Loss of Collectrin, an ACE2 Homologue, Uncouples Endothelial Nitric Oxide Synthase and Causes Hypertension and Vascular Dysfunction

Running title: Cechova et al.; Role of collectrin in blood pressure homeostasis

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Abstract

Background—Collectrin is an orphan member of the renin-angiotensin system and is a homologue of ACE2, sharing ~50% sequence identity. Unlike ACE2, collectrin lacks any catalytic domain. Collectrin has been shown to function as a chaperone of amino acid transporters. In rodents, the renal expression of collectrin is increased after sub-total nephrectomy and during high salt feeding, raising the question whether collectrin has any direct role in blood pressure regulation.

Methods and Results—Using a susceptible genetic background, we demonstrate that deletion of collectrin results in hypertension, exaggerated salt sensitivity and impaired pressure natriuresis. Collectrin knockout mice display impaired endothelial dependent vasorelaxation that is associated with vascular remodeling, eNOS uncoupling, decreased nitric oxide production and increased superoxide generation. Treatment with tempol, a superoxide scavenger, attenuates the augmented sodium sensitivity in collectrin knockout mice. We report for the first time that collectrin is expressed in endothelial cells. Furthermore, collectrin directly regulates L-arginine uptake and plasma membrane levels of CAT1 and y+LAT1 amino acid transporters in endothelial cells. Treatment with L-arginine modestly lowers blood pressure of collectrin KO mice.

Conclusions—Collectrin is a consequential link between the transport of L-arginine and eNOS uncoupling in hypertension.

Key words: hypertension, salt sensitivity hypertension, arginine, angiotensin-converting enzyme, amino acids, collectrin
Collectrin (Tmem27, or Nx-17) was first identified in a screen for genes that are upregulated during the hypertrophic phase after renal mass ablation, a model of chronic kidney disease (CKD)\(^1\). The protein product is a 222-amino acid transmembrane glycoprotein that shares ~50\% sequence identity with angiotensin-converting enzyme 2 (ACE2). Unlike ACE2, collectrin lacks any catalytic domain\(^2\). Collectrin is highly conserved among species, sharing >80\% identity between mouse, rat, and human. Collectrin has a discrete tissue distribution, with highest expression levels in the proximal tubule and collecting duct of the adult kidney, followed by pancreatic B-cells, liver, intestinal epithelial cells, and the retina. Taken together, these data suggest that collectrin has distinct effects specific to its tissue localization. In support, \textit{in vitro} and \textit{in vivo} studies reveal that collectrin is involved in insulin secretion\(^3\) and islet mass in the pancreas\(^4\). In the kidney, we and others demonstrated that deletion of collectrin results in severe generalized urinary amino acid wasting due to reduced expression of neutral and cationic amino acid transporters in the plasma membrane of the proximal tubule brush border\(^5\)\(^-\)\(^6\). These studies illustrate a role of collectrin as a chaperone for amino acid transporters. The mechanism by which collectrin mediates amino acid transporter trafficking is thought to be through its binding with the SNARE complex. In the pancreas, collectrin facilitates SNARE complex formation by interacting with snapin, a synaptosomal-associated protein\(^3\). In the collecting duct, collectrin interacts with snapin, SNAP-23, syntaxin-4 and VAMP-2\(^7\).

As a member of the ACE family, how collectrin is functionally integrated in the context of blood pressure regulation is not known. The collectrin gene is located on the X chromosome, where loci have been linked to HTN in both the human and rat species\(^8\)\(^-\)\(^9\). The expression of collectrin in the kidney is upregulated after 5/6 nephrectomy\(^1\) and during salt sensitive hypertension\(^7\), but the cause or effect remains to be established. Interestingly, in rat models of
salt sensitive hypertension, loci on the X chromosome have also been linked to HTN and hypertensive nephrosclerosis.

Taken together, the evidence suggests that collectrin plays a role in blood pressure regulation. We set out to determine the role of collectrin in blood pressure homeostasis in normal and high salt states. On a mixed genetic background, we found no blood pressure difference between collectrin knockout (KO) and wild-type (WT) mice. Due to the possibility of genetic background masking any blood pressure effect commonly observed in many mouse models of cardiovascular disease (CVD) and hypertension, we backcrossed the collectrin null mutation on the 129S6 strain that is susceptible to the development of hypertension and salt-sensitivity. Here, on the 129S6 background, we show that collectrin is a determining factor in the development of hypertension in conditions where L-arginine/nitric oxide/superoxide pathway is altered.

Methods

Animals

Mice deficient of collectrin were generated by homologous recombination in embryonic stem cells, as previously reported. To generate inbred 129S6 collectrin KO mouse line, the null mutation was backcrossed onto the 129S6 background for > 14 generations. The collectrin gene is located on the X-chromosome; thus WT littermates cannot be obtained for female knockouts. Therefore, only male KO mice and their WT littermates were used for all studies described herein. Animals were bred and maintained under local and National Institutes of Health guidelines.

Radiotelemetric BP monitoring in vivo.

With the exception of blood pressure (BP) measurement after sub-total nephrectomy, all BPs
were measured in conscious mice under unrestrained conditions by radiotelemetry.

Radiotelemetric units were implanted as previously described. See Supplemental Information.

**Measurement of tissue superoxide and nitric oxide levels**

For superoxide, the method was modified from previous studies. Briefly, Krebs-Hepes buffer (in mM: 99.0 NaCl; 4.69 KCl; 1.2 MgSO₄; 25.0 NaHCO₃; 1.03 KH₂PO₄; 5.6 D(+)-GLUCOSE; 20 Na HEPES; 2.5 CaCl₂; pH= 7.2-7.4 with 1N HCl) was oxygenated for 15 min in a water bath @ 37°C. A 3-5 mg piece of kidney cortical tissue was placed in oxygenated buffer and incubated for 30 min in the water bath @ 37°C, then placed into 5 μM Lucigenin (9,9’-Bis-N-methylacridinium nitrate, Sigma: M8010) solution prepared in Krebs-Hepes buffer for 5 minutes. Then, luminescence counts were taken 5 times for 1 minute each, averaged and corrected for baseline (5 μM Lucigenin in Krebs-Hepes buffer measured 5 times for 1 minute and averaged). Tissue was dried in the dry block at 60-70°C overnight. Results are expressed as counts/mg of dry tissue. Nitric oxide activity was measured by conversion of [³H] arginine to [³H] citrulline via a nitric oxide assay kit (R&D Systems) as previously described.

