Ligand trap for the Activin Type IIA receptor protects against vascular disease and renal fibrosis in mice with chronic kidney disease

Olga A. Agapova, Ph.D.1, Yifu Fang, M.D.1, Toshifumi Sugatani, D.D.S., Ph.D.1, Michael E. Seifert, M.D.1,3, and Keith A. Hruska, M.D.1,2,*

1Department of Pediatrics, Renal Division, Washington University, St. Louis, MO
2Department of Cell Biology, and Medicine, Washington University, St. Louis, MO
3Department of Pediatrics, Renal Division, Southern Illinois University, Springfield, IL

Abstract

The causes of cardiovascular mortality associated with chronic kidney disease (CKD) are partly attributed to the CKD-mineral bone disorder (CKD-MBD). The causes of the early CKD-MBD are not well known. Our discovery of Wnt (portmanteau of wingless and int) inhibitors, especially Dickkopf 1, produced during renal repair as participating in the pathogenesis of the vascular and skeletal components of the CKD-MBD implied that additional pathogenic factors are critical. In the search for such factors, we studied the effects of activin receptor type II A (ActRIIA) signaling by using a ligand trap for the receptor, RAP-011 (a soluble extracellular domain of ActRIIA fused to a murine IgG-Fc fragment). In a mouse model of CKD that stimulated atherosclerotic calcification, RAP-011 significantly increased aortic ActRIIA signaling assessed by the levels of phosphorylated Smad2/3. Furthermore, RAP-011 treatment significantly reversed CKD induced vascular smooth muscle dedifferentiation as assessed by smooth muscle 22α levels, osteoblastic transition and neointimal plaque calcification. In the diseased kidneys, RAP-011 significantly stimulated αklotho levels and it inhibited ActRIIA signaling and decreased renal fibrosis and proteinuria. RAP-011 treatment significantly decreased both renal and circulating Dickkopf 1 levels showing that Wnt activation was downstream of ActRIIA. Thus, ActRIIA signaling in CKD contributes to the CKD-MBD and renal fibrosis. ActRIIA signaling may be a potential therapeutic target in CKD.

Keywords

chronic kidney disease; vascular calcification; fibrosis; signaling

*Corresponding Author Keith A. Hruska MD, Renal Division, Department of Pediatrics, Washington University, Rm 5109 MPRB Building, 660 S. Euclid, Saint Louis, MO 63110, Phone: 314-286-2772, Hruska_k@kids.wustl.edu.

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Introduction

Kidney diseases are associated with high mortality rates related to their stimulation of cardiovascular disease.\(^1\) The kidney disease stimulation of cardiovascular risk extends to type 2 diabetes, where the presence of mild to moderate kidney disease increases atherosclerotic cardiovascular disease risk by 87%.\(^2\) Atherosclerotic calcification has become a standard biomarker of cardiovascular risk,\(^3,4\) and CKD increases atherosclerotic calcification.\(^5\) The causes of the increased cardiovascular risk associated with kidney diseases partly reside in the chronic kidney disease – mineral bone disorder (CKD-MBD) syndrome.\(^6\) Three non-traditional cardiovascular risk factors (hyperphosphatemia, vascular calcification, and elevated fibroblast growth factor 23 (FGF23) levels) have been discovered in the CKD-MBD,\(^7-9\) and their risk factor status confirmed in the general population.\(^10-12\) The CKD-MBD begins early in CKD (stage 2)\(^13-16\) consisting of arterial vascular cell dedifferentiation/calcification, an osteodystrophy, loss of klotho and increased FGF23 secretion.\(^13\) Progress into the causes of the CKD-MBD has been made,\(^16-19\) but they are mostly unknown. We have employed a murine model of type 2 diabetes, atherosclerosis and atherosclerotic calcification to demonstrate that CKD stimulates atherosclerotic calcification.\(^20,21\) The development of hyperphosphatemia further stimulates the process,\(^22,23\) but is not involved in its inception which begins much earlier in the course of CKD than hyperphosphatemia. Most recently we have focused on circulating factors produced by kidney disease that perturb normal physiologic systemic processes.

