Altered Subcellular Distribution of Na\textsuperscript{+},K\textsuperscript{+}-ATPase in Proximal Tubules in Young Spontaneously Hypertensive Rats

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Abstract—During early development of hypertension, the spontaneously hypertensive rat (SHR) demonstrates increased proximal tubule sodium reabsorption. Our previous observations of reduced Na\textsuperscript{+},K\textsuperscript{+}-ATPase catalytic \(\alpha\)1 and \(\gamma\) subunit transcript abundance in SHR proximal tubule led us to test the hypothesis that increased proximal tubule sodium reabsorption may be attributable to altered subunit protein abundance, post-translational modification, or a shift in subcellular \(\alpha\)1 and \(\gamma\) distribution toward the basolateral membrane. We now extend previous gene expression studies by analyzing total cellular \(\alpha\)1 and \(\gamma\) protein abundance in proximal tubule from SHR compared with matched Wistar–Kyoto (WKY) controls. We also used sucrose density-gradient centrifugation to isolate basolateral, early, and late endosomal membrane–enriched fractions as well as cell surface biotinylation to test the hypothesis of altered subunit subcellular distribution in the SHR proximal tubule. At 4 weeks of age, significantly greater amounts of \(\alpha\)1 were present in basolateral membrane–enriched fractions of SHR than WKY (21.1 ± 1.8\% versus 12.3 ± 1.8\%; \(P<0.005\)), and there was a concomitant reduction of \(\alpha\)1 in late endosomal membrane–enriched fractions of SHR (63.3 ± 2.7\% versus 74.8 ± 4.3\%; \(P<0.05\)). This finding was confirmed in cell surface biotinylation studies that showed higher \(\alpha\)1 (1.45 ± 0.1-fold greater; \(P<0.05\)) and \(\gamma\)-subunit (3.48 ± 0.7-fold greater; \(P<0.01\)) abundance in 4-week-old SHR proximal tubule plasma membrane compared with matched WKY samples. These studies support the hypothesis that development of hypertension in SHR may involve an altered subcellular distribution of proximal tubule Na\textsuperscript{+},K\textsuperscript{+}-ATPase subunits. (Hypertension. 2004;44:95-100.)

Key Words: hypertension, genetic rats, spontaneously hypertensive sodium Na\textsuperscript{+},K\textsuperscript{+}-transporting ATPase phosphorylation

Kidney transplantation studies have shown that hypertension in the spontaneously hypertensive rat (SHR) is a renal disorder and that total body sodium retention is a correlate of increasing blood pressure after renal transplantation from SHR donors into immunologically compatible normotensive recipients. Before onset of hypertension, SHR exhibits increased sodium and water reabsorption, and the proximal tubule (PT) has been implicated in this sodium retention. Fractional lithium reabsorption studies provide in vivo evidence confirming that increased renal sodium reabsorption in the SHR kidney is localized to the PT. The primary active transporter responsible for driving this reabsorption is Na\textsuperscript{+},K\textsuperscript{+}-ATPase. We have shown that transcript abundance of the catalytic \(\alpha\)1 and the \(\gamma\) subunit of Na\textsuperscript{+},K\textsuperscript{+}-ATPase in PTs is lower in SHR than in its normotensive control strain, Wistar–Kyoto (WKY). These differences precede onset of hypertension and may reflect altered renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase regulation in SHR that is involved in increased tubular sodium reabsorption. Increased Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity (measured indirectly as ATP hydrolysis under saturating substrate conditions) in PTs from young (5 to 10 weeks old) SHR has also been reported. However, this difference was absent in older (>10 weeks old) SHR. These results have been extended by Beach et al, who demonstrated greater ATP hydrolysis by Na\textsuperscript{+},K\textsuperscript{+}-ATPase in basolateral membrane (BLM) preparations from renal cortex dissected from 5-week-old SHR compared with WKY but no difference at 16 weeks.

Together, these data point to a regulatory defect in the function of Na\textsuperscript{+},K\textsuperscript{+}-ATPase within young SHR PT, which may contribute to hypertension pathogenesis. Such a defect may be attributable to enhanced translation of \(\alpha\)1 or \(\gamma\) transcripts, reduced degradation of proteins, or altered post-translational modifications, resulting in greater Na\textsuperscript{+},K\textsuperscript{+}-ATPase protein subunit abundance and function within young SHR PT. We also hypothesized that such a change might be attributable to altered subcellular targeting of Na\textsuperscript{+},K\textsuperscript{+}-ATPase so that a greater fraction of Na\textsuperscript{+},K\textsuperscript{+}-ATPase is directed to BLM in SHR, where it can participate in renal transepithelial sodium reabsorption.
A key mechanism of epithelial Na⁺,K⁺-ATPase regulation is trafficking protein between endosomal and BLM compartments. This process is controlled by α1 N-terminal serine (Ser-11 or Ser-18) phosphorylation in response to G-protein-coupled receptor activation (dopamine, serotonin, or angiotensin II). In this study, we followed up our study of α1 and γ subunit mRNA transcript abundance by comparing relative abundance of α1 and γ subunit protein between WKY and SHR PTs. We also examined Ser-18 phosphorylation state of α1 in these samples. In addition, we developed evidence in support of the hypothesis of altered Na⁺,K⁺-ATPase targeting by comparing protein abundance of α1 and γ subunits in various membrane compartments of the PT.