**Endothelium-dependent and -independent relaxation assays**

Vasoreactivity measurements were performed as previously described. See Supplemental Information.

**Isolation of mouse pulmonary endothelial cells**

Primary lung endothelial cells were isolated as previously described, and were used for subsequent L-arginine uptake assay. Details are in Supplemental Information.

**[³H] L-arginine uptake**

[³H]L-arginine uptake assay was performed following previously published protocol. ECs were grown in 24-well plates to near-confluence, washed in DPBS, and incubated in Na-free
Uptake Buffer for 5 min at 37°C. Some of the wells were then incubated with 0.5 ml/well of either warmed 50 uM L-arginine + 100 nM [³H]L-arginine, or warmed 20 mM L-arginine + 100 nM [³H]L-arginine for 1 minute. The wells were then washed 4x with ice-cold Na-free Uptake buffer and the cells were solubilized in 0.5 ml 0.2%SDS, 0.2N NaOH. Two 0.2 ml aliquots were counted using a Beckman Coulter scintillation counter and 5 ml Ultama Gold LLT scintillation fluid (Perkin-Elmer), and two 25 ul aliquots were assayed for protein concentration using the Pierce® BCA Protein Assay Kit (Thermo Scientific).

Statistics

Data are expressed as means ± SE. Statistical calculations were done using commercially available software packages (Minitab, Inc. and NCSS). Student’s t-test was used for comparisons between two groups, unless otherwise stated. Differences between matched samples were analyzed by paired t-test. ANOVA was performed to test for significant overall group differences when there are more than two groups. Endothelium-dependent and –independent relaxation assays were analyzed using the GraphPad Prism software. P < 0.05 was considered statistically significant. P-values were two-sided. Given the nature of the study, there was no adjustment for multiple comparisons.

Results

Deletion of collectrin causes HTN, augmented salt-sensitivity, and attenuated pressure-natriuresis response

By radiotelemetry monitoring, collectrin KO mice have normal diurnal variation (Supplemental Figure 1A) but significantly elevated systolic blood pressure (SBP) (Figure 1A) and mean arterial pressure (MAP) (Supplemental Figure 1B) during both light and dark cycles. The
average SBP over a 14 day period at baseline (on normal salt diet) was ~ 9 mm Hg higher in collectrin KO mice (Figure 1B), and is associated with a significant increase in heart mass (Figure 1A) that correlates well with SBP (Supplemental Figure 1C), suggestive of increased pressure load inducing cardiac remodeling. Since the expression of collectrin is upregulated in rodent models of CKD and salt sensitive hypertension, we therefore assessed the effect of collectrin on blood pressure in these models. After sub-total nephrectomy (Nx), collectrin KO mice had significant mortality within 2 weeks (Supplemental Figure 2A). However, after uni-nephrectomy (UNx), collectrin KO mice had normal survival, but developed much more severe hypertension than WT (Supplemental Figure 2B). We next determined the effect of collectrin on salt-sensitivity by feeding mice a high salt diet (HSD - 6% NaCl) for 2 weeks. Compared to WT mice which displayed a ~ 8 mm Hg increase in SBP from normal salt (NS) to HSD, collectrin KO mice displayed ~ 15 mm Hg increase in SBP (Figure 1B). In addition, collectrin KO mice had a significantly lower urinary sodium excretion by the third day (acute phase) of HSD (Figure 1B). There were no significant differences in food or NaCl consumption between the two groups. However, there was a transient increase in water intake, and a trend for increased urine volume in collectrin KO mice on day 1 (Table 1). These findings demonstrate an attenuated pressure-natriuresis response in collectrin KO mice. The significant rise in BP in collectrin KO mice after HSD was also accompanied by evidence of end organ damage, including increased cardiac fibrosis (Figure 2A), glomerular mesangial hypercellularity and thickened renal vascular walls (Figure 2B).

**Deletion of collectrin results in altered balance of nitric oxide and superoxide**

To begin to understand the mechanism contributing to hypertension and salt-sensitivity in collectrin KO mice, we examined whole kidney plasma membrane expression of renal epithelial
salt transporters and channels, including αENaC, NHE3, NKCC, Na-K-ATPase, and NaPi2, but found no differences. (Supplemental Figure 1D). There was no difference in baseline plasma renin concentration (p = 0.40, Supplemental Figure 1E). There were also no differences in mRNA expression levels of ACE2 or Mas receptor in the renal cortex, by real-time RT-PCR (Supplemental Figure 1F). We next measured urinary 8-isoprostane F2\alpha and nitrates/nitrites levels that are indices of superoxide (O2 •−) and nitric oxide (NO) generation, respectively, during the first 3 days of HSD. Collectrin KO mice excreted more than twice the levels of 8-isoprostane F2\alpha, and 40% less nitrates/nitrites compared to WT (Figure 3A). These data suggest that augmented renal O2 •− generation and/or impaired renal NO production contribute to altered blood pressure regulation and pressure natriuresis in collectrin KO mice.

Hypertension and salt sensitivity are pathological conditions commonly associated with a state of eNOS uncoupling 20-23. In the active form, eNOS is a homodimer that oxidizes L-arginine to L-citrulline and NO. Various perturbations, including decreased availability of L-arginine, can lead to the uncoupling of eNOS to the monomeric form that generates O2 •− instead of NO 24-25. We next compared baseline levels of eNOS dimer and monomer in the aorta and kidney cortex and medulla. In these tissues, eNOS dimer and dimer/monomer ratios were significantly lower in collectrin KO mice compared to WT (Figure 3B), suggesting that there is an altered balance of active and inactive eNOS in collectrin KO mice that favors a state of increased O2 •− generation and reduced NO synthesis. In support, we find a significant increase in baseline O2 •− and a significant decrease in baseline NO levels in whole kidney tissues in KO mice (Figure 3C). We next determined whether the superoxide scavenger Tempol would correct the augmented sodium sensitivity in collectrin KO mice. Treatment with Tempol (via osmotic pump) resulted in a mild but statistically insignificant lowering of blood pressure in both WT and
KO mice at baseline. Tempol did not attenuate salt-sensitivity in WT mice. However, tempol significantly attenuated the increase in blood pressure in collectrin KO during high salt feeding (Figure 3D), resulting in similar change in blood pressure from normal salt to high salt between WT and KO mice (Figure 3E). These data suggest that the mechanism for augmented sodium-sensitivity in collectrin KO mice is in part mediated by superoxide.

