{51} Blood supply to renal medulla is governed by descending vasa recta, the downstream of the efferent arterioles of juxtamedullary nephrons. Microalbuminuria would indicate the existence of damages in juxtamedullary afferent arterioles and glomeruli and therefore impairments of the downstream efferent arterioles and vasa recta. Thus, it may be speculated that microalbuminuria may reflect an impaired medullary circulation and therefore impaired pressure natriuresis, resulting in salt sensitivity of BP. Thus, the strain vessel injuries may partially explain the reason why salt sensitivity of BP by itself is an independent risk factor of CVE, particularly of stroke.\textsuperscript{52} Taken together, the strain vessel theory may be one reasonable explanation for the close inter-relationships among microalbuminuria, salt sensitivity of BP, and cerebrocardiovascular mortality and morbidity.

**Central Hemodynamic, Albuminuria, and CVE**

Central BP measured at the aorta differs from BP measured at the brachial artery. Because strain vessels arise directly from large high-pressure arteries, they would be greatly influenced by central hemodynamics. It is well known that increased arterial stiffness accelerates pulse wave velocity and results in higher systolic and lower diastolic BP in the central aorta. Such changes would accelerate strain vessel injuries in vital organs. Indeed, Munakata et al\textsuperscript{53} have shown that pulse wave velocity is associated with the degree of albuminuria in general and hypertensive populations. Liu et al\textsuperscript{54} have reported that the association between pulse wave velocity and albuminuria is strong in hypertensive and diabetic subjects. In addition, we have shown recently that central pulse pressure is closely related to changes in renal hemodynamics (resistive index of renal segmental artery) and urinary albumin excretion rate.\textsuperscript{55} It has been reported that a pulse wave velocity and altered central hemodynamics are associated with high rates of CVE independent of BP measure at the upper arm.\textsuperscript{56} Unlike other small vessels in peripheral circulation where blood flow and pressure are rather constant, the strain vessels are exposed to pulsatile pressure and flow, and therefore, stiffness of large arteries would have a great effect on the burden imposed on strain vessels.

**Evolutionary Point of View and Perspectives**

Why do we need such vulnerable structures as strain vessels or the RAS that may cause organ damage? From the evolutionary point of view, we speculate that unique structures, such as strain vessels in vital organs, as well as neurohormonal systems, such as the RAS, would have been essential for creatures on the land to survive in their natural environments. Given the difficult access to salt and a high risk of wound injuries, hypotension and hypoperfusion of vital organs were the principal challenges with which they had to
cope. For this purpose, the complex and well-organized renal structures and the site-specific vasoconstrictor and sodium-retaining actions of the RAS were all indispensable to maintain systemic circulation and a large GFR, while avoiding ischemic renal injuries. In addition, in the face of hypotension, a special circulatory system was needed to maintain enough blood flow to crucial sites for survival, such as basal ganglia and brain stem that are the centers of motor, circulatory, and respiratory functions. The strain vessel would be one such system because it branches off directly from the large high-pressure artery and maintains high vascular tone at baseline. When systemic BP is dropped, vasodilation of strain vessels would efficiently deliver blood to the downstream tissues. All the above mentioned structures and functions of vital organs may have been acquired during evolution of life to cope with hypotension. For example, the fact that the JGA appears first in amphibian species in evolution suggests that the transition from aquatic life to terrestrial life required this particular structure and its functions.57

In modern society, lifestyle-related diseases, such as hypertension, obesity, and diabetes mellitus, have become widespread because of excessive intakes of calories and salt and reduced physical activity. This situation was unexpected, viewed from the process of evolution. In particular, hypertension and arteriosclerosis impose the greatest threat on strain vessels as they are greatly affected by systemic (or central) hemodynamics. Without sufficient evolutionary time to adapt to the modern diets of the rapidly developed industrialized societies, the organisms were not designed to cope with high-salt intake, hypertension, and obesity, as reviewed elsewhere.58–60 In other words, although we human beings enjoy the benefits of many developments after the industrial revolution, we have to keep in mind that our fate is still governed by the natural laws of evolution.

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Figure S1. Anatomy of the superficial nephron and juxtaglomerular apparatus. Abbreviations are: Af-Art, afferent arteriole; Ef-Art, efferent arteriole; MD, macula densa; DCT, distal convoluted tubule; CNT, connecting tubule.
S2. Schematic representation of the pipette arrangement used for perfusion of an afferent arteriole (Af-Art) and attached macula densa (MD) (above) and a photograph of a preparation (below). Abbreviations are: TALH, thick ascending limb of Henle’s loop; GL, glomerulus; DCT, distal convoluted tubule; Ef-Art, efferent arteriole; Hold-Pip, holding pipette; Perf-Pip, perfusion pipette; Exch-Pip, exchange pipette; Pre-Pip, pressure pipette. (Modified from reference 6 and 24)
Figure S3. Photographs of preparations of double afferent arteriolar perfusion (top), orthograde perfusion of efferent arteriole (middle) and retrograde perfusion of efferent arteriole (bottom). Abbreviations are same as in Figure S2. (Reproduced from references 29 and 31)
Figure S4. Anatomic structures of the renal vasculature and the tubular segments. In the outer medulla the long loops of the juxtamedullary nephrons lie closest to the vascular bundle, while shorter loops arising from superficial glomeruli are more peripheral, and therefore, closer to the collecting ducts. The afferent arteriole of juxtamedullary nephron is the first branch of the interlobular artery. (Reproduced from reference 3)
Figure S5. Strain vessels in the kidney and brain. In an early stage of renal injury in lifestyle-related disease (i.e. hypertension, diabetes and obesity), arteriolar damage occurs mostly in juxtamedullary afferent arteriole because it is exposed a high pressure and it has to maintain a high vascular tone in order to create a large pressure gradient between arcuate artery and glomerulus. The juxtamedullary glomerular damage follows the arteriolar damage, resulting in albumin leakage from the glomerulus. More superficial glomeruli are intact because the feeding pressure of the afferent arterioles is substantially low. Thus, albumin leaked out of the juxtamedullary glomerulus is diluted by albumin-free urine from other nephrons, resulting in microalbuminuria in the final urine. Analogous to the vascular structure of the kidney is the perforating artery of the brain. The similarity of vascular structure of the brain and kidney seems to be a basis for close associations between microalbuminuria and stroke in life style-related diseases.