Multiple investigators and we have shown that kidney diseases reactivate developmental programs involved in nephrogenesis during disease stimulated renal repair.\(^24-28\) Among the nephrogenic factors reactivated in renal repair, the Wnt (portmanteau of Wingless and Integrated) family is critical for tubular epithelial reconstitution,\(^27-29\) and fibrosis. In the control of Wnt function, canonical signaling transcriptionally induces the expression of a family of Wnt inhibitory proteins which are secreted proteins that serve to restrict the distances of Wnt stimulation to autocrine or paracrine factors.\(^30-34\) The Wnt inhibitors are circulating factors, and the family includes the Dickkopfs (Dkk). We have shown that various forms of kidney disease increase renal expression of Wnt inhibitors including the Dkk family and increase their levels in the systemic circulation.\(^17,25\)

Neutralization of a key Wnt inhibitor elevated in the circulation in CKD, Dkk1, was efficacious in the CKD-MBD. Dkk1 neutralization inhibited CKD induced vascular dedifferentiation, vascular calcification, and renal osteodystrophy.\(^17\) This effect was surprising since Wnt signaling in the vascular smooth muscle cell (VSMC) is implicated in stimulating osteoblastic transition and vascular calcification.\(^35,36\) However, recent studies demonstrate that inhibition of Wnt signaling stimulates lipid accumulation in atherosclerotic plaques,\(^37\) and that Dkk1 mediated inhibition of aortic Wnt7b stimulates smad mediated aortic endothelial-mesenchymal transition (EndMT) and vascular calcification.\(^38\) EndMT is a developmental physiologic process involved in the development of the cardiac valves, the cardiac septum and the aortic root,\(^39,40\) and it may\(^41\) or may not\(^42\) contribute to cardiac fibrosis in various adult disease states. Since EndMT is a process driven by Smad transcription factors activated by receptors for the transforming growth factor beta (TGFβ) superfamily,\(^43\) we investigated whether changes in TGFβ superfamily receptor function are...
involved in the pathogenesis of the CKD-MBD. Our initial strategy was to focus on the function of putatively important TGFβ superfamily receptors. The superfamily ligands generally bind to type two receptors which associate and activate type one receptors initiating signal transduction. Besides TGFβ receptor II (TGFβRII), bone morphogenetic protein type 2 receptor (BMPRII), activin receptor type 2A (ActRIIA) and activin receptor type 2B (ActRIIB) are the most important type 2 receptors of the superfamily.

Here we report the effects of a ligand trap for the activin type 2A receptor, ActRIIA, on CKD stimulated atherosclerotic calcification and renal αklotho levels, because the latter is a component of the CKD-MBD and has been implicated in vascular calcification.44 Aortic VSMC ActRIIA levels and ActRIIA signaling were decreased by CKD. The ActRIIA ligand trap increased aortic ActRIIA signaling measured by Smad activation, blocked CKD stimulated vascular smooth muscle osteoblastic transition, and decreased atherosclerotic vascular calcification. In the kidney, we found that the ligand trap increased renal αklotho expression. Furthermore in the kidney, ActRIIA levels were not changed by CKD, and the ligand trap decreased renal ActRIIA signaling. The ligand trap also decreased renal Wnt activation and decreased circulating Dkk1. Renal fibrosis and proteinuria were decreased by the ActRIIA ligand trap. The compiled result was that RAP-011 decreased vascular calcification, and renal fibrosis.

Results

Experimental Design, and Kidney function in a model of CKD

To study the effect of the ActRIIA ligand trap on the CKD-MBD in early CKD, we used the high fat fed ldlr−/− mouse with ablative CKD treated with a ligand trap for the receptor. The experimental design of the ActRIIA ligand trap experiments in our model is shown in Figure 1. The high fat fed ldlr−/− mouse is a well characterized model of atherosclerotic vascular calcification requiring both the diet and the genotype to produce atherosclerotic vascular calcification.20,45 We have used the model extensively to study stimulation of atherosclerotic calcification by CKD.13, 17, 20, 21, 23, 46-48 In the present studies, kidney function was reduced to approximately 30% of normal (CKD) in ldlr−/− high fat fed mice by renal cortical injury and contralateral nephrectomy (supplemental Figure 1). The CKD mice were hyperphosphatemic (Table 1)