**Loss of collectrin causes impairment in endothelium-dependent vasorelaxation**

The expression of collectrin in endothelial cells has not been previously reported. Here, we show that collectrin is expressed in endothelial cells in the thoracodorsal (TD) and renal resistance vessels (Figures 4A-E & Supplemental Figures 3A-B). We next queried whether loss of collectrin could lead to endothelial dysfunction, and compared resistance vessel relaxation capacity of 3rd order mesenteric arteries between WT and KO mice fed on NS diet. Collectrin KO mice have severely impaired endothelium-dependent relaxation (EDR) of 3rd order mesenteric arteries in response to acetylcholine (Figure 4F). Very similar differences in response to acetylcholine were also observed in thoracodorsal arteries between the two groups (Figure 4F). Transmission electron microscopy (TEM) revealed that the TD arteries of collectrin KO mice are markedly remodeled, with vacuolization of ECs and hypertrophic and disorganized smooth muscle layer (Figure 4G). To determine whether the vascular smooth muscle cells play a role in the impaired vasorelaxation in collectrin KO mice, we performed endothelium-independent relaxation assay using sodium nitroprusside (SNP). As shown in Figure 4H, WT and collectrin KO mesenteric and TD arteries had virtually identical vasodilatory responses to SNP, suggesting the impaired vasodilation in the collectrin KO mice is primarily dependent on the endothelium.

**Collectrin regulates L-arginine uptake and plasma membrane expression of L-arginine**
amino acid transporters in endothelial cells

Our data raise the central question whether collectrin regulates blood pressure through its role in amino acid transport, particularly L-arginine. In this regard, KO mice have altered blood pressure homeostasis that is associated with abnormal NO synthesis and O$_2^\bullet -$ generation. In several cell types, including the endothelial cells (ECs) and renal epithelium, the synthesis of NO requires the de novo import of extracellular L-arginine $^{26-28}$. L-arginine transport into cells is mediated by different classes of amino acid transporters that are defined by their ion dependency, substrate specificity, and relative affinity $^{29-30}$. In ECs, the system y$^+$, a sodium-independent system, accounts for $\sim$60% of L-arginine transport, and system $y^+$L, a sodium-dependent system, accounts for $\sim$40% of L-arginine transport $^{26,31-32}$. System y$^+$ selectively mediates the cellular transport and exchange of cationic amino acids, including L-arginine, whereas system y$^+$L transports and mediates the exchange of both cationic and neutral amino acids $^{26,28}$. EDR has been shown to be impaired in a patient with L-arginine deficiency who was shown to be a compound heterozygote for two mutations in the $SLC7A7$ gene that encodes for the $y^+$LAT1 amino acid transporter. The patient’s EDR was corrected with L-arginine infusion $^{33}$. Thus, a defect in L-arginine uptake may lead to endothelial dysfunction. To determine whether collectrin-deficient ECs have impaired L-arginine uptake, we isolated primary ECs from WT and collectrin KO mice from the pulmonary artery using FACs sorting $^{17}$. Collectrin KO ECs display significantly lower $[^3]$H-L-arginine uptake than WT ECs (Figure 5A). To rule out the possibility of altered structural integrity of collectrin-deficient endothelial cells (Figure 4G) influencing L-arginine uptake, and to demonstrate direct cause and effect of collectrin on L-arginine uptake, we used a primary human coronary EC (HCEC) line and successfully overexpressed collectrin (Figure 5B). HCECs overexpressing collectrin have significantly higher levels of $[^3]$H-L-arginine

uptake compared to control cells (Figure 5B).

We previously reported that collectrin KO mice have generalized urinary amino acid wasting, and decreased protein expression of the apical amino acid transporters in plasma membrane fractions of the renal proximal tubules, suggesting that collectrin is a chaperone that mediates the trafficking of amino acid transporters to the plasma membrane. We next examined whether collectrin affects the expression of the $y^+$ (CAT1) and $y^+$L (y$^+$LAT1) amino acid transporter systems that are known to regulate L-arginine transport in ECs. We demonstrate that knockdown of collectrin results in a significant decrease in expression of CAT1 and y$^+$LAT1 transporters in HCEC plasma membrane fractions, and decreased eNOS dimerization (Figure 5C). Conversely, overexpression of collectrin significantly increases the expression levels of these transporters, and increases eNOS dimerization (Figure 5D). We also examined whether loss of collectrin also affects kidney plasma membrane expression of the $y^+$LAT1 transporter that is localized to the basolateral membrane in renal epithelial cells. We found a significant decrease in expression of $y^+$LAT1 transporter in whole kidney plasma membrane fractions from KO mice (Supplemental Figure 4). We next queried whether supplementation with L-arginine would ameliorate or decrease blood pressure in collectrin KO mice. We administered L-arginine in drinking water (50 mg/kg/day). As demonstrated in Figure 5E, by the end of 1 week of treatment with L-arginine, a modest but significant lowering of SBP in collectrin KO mice was observed. There was no further lowering of SBP beyond 1 week of L-arginine treatment.