Methods

An expanded Methods section is available in an online supplement available at http://www.hypertensionaha.org.

Animals

Studies were performed on male SHR and WKY of the Heidelberg substrains from colonies maintained in our animal facility. Animals were studied at 4 and 16 weeks old.

Preparation of Subcellular Membrane Fractions

PT cells were isolated using the method described by Seri et al. PTs were disrupted and nuclei were removed by centrifugation and the postnuclear supernatant (PNS) collected. Early endosomes (EEs) and late endosomes (LEs) were fractionated from the PNS by sucrose flotation–gradient centrifugation using the method described by Gorvel et al. A third fraction containing cell ghosts, mitochondria, and BLM was also collected. BLM was further enriched using the technique described by Hammond et al. Protein amount per fraction was determined using the bicinchoninic acid assay (Pierce).

Identification of EE-, LE-, and BLM-enriched preparations was verified by enrichment of marker proteins (rab5a; Santa Cruz Biotechnology) for EE, insulin-like growth factor receptor II (Transduction Laboratories) for LE, and rat organic anion transporter I (Chemicon) for BLM.

DNA Isolation

Genomic DNA was purified from PTs using the Puregene DNA Purification kit following the protocol of the manufacturer (Genta Systems). After PT lysis, an aliquot was removed for protein concentration determination by the Bradford assay (Bio-Rad), and DNA was isolated from the remaining lysate. DNA recovery was measured by 260-nm absorbance. Protein recovery was standardized per microgram of DNA.

Cell Surface Biotinylation

Cell surface biotinylation was performed using a modification of the technique described by Enfors et al. Brieﬂy, freshly isolated PTs were diluted (1 mg of cell protein) to 970 µL of PBS, pH 8.0. EZ-Link Sulfo-NHS-Biotin (Pierce) was added to cells to a ﬁnal concentration of 1.5 mg/mL. After biotinylation, cells were disrupted, and biotinylated proteins were collected using streptavidin-coated paramagnetic beads (Promega). Paramagnetic beads were washed, and isolated proteins were separated from the biotin-streptavidin bead complex by incubation at 60°C for 15 minutes in Laemmli buffer.

Western Blots to Quantitate Protein Abundance

For subcellular fractions, PNS, and cell lysates, a uniform amount of protein was loaded into each lane. For biotinylated protein preparations, equal volumes (7.5 µL) were loaded. We conﬁrmed that comparable amounts of protein had been loaded onto each gel by silver staining. Na⁺,K⁺-ATPase α1 protein was identiﬁed by a polyclonal antiserum raised against an Na⁺,K⁺-ATPase α1 oligopeptide.
protein/DNA in the PT was compared between strains. No differences between strains were detected (Figure 2).

To investigate the hypothesis that SHR PT Na⁺,K⁺-ATPase has a greater distribution to the BLM, we investigated subcellular distribution of PT α1 in SHR and WKY BLM-, LE-, and EE-enriched fractions at 4 and 16 weeks of age. As expected, the BLM fraction recovered the greatest quantity of total protein, followed by the LE and EE. There was no strain-specific difference in total protein recovered for each fraction (data not shown). At 4 weeks, there was a greater percent abundance of α1 in BLM, a lower percent abundance in LE, and no difference in percent abundance in EE (BLM: WKY 12.3±1.8%, SHR 21.1±1.8%, P<0.005; LE: WKY 74.8±4.3%, SHR 63.3±2.7%, P>0.05; EE: WKY 13.1±3.8%, SHR 15.7±2.4%, NS; mean±SEM; Figure 3A). At 16 weeks, no significant difference between strains in percent abundance of α1 in any of the fractions was observed (BLM: WKY 24.3±10.1%, SHR 28±7.5%, NS; LE: WKY 73.1±9.4%, SHR 71.3±7.55%, NS; EE: WKY 2.5±1.2%, SHR 0.7±0.7%, NS; mean±SEM; Figure 3B). A representative Western blot showing the distribution of α1 protein in subcellular fractions is shown in Figure 3C. The observation of greater α1 abundance in SHR BLM was supported by direct comparison of multiple independent BLM samples from 4-week-old WKY and SHR animals that were analyzed in a single Western blot (SHR 4.87±0.96; P<0.05; fold difference±SEM; Figure 4A and 4B).