Activin Receptor Type IIA (ActRIIA) levels in CKD

Aortas from the high fat fed ldlr−/− mice with CKD were analyzed for TGFβ superfamily type II receptors which are the ligand binding component of the superfamily receptor heteromultimers composed of type II and type I (ALK) receptors. The Activin type II receptor A (ActRIIA) was expressed in aortic vascular smooth muscle cells (VSMC) of the ldlr−/− high fat fed mice (Fig. 2A,B). CKD induced ActRIIA down regulation in the aorta (Fig. 2A,B). This is consistent with internalization and degradation of ActRIIA produced by high circulating ligand levels reported in other tissues.49, 50 Endothelial cell ActRIIA was not detected by immunochemical and immunofluorescent detection. (Fig. 2B).
Vascular effects of the ActRIIA ligand trap in CKD

The effects of CKD-induced suppression of ActRIIA levels were analyzed in aortic homogenates from CKD mice treated with vehicle or the ActRIIA ligand trap (RAP-011) (Fig. 3). First, CKD stimulated osteoblastic transition was assessed by expression of mRNA for Runx2 and alkaline phosphatase (Alpl) in the aortas of ldlr−/− high fat fed mice. CKD stimulated their expression and RAP-011 treatment reversed the effects of CKD (Fig. 3A). Both Runx2 and Alpl expression represent biomarkers of osteoblastic transition in the aorta that were reversed by RAP-011 treatment. Next, aortic mRNA of smooth muscle 22α or transgelin (Tagln), a biomarker of differentiated vascular smooth muscle cells, was decreased by CKD and stimulated by RAP-011. CKD also caused decreased aortic myocardin (Myocd) mRNA expression, the vascular smooth muscle cell specific transcription factor, but myocardin was not affected by RAP-011 treatment. In terms of the effects of CKD and RAP-011 treatment on aortic protein levels of the respective mRNAs studied in Figure 3A, CKD increased aortic Runx2 and Alpl levels and RAP-011 normalized them (Fig. 3B, data for Alpl not shown). CKD decreased the aortic levels of Tagln and alpha smooth muscle actin (αSMA), another biomarker of differentiated vascular smooth muscle cells, and RAP-011 treatment increased them (Fig. 3B, data for Tagln not shown). Myocardin levels were not changed by CKD or RAP-011 treatment.

The ldlr−/− high fat fed mouse has atherosclerotic calcification stimulated by CKD as described above. In the studies reported here, CKD caused accumulation of calcium deposits in the aortic atheromas in CKD vehicle treated mice (CKD V) (Fig. 4A) and increased total tissue calcium levels (Fig. 4B). Visible calcium deposits were not present in CKD mice treated with RAP-011 (CKD RAP-011), and RAP-011 decreased aortic tissue calcium content to levels observed in wild type and sham mice, significantly below those present at the time of institution of RAP-011 treatment, the CKD group (Fig. 4B). The dose of RAP-011 used here, 10mg/kg twice weekly subcutaneously, was used to relate to prior toxicology, pharmacodynamic and efficacy studies. Subsequent to the studies in Figure 4B, we performed a dose response study and equivalent or better reductions in aortic tissue calcium content resulted when doses of 5mg/kg and 1mg/kg twice weekly subcutaneously were used (data not shown).