Discussion

Collectrin is an “orphan” member of the renin angiotensin system with a previously unrecognized role in cardiovascular and renal physiology. We demonstrate that loss of collectrin
results in hypertension, altered pressure natriuresis, and superoxide-dependent augmented salt sensitivity. We further demonstrate that deletion of collectrin results in endothelial dysfunction that is associated with eNOS uncoupling, increased $O_2 \cdot -$ production and decreased NO availability, likely due to impaired cellular uptake of L-arginine substrate. Our data suggest that collectrin plays a protective role against conditions that predispose to the hypertensive state by maintaining a balance of NO and $O_2 \cdot -$ through its role in facilitating the cellular uptake of L-arginine. It is worth noting that we previously reported collectrin KO mice have normal development and are able to compensate for renal wasting of amino acids by increased liver synthesis of non-essential amino acids and are able to maintain normal serum levels of all amino acids including, L-arginine $^5, ^{36}$. Thus, the effect of collectrin deficiency on blood pressure is unlikely from renal wasting of L-arginine per se. Our data suggest that collectrin may regulate blood pressure specifically through its role chaperoning L-arginine transporters expressed in ECs, and possibly in renal epithelial cells, for the intracellular uptake of L-arginine. The partial blood pressure lowering response to L-arginine infusion supports the notion of competitive inhibition of L-arginine uptake by other extracellular cationic amino acids such as lysine $^{28}$. Accordingly, administration of L-arginine could overwhelm the competitive inhibition of extracellular lysine. However, the lack of normalization of blood pressure by L-arginine infusion implicates a severe deficit in the functional activity of the CAT1 and $\gamma^L$ system transporters due to their diminished plasma membrane expression. It is also possible that collectrin interacts with other machinery that regulates eNOS enzymatic activity. In this regard, endothelial caveolae containing caveolins are important in determining intracellular localization of eNOS and enzymatic activity $^{37}$. Interestingly, endothelial caveolae contain some members of the SNARE complex $^{38}$ with which collectrin interacts $^{3, 7}$. Our study suggests that the altered balance of NO
and O$_2$ •− plays a partial role in the elevation of blood pressure and augmented sodium sensitivity in collectrin knockout mice. It is likely that other yet to be determined mechanisms are also involved.

Our findings are consistent with the clinical observation that mutations resulting in dysfunction of the y$^+$LAT1 transporter causes impaired EDR $^{33}$. The reduction in L-arginine transport via the y$^+$L system has also been observed in HTN in both humans and animals $^{39-40}$. In addition, the Dahl salt-sensitive (SS) versus salt-resistant (SR) rats are a classical genetic model that links salt sensitivity hypertension with alteration in the O$_2$ •−/NO states. Dahl SS rats consuming high-salt diet exhibit increased urinary excretion of 8-isoprostane, an index of oxidative stress, and significant reductions of kidney and aorta eNOS $^{41}$. Infusion of L-arginine into the renal medullary interstitium of Dahl SS rats prevents the development of HTN during high-salt feeding $^{42}$. The genetic basis for their phenotype is unclear. Our studies demonstrate that collectrin-deficient mice have a phenotype very similar that of the DSS rats.

While an 8-9 mm Hg baseline increase in SBP in collectrin KO may be viewed as modest, it is worth noting that this has significant physiologic effects, since collectrin KO mice have increased heart mass and vascular remodeling at baseline. Results from numerous clinical trials show that even a 2-3 mm Hg increase in SBP confers a significant increase in cardiovascular disease risks. In addition, on HSD, collectrin KO mice have ~15 mm Hg higher SBP than WT mice on HSD, along with increased cardiac fibrosis and mesangial hypercellularity in the glomerulus. This augmented sodium sensitivity is in part mediated by superoxide.

As mentioned above, our previous study using mice on a mixed genetic background showed that collectrin KO mice have similar basal blood pressure as WT mice, and they did not exhibit sodium sensitivity $^7$. This demonstrates that the role of collectrin on blood pressure
regulation in normal and pathophysiologic states is dependent on genetic background. The dependency on a susceptible genetic milieu is similar to many rodent models of human diseases. For example, the Ace2 and the frequently used Apoe knockout mouse models require the C57BL/6 strain for development of HTN \textsuperscript{14} and abdominal aortic atherosclerotic disease \textsuperscript{43-44}, respectively. The requirement of a proper genetic background does not diminish the significance of the effect of a gene, but highlights the involvement of epigenetic and/or multigenic effects. In essential or salt-sensitive hypertension, the pathogenesis is likely mediated by small effects of many genes, rather than a rare large effect of one single gene.

It is worth noting that our studies were conducted using male mice, since collectrin is on the X-chromosome and therefore wild-type littermates cannot be obtained for female knockout mice. It remains to be determined whether collectrin has the same effect on blood pressure and SSH in female mice. Interestingly, in the Sabra rat model of SSH, loci on the X chromosome have also been linked to salt-sensitivity in females but not in males \textsuperscript{10}. Due to discrepancy of the position of the rat microsatellite markers that differ significantly from 2003 to 2012, it is unclear whether collectrin lies within any of these loci. In humans, it is not known whether there are any loci on the X chromosome that contribute to sex-linked differences in heritability of hypertension, since not until more recently genome wide association studies (GWAS) have largely excluded X chromosome variants from analyses.

Although our results may suggest that the ED in collectrin KO mice could contribute to their HTN, they are interpreted with caution, since impairment in endothelial function may also be a consequence of HTN that induces remodeling of the endothelium. We cannot rule out possible effects of collectrin on circulating or other vasoactive mediators released by the endothelium, such as endothelium-derived hyperpolarizing factor (EDHF), prostacyclin and...
EETs. Endothelial specific deletion of collectrin will provide direct proof of causality, and will be a focus of future studies.

Our studies raise a key question as to which specific collectrin expressing cell plays a major role in the BP regulation. Guyton and colleagues advanced the paradigm that the kidney’s substantial capacity for sodium excretion provides a compensatory system of virtually infinite gain to oppose processes causing elevation in BP, including increases in peripheral vascular resistance. It follows that, in collectrin KO mice, even if the initial cause of HTN is due to increased peripheral vascular resistance from reduced vasorelaxation, a defect in renal excretory function would be a pre-requisite for the sustained chronic increase in BP. The altered pressure-natriuresis observed in collectrin KO mice suggests that the altered kidney function could result from a defect in renal epithelial function and/or renal hemodynamics. We found no alteration in the expression of renal epithelial sodium transporters or channels in the kidney in collectrin KO mice at baseline, but we cannot exclude improper localization of these transporters or channels. We postulate that the impaired pressure natriuresis is due, at least in part, to abnormal regulation of renal vascular resistance, possibly due to a deficiency in nitric oxide. These possibilities will be a major focus in future studies. Collectrin is also expressed in the basolateral surface of the collecting duct epithelium (and our unpublished data), where neuronal NOS (nNOS) is localized and has been shown to be the source of NO that mediates medullary blood flow and natriuresis. Whether collectrin affects the dimerization and function of nNOS in the collecting duct remains to be explored. Ultimately, it will be necessary to determine the precise cell lineage - in the kidney (and in which compartment - vascular vs. proximal tubule or collecting duct epithelium, or both) or outside the kidney, or both – in which BP is regulated by the actions of collectrin.
In summary, our studies have unveiled collectrin as a determining factor in the development of HTN in conditions where L-arginine/NO/O_2• - pathway is altered. Loss of collectrin function may also impact other physiological and pathophysiological states that are influenced by nitric oxide and/or superoxide signaling.