To confirm the greater abundance of α1 in BLM of young SHR, PTs were recovered from a different set of young SHR and WKY, plasma membrane proteins were isolated by cell surface biotinylation, and α1 abundance was compared. Results show a significantly greater abundance of α1 in SHR plasma membrane than WKY (SHR 1.45±0.1; P<0.05; fold difference±SEM; Figure 4C and 4D). Thus, 2 different methods to analyze cell surface α1 abundance showed a greater amount in SHR. Cell surface biotinylation also confirmed the observation of no difference in α1 abundance in 16-week-old SHR BLM (SHR 0.81±0.13; NS; fold difference±SEM; Figure 4C). A significantly greater abundance of γ-protein is also observed in PT plasma membrane derived from 4-week-old SHR but not from 16-week-old SHR (4-week-old SHR 3.48±0.7, P<0.01; 16-week-old SHR 1.0±0.28; NS; fold difference±SEM; Figure 5A and 5B). These results point to a dysfunction in regulation of subcellular distribution of Na⁺,K⁺-ATPase α1 and γ-subunits in young SHR.

Numerous studies indicate that distribution of rat Na⁺,K⁺-ATPase between BLM and endosomal compartments is controlled by phosphorylation of α1 Ser-11 or Ser-18 by stimulation of G-protein–coupled receptors and kinase activation.9–11 Examination of the phosphorylation state of α1 Ser-18 in PTs using the McK1 monoclonal antibody revealed that at 4 weeks of age, relative abundance of non-Ser-18–phosphorylated α1 in PNS derived from SHR PTs was greater compared with WKY (SHR 3.45±0.85; P<0.05; fold difference±SEM; Figure 6A and 6B). At 16 weeks of age, there was no difference between SHR and WKY in abundance of non-Ser-18–phosphorylated α1 in PNS (SHR 1.41±0.22; NS; fold difference±SEM; Figure 6A). Therefore, in young SHR, there is an alteration of phosphorylation state of an α1 serine residue that has been implicated in subcellular distribution of this subunit.
Young SHR demonstrates greater renal sodium retention and PT Na\(^+\)/K\(^-\)-ATPase activity concurrent with elevation of blood pressure.\(^4,7,8,20\) Previously, we observed that transcript abundance of genes encoding 2 of the subunits of Na\(^+\)/K\(^-\)-ATPase was consistently lower in SHR PT than in the normotensive strain.\(^6\) This observation, coupled with reports of increased PT sodium reabsorption and Na\(^+\)/K\(^-\)-ATPase activity, leads to our hypothesis that Na\(^+\)/K\(^-\)-ATPase is mistargeted in SHR so that there is a greater abundance of Na\(^+\)/K\(^-\)-ATPase in BLM, the site where active sodium reabsorption occurs.\(^21\)

This study extends our earlier Na\(^+\)/K\(^-\)-ATPase \(\gamma\)-subunit transcript abundance study\(^6\) to the protein level. Lack of correlation between the relative transcript and protein abundance of the young SHR and WKY reported here may be a result of altered Na\(^+\)/K\(^-\)-ATPase subunit mRNA kinetics (message half life, translational efficiency) or lower protein turnover rate. Membrane fractionation results provide an explanation (greater distribution of Na\(^+\)/K\(^-\)-ATPase subunits to the BLM) for the greater Na\(^+\) reabsorption exhibited by young SHR despite no difference in overall \(\alpha\) protein abundance.