ActRIIA signaling in the aorta

Despite the decrease in ActRIIA in CKD, there remained easily detected ActRIIA in the VSMC potentially available for signaling (Fig. 2B). Canonical signal transduction by the TGFβ superfamily involves ligand binding to type II receptors activating their serine/threonine kinase activity and stimulating association and phosphorylation of type I receptors, the Alk kinases (see diagrammatic representation in supplemental Figure 2). There are seven Alk kinases utilized by the TGFβ superfamily, and Alk4 (ActRIB) is the type I receptor most often associated with ActRIIA signaling. Aortic homogenates isolated from CKD mice revealed that the decrease in ActRIIA levels, which were shown quantitatively in Figure 2 above, were not associated with decreased tissue levels of Alk4, and Alk1 (Fig. 5A). Alk5 or Alk2, other type I receptors associated with ActRIIA signaling, were not detectable. ActRIIA activity was assessed by measuring the effect of receptor heteromultimerization, i.e., phosphorylation of regulatory Smads. CKD decreased aortic
phosphosmad 2/3 levels (activated smad 2/3), and RAP-011 increased them compared to CKD, even though total Smad2/3 levels were decreased (Fig. 5A, B). We also analyzed noncanonical ActRIIA signaling (supplemental Figure 2), and found that map kinase (phospho-Erk1/2) was also decreased by CKD and not further affected by RAP-011 (Fig. 5A), and that vascular smooth muscle levels of p38 and JNK were very low. Also, RAP-011 did not affect p-AKT levels indicating that aortic AKT/PI3 kinase was not affected by ActRIIA signaling (data not shown). In summary, aortic ActRIIA signaling (phosphosmad 2/3) was decreased by CKD and stimulated by the ligand trap associated with suppression of osteoblastic transition in the atherosclerotic aortas by the ligand trap (Fig. 3). When RAP-011 was administered to wild type mice, the reduction in total Smad 2/3 levels observed in CKD mice were reproduced (Fig 5C). However, WT mice had very low levels of ActRIIA and p-Smad 2/3 which were not affected by RAP-011. RAP-011 was well tolerated in mice with normal kidney function, and there was no discernible effect of RAP-011 on appearance, activity, food intake, and weight gain on the CKD mice. There was the expected increase in hematocrit, but this was an efficacious side effect.

Because the effects of RAP-011 treatment on the aorta were similar to those of Dkk1 inhibition, another non-canonical ActRIIA signaling pathway was examined, i.e. - the Wnt pathway (supplemental Figure 2). Aortic β-catenin, the major canonical Wnt induced transcription factor, was localized by immunofluorescence to endothelial cells, and was not detectable in VSMC (Fig. 6A). But because Dkk1 is a transcriptional target of canonical Wnt signaling, and its mRNA is detectable in VSMC in vivo reflecting low level Wnt activity, we analyzed Dkk1 levels in the VSMC as a biomarker of Wnt activity. We found that CKD increased VSMC Dkk1 levels, and RAP-011 treatment decreased Dkk1 levels in our CKD mice (Fig. 6B). The decrease in aortic Dkk1 levels by RAP-011 suggests that vascular smooth muscle Wnt signaling was inhibited by the ActRIIA ligand trap. In addition, the effects of the ligand trap to decrease renal Wnt activity as shown below lead to a major decrease in circulating Dkk1 levels (Fig. 6C).

Effects of the ActRIIA ligand trap on αklotho and renal ActRIIA signaling

Because αklotho is a critical component of the CKD-MBD and a pathogenic factor in vascular calcification, we examined renal αklotho levels and the effects of CKD and RAP-011 treatment. Recent studies demonstrate that kidney tubules are the source of most αklotho. As shown in Figure 7A, we found a significant increase in renal αklotho levels induced by RAP-011 treatment. Therefore, we studied renal ActRIIA levels and ActRIIA signaling to examine whether the effect of CKD to downregulate the receptor and decrease signaling observed in the aorta were reproduced in the kidney. Renal ActRIIA levels were not affected by CKD (Fig.7B), and a primary ActRIIA ligand, activin A, was strongly induced in the CKD mice, and was suppressed by RAP-011 treatment (quantitation of activin A levels is shown below in Fig. 9). Detecting type I receptor activation was impaired by the lack of available phospho-Alk antibodies, requiring an experimental design wherein a phosphoserine antibody was used to immunoprecipitate and immunoblots of the precipitate were performed with anti-Alk antibodies. We were unable to do this in the aorta due to insufficient amounts of tissue, but we were able to perform these experiments using kidney homogenates. Alk4 phosphorylation was not significantly altered in the diseased kidneys of
our CKD model or by RAP-011 treatment (Fig. 7B), but renal phosphosmad 2/3 was increased by CKD and decreased by RAP-011 (Fig. 7B,C) indicating that the component of renal Smad 2/3 activation in CKD mediated through ActRIIA involved an Alk different from Alk4 (Fig. 7B). Furthermore, psmad 2/3 transcriptional targets, fibronectin and type 1 collagen (Col1A1) were stimulated by CKD and inhibited by RAP-011 treatment (Fig. 7B,C).