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**Conflict of Interest Disclosures:** None.

**References:**


32. Kilberg MS, Stevens BR, Novak DA. Recent advances in mammalian amino acid transport.


**Table 1.** Metabolic Cage Studies During the First Three Days on High Salt Diet

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<th>Day 1</th>
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Data are expressed as means ± SE. Initial body weight of WT mice was 26.2 ± 1.4, and of KO mice was 26.9 ± 0.88, p = NS. * p = 0.03.

**Figure Legends:**

**Figure 1.** Deletion of collectrin causes HTN, augmented SS, and attenuated pressure-natriuresis response. (A) Left, average SBP of WT and KO mice during 12 hrs periods of light and dark cycles over 14 days; * p ≤ 0.001. Right, heart weight/body weight ratios, * p = 0.016. (B) Top
left, average SBP over 14 days of normal salt (NS) and HSD; * p < 0.02. Top right, the change in SBP from NS to HSD was higher in KO mice, p=0.01. Bottom left, daily SBP at baseline (Days 9 – 14) and after HSD (Start and Days 1-14). Bottom right, average daily urinary sodium excretions during the first 3 days (acute phase) of HSD are significantly lower in the KO mice on the third day, p = 0.018. N ≥ 6 all studies, values are expressed as mean ± SE.

**Figure 2.** Evidence of end-organ damage in collectrin knockout mice. (A) Masson’s Trichrome staining of cross sections of cardiac ventricles (at 20X) from 3 mice in each group following high salt diet, showing increased fibrosis in KO mice. (B) Renal histology by light microscopy. Hematoxylin and eosin stain demonstrates normal glomeruli (arrowheads) and renal interlobar artery in WT mice, but diffuse glomerular mesangial hypercellularity and thickened vascular smooth muscle wall of interlobar artery in KO mice. Upper panels at 20X magnification, glomeruli inserts 40X. Lower panels 40X.

**Figure 3.** Deletion of collectrin results in altered balance of nitric oxide and superoxide. (A) Comparison of urinary 8-isoprostane F$_{2\alpha}$ and nitrates/nitrites levels, averaged over the first 3 days of HSD: 8-isoprostane F$_{2\alpha}$: p = 0.008; Nitrates/nitrites: p = 0.03, n ≥ 4 each (B) Comparison of active (dimer) and inactive (monomer) forms of eNOS. Left, immunoblot of aorta and kidney cortex and medulla at baseline. Right, densities of eNOS dimer and monomer bands, normalized to tubulin, , # p ≤ 0.04, $\Psi$ p ≤ 0.002. (C) Baseline renal tissue levels of superoxide and nitric oxide. Left: superoxide levels: * p=0.02. Right: nitric oxide levels; * p = 0.025. (D) Tempol attenuates the augmented sodium sensitivity in collectrin KO mice. Normal Salt: # p < 0.001; $\Psi$ p = 0.02. High Salt: $\delta$ p = 0.01, $\Psi$ p ≤ 0.02; N ≥ 6 in each group. (E) Tempol decreases change
in blood pressure in KO mice after high salt. Change in SBP: For WT + Tempol vs. KO + Tempol p = 0.52; For KO vs KO + Tempol, * p ≤ 0.04. Mice without tempol treatment are same mice represented in Figure 1B.

**Figure 4.** Collectrin is expressed in endothelial cells and loss of collectrin causes impairment in endothelium-dependent vasorelaxation. (A-D) Immunoperoxidase (IP) staining showing collectrin is expressed ECs in WT mouse in the renal (A) interlobar artery, (B) vasa recta, (C) interlobular artery with internal size marker of 50 µm. PT is proximal tubules. (D) Enlarged interlobular artery from (C). Arrows point to endothelial layer. Images were taken at 100X. (E) IP staining showing collectrin is expressed in ECs in the thoracodorsal (TD) artery at 40X and 100X. (F) EDR assay. Upper: 3rd order mesenteric arteries from KO mice have severely impaired EDR in response to acetylcholine (Ach) after pre-constriction with phenylephrine, * p < 0.02. Lower: TD arteries from KO mice also demonstrate similar severe impairment in EDR in response to acetylcholine, * p < 0.003. (G) TEM: At baseline, KO TD arteries display marked thickening and remodeling of vessel wall. L is lumen. ECs are vacuolized in KO mice. (H) Endothelium-independent relaxation assay with sodium nitroprusside (SNP). No differences are observed in response to SNP between WT and KO 3rd order mesenteric arteries (upper) and TD arteries (lower); p ≥ 0.6.

**Figure 5.** Collectrin regulates L-arginine uptake and plasma membrane expression of L-arginine amino acid transporters in endothelial cells. (A) Primary pulmonary ECs from KO mice have significantly lower [³H]L-arginine uptake, *p=0.01, n=6 separate wells each group, done in duplicates. (B) Upper, immunoblot of collectrin in primary human coronary ECs (HCECs): left -
untransfected, middle - empty pBK-CMV vector, right - transfected with pBK-CMV collectrin expression vector. Lower, $[^3]$H]-L-arginine uptake in primary HCECs transfected with empty (control) or collectrin expression vector, *p = 0.003, n=6 separate transfection experiments performed in duplicates. (C-D) Immunoblot comparing expression of collectrin and L-arginine amino acid transporters CAT1 and y+LAT1 in plasma membrane fractions of primary HCECs: (C) Upper panel, control vs. collectrin knockdown conditions with siRNA. Middle panel, relative densities, normalized to tubulin, *p < 0.05. Lower panel, immunoblot of eNOS dimers and monomers. (D) Upper panel, control vs. collectrin overexpression conditions. Middle panel, relative densities, normalized to tubulin, *p ≤ 0.04. Lower panel, immunoblot of eNOS dimers and monomers. (E) Blood pressure response to treatment with L-arginine; n = 5, *p = 0.008 by paired analysis.
Figure 1A