Whether there is a primary alteration in Na\(^+\)/K\(^-\)-ATPase causing altered distribution and greater Na\(^+\) transport in young SHR PT is unknown. A primary defect in a transporter resulting in altered subcellular distribution and pathogenesis of disease is not unprecedented. Mutations in either the \(\beta\)-subunit of another P-type ATPase, H\(^+\)/K\(^-\)-ATPase, or the amiloride-sensitive sodium channel (ENaC) result in increased expression of each transporter in the plasma membrane of gastric parietal cells or Xenopus oocytes, respectively.\(^22,23-25\) Therefore, mutations affecting Na\(^+\)/K\(^-\)-ATPase subcellular distribution analogous to those found in H\(^+\)/K\(^-\)-ATPase or ENaC may also contribute to hypertension in this model. Our own limited survey of key internalization motifs of Na\(^+\)/K\(^-\)-ATPase has uncovered only synonymous single nucleotide polymorphism (data not shown). A more extended sequence comparison, including related genes involved in BLM insertion and removal, may provide new insight into this regulatory abnormality.
Integration of basolateral sodium transport with apical sodium entry and the emerging role of intracellular sodium ion concentration as a modifier of sodium reabsorption regulatory mechanisms add complexity to interpretation of the present findings. There is evidence that the greater distribution of Na⁺,K⁺-ATPase subunits to young SHR PT plasma membrane may occur concurrently with elevation of intracellular sodium concentration ([Na⁺]). SHR PT exhibits an age-dependent change in apical sodium–hydrogen exchanger (NHE3) activity similar to that observed for Na⁺,K⁺-ATPase activity and distribution (greater in young SHR only). Increased PT NHE3 activity may lead to increased [Na⁺], allosteric increase of Na⁺,K⁺-ATPase activity, and increased Na⁺ reabsorption. Redistribution of Na⁺,K⁺-ATPase to the plasma membrane may be one aspect of increased activity. In support of this, Ibarra et al have shown that elevating [Na⁺], results in a predominantly non-Ser-18–phosphorylated α1. Thus, the greater abundance of non-Ser-18–phosphorylated Ser-18 α1 (Figure 6) in young SHR may be in response to an elevated [Na⁺], caused by overactive NHE3. The rendering of α1 as predominantly Ser-18 nonphosphorylated may antagonize the effect of dopamine-dependent Ser-18 phosphorylation and subsequent endocytosis. Alternatively, the greater abundance of nonphosphorylated Ser-18 α1 may be attributable to the observed failure of dopamine to stimulate protein kinase C in SHR PT. Clearly, regulation of Na⁺,K⁺-ATPase is highly dynamic with multiple levels of regulation working together to control sodium transport.

Functional studies have revealed that γ enhances affinity of Na⁺,K⁺-ATPase for ATP. It has been postulated that the purpose of increased ATP affinity afforded by γ is to maintain Na⁺,K⁺-ATPase activity in energy depleted/anoxic regions of the kidney, where γ-subunit protein is most abundant. There is evidence that SHR PT is in an energy-depleted state relative to WKY. Welch et al have shown a reduced partial pressure of oxygen in SHR PT compared with WKY. Greater γ-protein abundance apparent in SHR PT and plasma membrane reported here may be a homeostatic response to sustain Na⁺,K⁺-ATPase activity in these conditions. At 4 weeks of age, this occurs despite the fact that SHR PT Na⁺,K⁺-ATPase activity is greater. Obvious questions are whether increased γ-subunit abundance in SHR is in response to anoxia or contributes to anoxia by increasing Na⁺,K⁺-ATPase ATP usage. A better understanding of γ-subunit and Na⁺,K⁺-ATPase function in the PT is necessary to interpret the relevance of these findings. However, we hypothesize that greater γ-subunit protein abundance in young SHR PT plasma membrane may lead to increased interaction with α1, resulting in elevated activity caused by a greater affinity for ATP.

Magyar et al analyzed Na⁺,K⁺-ATPase activity after fractionation of PTs derived from young and mature SHR and Sprague–Dawley rats by sorbitol density–gradient centrifugation. Comparison of Na⁺,K⁺-ATPase activity by strain showed a significantly greater activity within fractions enriched in Na⁺,K⁺-ATPase in young SHR compared with young Sprague–Dawley rats but no difference in total PT Na⁺,K⁺-ATPase α1 or β-subunit abundance. Lack of correlation between Na⁺,K⁺-ATPase activity and subunit abundance was interpreted as a higher activity per transporter. Methodologies we used extend sorbitol density–gradient separation by identifying the location of key subcellular membrane compartments and permit us to perform a comparative examination of Na⁺,K⁺-ATPase distribution between them. Our findings support and extend those of Magyar et al and provide one explanation (increased basolateral Na⁺,K⁺-ATPase abundance) for higher Na⁺,K⁺-ATPase activity per transporter in young SHR.

In conclusion, we provided direct evidence of an alteration within young SHR PT in regulation of Na⁺,K⁺-ATPase at levels of subunit abundance, subcellular distribution, and phosphorylation. These findings provide a cellular mechanism by which increased renal sodium reabsorption, and consequently elevated blood pressure, in SHR may be generated.

**Perspectives**

The SHR is a model of renal polygenic hypertension with increased PT sodium reabsorption. Our results reveal multiple alterations in Na⁺,K⁺-ATPase regulation. We report alterations in subcellular distribution and post-translational modification of catalytic α1 and in protein abundance and subcellular distribution of the regulatory γ-subunit in SHR PT. It is unclear whether these differences are related through a common alteration or are the result of multiple levels of regulation simultaneously impinging on Na⁺,K⁺-ATPase. It will be valuable to determine mechanisms responsible for this altered regulation because they may reflect primary regulatory abnormalities linking increased renal sodium reabsorption to development and maintenance of hypertension.

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**References**


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