Because increased pSmad 3 is associated with renal fibrosis, and since ActRIIA signaling stimulated by activin A is a critical factor in fibrosis 58, 59 including renal fibrosis, 60, 61 we examined the effects of the ActRIIA ligand trap on renal fibrosis. Figure 8A shows the decrease in renal fibrosis in Trichrome stained kidney cortex sections from CKD RAP-011 treated mice (c and d) compared to CKDV treated mice (a and b). Supplemental Figure 3 shows low magnification coronal remnant kidney sections with arrows showing from where the high power sections in Figure 8A were taken, away from the scar reactions to the electrocautery delineate by arrow heads. Quantitation of fibrosis by interstitial volume analysis is shown in Figure 8B. Furthermore, RAP-011 decreased the proteinuria stimulated by CKD (Fig. 8C) consistent with the decrease in renal phosphosmad 2/3 and fibrosis induced by RAP-011 treatment.

Potential ActRIIA ligands in CKD

There are multiple potential ActRIIA ligands including activins A and B, growth and differentiation factor 11 (GDF11), bone morphogenetic proteins 9 and 10 (BMP9 and 10), and other BMPs, especially 7, which have lower affinity for the receptor. But a primary ActRIIA ligand is activin. We do not know the ligand/s involved in disordered ActRIIA signaling in CKD, which we have just begun to study as a result of the findings reported here. Using two models of CKD, we found that systemic circulating activin A levels were 10 fold elevated in the high fat fed ldlr−/− ablative CKD model and fivefold elevated in the Col4A5 Alport's syndrome mouse model (Fig. 9A). Activin B, GDF11, and BMP9 levels were not affected by CKD in these models. Physiologically, there is little free activin in the circulation due to levels of inhibitors stoichiometrically equaling activin levels. Activins associate with circulating inhibitory factors, follistatin and follistatin like 3 (fstL3), 62 and inhibin whose circulating levels (supplemental Figure 4) and tissue levels were not affected or decreased by CKD. The stoichiometry of follistatin, fstL3, and inhibin (sum of 620pg/ml in supplemental figure 4, plus 400pg/ml of unmeasured inhibin 63) to Activin A levels (>5000pg/ml) in the circulation suggests that CKD produces significant free activin levels, a pathologic event making activin A an active circulating factor in CKD. While the other potential ActRIIA ligands affected by CKD require additional study, this data indicates the potential that kidney diseases produce one or more circulating ActRIIA ligands that could potentially downregulate vascular ActRIIA.

To analyze the source of the increase in circulating activin, we analyzed kidney tissues from our ldlr−/− atherosclerotic calcification model for activin A (a homodimer of Inhibin betaA (Inhba)). Inhba mRNA was increased by CKD in the kidney (Fig. 9B), and activin (inhibin β-A) protein levels were increased (Fig. 9C). Localization of renal activin expression was found in the peritubular myofibroblasts of CKD mice (Fig. 10). However, these data do not
establish that activin is the ligand involved in disordered ActRIIA signaling in CKD, and additional research involving production of myofibroblast activin deficient mice and ActRIIA cell specific deficient mice will be necessary to establish this issue.

Discussion

Our results report reversal of CKD induced stimulation of aortic vascular smooth muscle dedifferentiation, osteoblastic transition and neointimal calcification by a ligand trap for ActRIIA, RAP-011. These results build on our recent demonstration that kidney diseases increase circulating Wnt inhibitors, that systemic Wnt inhibition contributes to CKD induced vascular and skeletal disease, and that neutralization of elevated Dkk1 prevents the vascular calcification and renal osteodystrophy of early CKD. The effects of Dkk1 neutralization to decrease vascular calcification were surprising because several investigators had linked vascular smooth muscle Wnt activity to vascular calcification. However, the recent demonstration that Dkk1 mediated inhibition of Wnt7b caused EndMT lead us to question whether EndMT was a mechanism of vascular disease and vascular calcification in CKD. EndMT is a Smad mediated process. Regulatory Smads are transcription factors activated by members of the TGFβ superfamily that includes the TGFβs, bone morphogenetic proteins (BMPs), activins/inhibins, and growth and differentiation factors (GDFs). We employed a ligand trap for the ActRIIA receptor to interrogate the role of Smad stimulated pathways in the vasculature during CKD. We found that treatment with the ActRIIA ligand trap decreased atherosclerotic calcification in the aorta, decreased expression of the osteoblastic proteins, Runx2 and Alpl, and increased levels of smooth muscle cell specific proteins, sm22α and αSMA (alpha smooth muscle actin). Since the RAP-011 ligand trap increased aortic p-Smad 2/3 levels, these data suggest that downregulation of vascular ActRIIA signaling in CKD contributes to altered VSMC biology involved in upregulation of Runx2 and osteochondrogenic transition critical to atherosclerotic calcification.