- Systolic Blood Pressure (mm Hg)
  - Light cycles
  - Dark cycles

- Heart weight/Body Weight (mg/g)
  - WT
  - KO
Figure 1B
Figure 2A
Figure 3A

Urinary 8-Isoprostanate (ng/mg creatinine)

WT KO

Urinary Nitrates/Nitrites (umol/day)

WT KO

*
Figure 3B
Figure 3C

Superoxide (counts/mg tissue)

WT KO

Nitric Oxide (nmol/mg protein)

WT KO

*
Figure 3D
Figure 3E

Without Tempol

With Tempol

Change in Systolic BP (mm Hg) with High Salt Diet

* *
Figure 4A-D

Renal Interlobar Artery

Renal Vasa Recta

Renal Interlobular Artery
Figure 4E
Figure 4F
Figure 4H
Figure 4H, cont’d
Figure 5A

[Diagram showing the comparison of [3]L-Arg uptake (pmol/mg protein/min) between WT and KO strains. The graph indicates a significant difference (*) between the two groups.]
Figure 5B

[Graph showing L-Arg uptake (pmol/mg protein/min) for Untransfected, Empty Vector, and Transfected conditions.]

[Western blots for Collectrin and Tubulin under different conditions.]
Collectrin CAT1 y+LAT1

Relative Densities (Normalized to Tubulin)

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Figure 5C
Figure 5D
Figure 5E
Loss of Collectrin, an ACE2 Homologue, Uncouples Endothelial Nitric Oxide Synthase and Causes Hypertension and Vascular Dysfunction


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SUPPLEMENTAL MATERIAL

Supplemental Experimental Procedures

**Radiotelemetric blood pressure monitoring *in vivo***. Briefly, mice were anesthetized with 2% isoflurane in medical oxygen (95% O_2 and 5% CO_2). A catheter and sensor (TA11PA-C10, Data Sciences International (DSI), St. Paul, MN) was implanted in the left carotid artery, and the radiotransmitter was placed in a subcutaneous pouch along the flank. A post-operative analgesic, buprenorphine, 0.1 mg/kg body weight, sc., and 0.25% bupivacaine, 0.02-0.05 mg/kg, sc., was given to relieve pain. Mice were allowed to recover for 7 days after surgery to regain their normal circadian rhythms before blood pressure measurements and experiments were initiated. While blood pressure was being monitored, mice were housed in a quiet room in individual cages placed above the telemetric receivers with an output to a computer. Blood pressures were collected every 1 hour for 5 minutes, processed, and analyzed using Dataquest A.R.T. 20 software (DSI).

**Urine studies.** Mice were housed individually in metabolic cages and urine was collected over a 24-hour period. Urinary sodium was measured using flame photometer (IL-943, Instrumentation Laboratory, Lexington, MA). Urinary 8-isoprostane F_{2a} was measured using commercially available ELISA kit from Oxford Biomedical Research, Inc. (Oxford, MI, Product number: EA 85) and urinary nitrates/nitrites were measured using commercially available Colorimetric Assay kit from Cayman Chemical Company (Ann Arbor, MI, Product number 780001), according to manufacturer’s instructions.

**Renal Ablation**: Under general anesthesia, (1.5 % isoflurane), the right kidney was removed (for both sub-total nephrectomy or uni-nephrectomy), and the blood supply of the upper half of
the left kidney was interrupted by ligation of the upper branch of the two main branches of the left renal artery (for sub-total nephrectomy) as previously reported (1).

**Measurement of blood pressure using tail cuff manometry.** Blood pressures in mice subjected to nephrectomy were measured using a computerized tail cuff system (Hatteras Instruments, Cary NC) that determines tail blood flow using a photoelectric sensor. This system allows for blood pressures to be measured in four mice simultaneously and minimizes the potential for observer bias. Mice are trained for blood pressure measurement everyday for 2 weeks, then systolic blood pressures are recorded daily for 2 weeks.

**Measurement of plasma renin concentration.** Plasma renin concentration was measured by radioimmunoassay of angiotensin I by use of the antibody-trapping technique (2). Only results with linearity in 3 serial dilutions were accepted. Renin values were standardized with renin standards obtained from the National Institutes for Biological Standards and Control (Potters Bar, Hertfordshire, UK) and are expressed in milliGoldblatt units per milliliter (mGU/ml).

**Tempol administration:** Tempol (4-hydroxy-Tempo, Aldridge-Sigma), was prepared in sterile saline and infused in mice using Alzet mini-osmotic pump, model 2004 (Durect Corporation, Cupertino, CA) at rate 200 nmol.kg⁻¹.min⁻¹ in a 28 day pump. Pump was implanted under the skin on the back of anesthetized mouse and wound was closed by surgical clips. Mice were fed high salt diet beginning on day 10 after Tempol initiation.

**Endothelium-dependent and -independent relaxation assays.** Vasoreactivity measurements were performed as previously described (3). Mice were sacrificed with CO₂ and both thoracodorsal (TD) arteries and the whole mesentery were isolated and placed in Kreb’s Hepes solution or MOPS buffer respectively. The TD and 3rd order mesenteric arteries were
free of surrounding tissue and cannulated at both ends on glass micropipettes, secured with a 10-0 nylon monofilament suture in a pressure myograph (Danish MyoTechnology, DMT). The TD and 3rd order mesenteric arteries were maintained at 37°C in a no-flow state and held at a constant transmural pressure of 80 mmHg and 75mmHg respectively (3-4). TD arteries and mesenteric arteries were pre-constricted with phenylephrine (PE, 10 µM), respectively and the internal diameter was measured in response to cumulative concentrations of Ach or SNP (10⁻⁹ to 10⁻³ M). Vessel diameter was quantified after each dose of Ach or SNP using the slide book software or the DMT vessel acquisition software as previously described (3, 5).

**Isolation of mouse pulmonary endothelial cells.** Primary lung endothelial cells were isolated as previously described (6). Tissue culture reagents were from Invitrogen unless otherwise noted. Lungs from 3 to 4 mice per group were first perfused with PBS containing 100 ug/ml heparin (Sigma) and 1% Anti-Anti and Pen/Strep (200 U/ml penicillin, 200 ug streptomycin, and 25 ng Amphotericin B.), minced, and digested in PBS containing 100 ug/ml heparin, 1% Anti-Anti and Pen/Strep, 0.1% collagenase (Sigma), and 5 mM CaCl₂ at 37°C for 30-60 min. After filtration through a 100 um cell strainer (BD Falcon), the cells were pelleted at 500 xg for 10 min, resuspended and washed twice in DMEM low glucose with 20 mM HEPES and 1% Anti-Anti and Pen/Strep, then washed once in DMEM low glucose, 20 mM HEPES, 1% Anti-Anti and Pen/Strep, and 20% FBS. Finally, the cells were resuspended in 3 ml of Complete Media, (consisting of DMEM low glucose containing 20% FBS, 20 mM HEPES, 2 mM each Glutamine and Sodium Pyruvate, 1% each Non-Essential amino acids, Anti-Anti, and Pen/Strep, 100 ug/ml each ECGS (endothelial cell growth supplement, BD Biosciences) and heparin, and 3.6 ug/ml thymidine), placed into one well of a 6 well plate, and incubated at 37°C in 95% O₂ 5% CO₂, 80% humidity. The red blood cells and other non-adherent cells were washed off after 1 day. The remaining cells were cultured in Complete Media with 10 ug/ml Dil-Ac-LDL (Biomedical
Technologies) for 3-4 hrs, then removed from the wells using Trypsin/EDTA and sorted by FACS. The positive cells were recovered and expanded in Complete Media in 6-well plates.

**Construction of collectrin over-expression vector.** Total RNA was extracted from whole mouse kidney tissue using TRIzol reagent (Invitrogen, NY, USA) and a spin-column purification kit (RNeasy Mini Kit; Qiagen, CA, USA) according to manufacturer protocols. cDNA was then generated using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad Laboratories, CA, USA) and was subsequently used as template for PCR. 5'- and 3'- PCR primers (Eurofins MWG Operon, AL, USA) were designed to amplify the collectrin cDNA and incorporate a 5'- *NheI* restriction site and a C-terminal *myc* epitope tag and 3'-*EcoRI* restriction site. The resultant PCR product was gel-purified (QIAquick Gel Extraction Kit; Qiagen, CA, USA), subjected to restriction enzyme digest (New England Biolabs, MA, USA) and cloned into the pBK-CMV expression vector (Stratagene, CA, USA) using standard techniques. Purified mini-prep plasmid samples (QIAprep Miniprep Kit; Qiagen, CA, USA) were sequenced for verification (UAB DNA Sequencing Core Facility; Birmingham, AL, USA).

**Knockdown and over-expression of collectrin in primary human coronary endothelial cells.** Primary human coronary endothelial cells (HCECs) (Lonza Group Ltd., Switzerland) were grown in MCDB 131 (GIBCO) supplemented with FBS, hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, hEGF, GA-1000 (Lonza Group Ltd., Switzerland) and 1% L-glutamine (GIBCO) in 75-cm² Corning tissue culture flasks at 37°C in a humidified environment of 5% CO₂ and air. Cells were maintained at subconfluent level and passaged using trypsin/EDTA (GIBCO). For siRNA studies, high-performance purity grade (>90% pure) small interfering RNAs (siRNAs) against collectrin (Collectrin-siRNA) and siRNAs with a nonsilencing oligonucleotide sequence (nonsilencing siRNA), used as a negative control (control-siRNA), were obtained from Ambion, Inc. Cells were seeded at a density of 3×10⁴ cells per well in 6-well plates and grown in MCDB
131 with the additives. One day after seeding, cells were transfected with 20 umol of control- or collectrin-siRNA using siPORT NeoFX Transfection Agent (Ambion) per manufacturer’s instructions. Seventy-two hours post transfection, cells were harvested for Western immunoblotting. For over-expression studies, 2 ug of plasmid DNA construct (PBK-CMV-collectrin cDNA construct to overexpress collectrin and PBK-CMV empty vector for control) were transfected using Transfectin lipid reagent (BIO-RAD) into HCECs, seeded at a density of 3×10⁴ cells and grown in 6-well plates. At 48 hours after transfection, the cells were harvested for Western immunoblotting and L-Arg uptake studies.

**Western immunoblotting.** Unless otherwise noted, tissues or cells were rapidly obtained and placed into ice-cold isolation buffer (10 mM Tris, 250 mM sucrose and 5 mM EDTA, pH 7.4) with protease inhibitor cocktail (Sigma-Aldrich). Homogenates were then rapidly processed as described (7-8). Briefly, tissues or cells were homogenized for 10 s and lysates were spun at 3000 g for 10 minutes at 4°C. The supernatant was saved on ice, and the pellet was resuspended, homogenized and centrifuged. The two supernatants were combined and spun at 16,000 g for 30 minutes at 4°C. The resulting supernatant represented the intracellular fraction. Pellets were resuspended in isolation buffer and represented the plasma membrane fraction. Protein concentrations of each fraction were determined by the BCA assay (Bioassay Systems). Twenty µg of total protein was loaded onto 10% SDS-PAGE gels and then transferred to PVDF membranes (Invitrogen) per manufacturer instructions. For eNOS, homogenization buffer of 50 mmol/l Tris-HCl, pH 7.6, 100 mmol/l NaCl, 2 mmol/l EDTA, 2 mmol/l EGTA, 1 mmol/l DTT, 1 mmol/l PMSF, 1% Triton X-100, and 6% SDS-PAGE gels and nitrocellulose membrane were used. Membranes were blocked using Blocking Solution (Invitrogen) for 1 hr at room temperature. Membranes were then incubated at 4°C overnight with primary antibody diluted in blocking buffer as follows: rabbit anti-collectrin antibody (1:1000) (custom made for Le’s lab by
Covance Immno Technologies, Denver, PA) by generation against the synthetic peptide (NDAFMTEDERLTPL) as previously reported (9), rabbit anti-eNOS (BD Biosciences) at 1:1000, anti-CAT1 antibody (Santa Cruz Biotechnology) at 1:1000, anti-y+LAT1 antibody (Santa Cruz Biotechnology) at 1:1000, anti-y+LAT2 (Santa Cruz Biotechnology) antibody at 1:1000, mouse anti-tubulin at 1:2000 (Santa Cruz). Antibody to αENaC (at 1:500) was a kind gift from Dr. Susan Wall (Emory University) and antibodies to NHE3, NKCC, Na-K-ATPase, and NaPi2 (at 1:1000) were kind gifts from Dr. Mark Knepper (National Institute of Health) (10-11). Secondary antibody incubation was performed for 30 minutes at room temperature with anti-rabbit and anti-mouse conjugated with alkaline phosphatase. Blots were visualized using WesternBreeze chemiluminescent detection kit (Invitrogen).

**Immunohistochemical and immunofluorescence studies.** Chromogenic immunohistochemical staining for collectrin was done on paraffin-embedded, formalin-fixed 4 μm cross-sections of kidney and thoracodorsal artery from WT mice using the Dako EnVision+ System-HRP (DAB) Kit, (using Rabbit anti Collectrin Ab), and the Dako Mayer’s Hematoxylin Histological Staining Reagent (Dako North America, Inc.) according to the manufacturer’s instructions. In short, the tissue was re-hydrated and antigens were unmasked using Antigen Unmasking Solution (low pH) (Vector Laboratories, Inc.) by heating in a microwave oven. Incubation was for 40 min at room temperature for the primary collectrin antibody (1:500), then the peroxidase labeled polymer; 10 min at room temperature for the DAB+ Chromogen; and 1-2 min with the Hematoxylin counterstain. The tissue sections were covered with coverslips, and sealed with VectiShield Hard Mount (Vector Laboratories, Inc.). Immunofluorescent staining was performed on paraffin-embedded, formalin-fixed 4 μm cross-sections of kidney from wild-type mice that were obtained using a microtome (RM2125, Leica Microsystems Inc., Bannockburn, IL). The sections were re-hydrated and antigen retrieved with microwave heat for
15 min in TEG buffer (10mM Tris and 0.5mM EGTA, pH 9.0). After neutralization with NH₄Cl buffer, the sections were blocked with 1% BSA, 0.2% gelatin, and 0.05% saponin in PBS before incubation overnight with primary antibody diluted in 0.1% BSA and 0.3% Triton X-100 in PBS. The primary antibody against collectrin was obtained as described above, and against CD105 (Biolegend) specific for endothelial cells). After rinsing with 0.1% BSA, 0.2% gelatin, and 0.05% saponin in PBS, the sections were reacted 1 hr with secondary antibody diluted in 0.1% BSA and 0.3% Triton X-100 in PBS. The secondary donkey anti-rabbit antibodies used were Alexa555- or Alexa647-conjugated (Invitrogen Corp., Carlsbad, CA). After washes with PBS, the sections were mounted in Vectashield solution containing DAPI to stain nuclei (H-1500, Vector Labs., Burlingame, CA). Confocal fluorescence images were taken using a Zeiss LSM 510 microscope and software (Carl Zeiss MicroImaging, Inc., Thornwood, NY).
Supplemental References


Supplemental Figure Legends.

Supplemental Figure 1. Comparison of SBP and MAP of Collectrin KO and WT mice. (A) KO mice have normal diurnal variation during 12 hrs light and dark cycles. (B) MAP of KO mice are significantly elevated during both light and dark cycles; Light: WT 111.1 ± 1.4, KO 117.6 ± 1.3 mm Hg, p = 0.002, Dark: WT 122.0 ± 1.6, KO 128.1 ± 1.4 mm Hg, p = 0.007, n = 6 each. (C) Correlation of SBP and heart weight/body weight ratio, R² = 42%, p = 0.003. Data points are from both WT and KO mice. (D) Immunoblot of plasma membrane fractions of whole kidney homogenates demonstrating that renal epithelial salt transporters or channels such as NKCC, NHE3, Na-K-ATPase, α-ENaC and NaPi2 transporters are not altered in collectrin-KO mice at baseline. Tubulin is loading control. Densities, normalized to tubulin, were not statistically significant. (E) Baseline plasma renin concentration, expressed as x 10⁻⁵ Goldblat Unit (GU)/mL. WT 575 ± 172 vs KO 404 ± 81 x 10⁻⁵ GU/mL, n ≥ 6, p = 0.40. (F) By real-time RT-PCR, baseline mRNA levels of ACE2 and mas receptor in the renal cortex are not different between wild-type and collectrin KO mice.

Supplemental Figure 2. (A) Survival rate of Collectrin KO and WT mice after sub-total nephrectomy, performed by removing the right kidney and ligating the arterial supply of the upper half of the left kidney. In the WT group, there is 100% (10 out of 10) survival on day 21. In KO group, 4 out of 7 mice died by day 21, giving a survival rate 43%; p<1x10⁻⁶. (B) SBP of collectrin KO mice is significantly increased after uni-nephrectomy compared to WT mice (WT 151 ± 2, n=9; KO 165 ± 2 mm Hg, n=7, p=0.0007).

Supplemental Figure 3. Expression of collectrin in vasculature. (A) Upper, immunoblot of collectrin protein in 3 out of 3 separate pure EC lines isolated from pulmonary artery of WT mice. Lower, immunoblot showing collectrin protein is expressed in the aortas from 6 WT mice. (B)
Confocal microscopy of the renal vasculature: red - antibody for collectrin, blue - CD105 antibody specific for ECs, fuchsia – merged pannels. Upper, the large renal vessels. Bottom, the sinus/inner stripe of the renal medulla where vascular bundles and capillary plexuses are found.

**Supplemental Figure 4.** Immunoblot of y+LAT1 transporter in plasma membrane fractions from whole kidney with tubulin as a loading control. N = 3 mice in each group. By densitometry, KO kidney PM fractions express significantly less y+LAT1, p = 0.01.
Supplemental Figure 1A

Days by 12 Hour Light and Dark Cycles

Supplemental Figure 1B

Mean Arterial Pressure (mm Hg)

Light

Dark
Supplemental Figure 1C

![Graph showing Systolic Blood Pressure (mm Hg) vs. Heart Weight/Body Weight Ratio (mg/g).](image)

Supplemental Figure 1D

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Supplemental Figure 1E

Supplemental Figure 1F
Supplemental Figure 2A

Percent Survival

Days

Supplemental Figure 2B

Systolic Blood Pressure (mm Hg)

WT
KO

*
Supplemental Figure 3A

Pulmonary Endothelial Cells
Whole Aorta Lysate

Supplemental Figure 3B

Renal Interlobar Artery
Renal Sinus
Supplemental Figure 4

WT          KO

\( y^* \text{LAT1} \)

\text{Tubulin}