When we attempted to study this biology in VSMC cell culture we discovered that murine VSMCs in culture do not express ActRIIA protein despite the mRNA being transcribed (data not shown). This again is consistent with the dedifferentiated state that characterizes VSMC cell cultures and suggests that ActRIIA signaling may be important in VSMC differentiation as our data in vivo suggests (the ligand trap treatment increased VSMC sm22α (tagln) and αSMA).

We found that CKD was associated with reduction of ActRIIA protein levels in the aortas of our mouse model of atherosclerotic calcification, and that this correlated with decreased activated Smad 2/3 levels (phosphosmad 2/3) and decreased MAP kinase (phosphoERK 1/2) indicating that CKD produced inhibition of aortic ActRIIA signaling. The finding that the ligand trap actually increased ActRIIA signaling (increased aortic p-Smad 2/3 levels) suggests that ActRIIA was suppressed in CKD mice due to high levels of a ligand inducing receptor desensitization possibly through endocytosis and degradation (supplemental Figure 5). The ActRIIA receptor undergoes ligand stimulated endocytosis during signal transduction, and can be down regulated during the signal transduction process depending on the effects of activin receptor interacting proteins (ARIP1 and ARIP2). While ARIP1 and ARIP2b and 2c have been associated with Smad binding and effective signaling.
ARIP2 is associated with receptor endocytosis, stimulation of receptor degradation and decreased signaling.\(^{50, 68}\) Downregulation of vascular ActRIIA by ligand stimulated endocytosis is consistent with the decrease in aortic ActRIIA in the CKD mice because of the high levels of circulating activin A produced by CKD. High activin levels have been shown to stimulate endocytotic mediated degradation of ActRIIA through the effects of ARIP2.\(^{68}\) In addition, ARIP2 is more highly expressed in the vasculature related to ARIP1, while in the kidney ARIP1 expression is much higher than ARIP2 (supplemental Figure 6). Results similar to the VSMC effects of CKD on ActRIIA levels, have been produced in testicular cells exposed to high activin levels.\(^{49}\) These data suggest that CKD may produce vascular ActRIIA downregulation through production of a ligand in the circulation since vascular tissue ligands of ActRIIA were also downregulated in CKD as shown for the primary ActRIIA ligand, activin A (Fig. 5). This led us to analyze ActRIIA signaling and potential ligands in the diseased kidney.

In the kidney we found that ActRIIA levels were not affected by CKD or RAP-011 treatment consistent with a different ARIP involved in renal ActRIIA signaling than that involved in the aorta consistent with the high level of renal ARIP1 mRNA shown in supplemental Figure 6. In the kidney, there was a major induction of activin A, a primary ligand for ActRIIA, by CKD and a coordinate increase in activated Smad 2/3 (p-Smad 2/3). RAP-011 treatment decreased renal activin A expression and decreased p-Smad 2/3. This indicates that in the kidney ActRIIA signaling was increased by CKD and reduced by RAP-011 treatment. Furthermore, CKD induced Wnt activation measured by increased renal levels of Dkk1 was inhibited by RAP-011. In agreement with these results, renal fibrosis, proteinuria, type 1 collagen levels and fibronectin levels were increased by CKD consistent with the effects of increased p-Smad 3 induced gene transcription, and decreased by RAP-011 treatment. Importantly, RAP-011 treatment increased renal production of αklotho, which may have contributed to its efficacy in inhibiting atherosclerotic vascular calcification.\(^{16}\)

In the diseased kidney, the profibrogenic effects of Wnt activation are marked by increased expression of the Wnt inhibitor, Dkk1.\(^{24, 25, 28}\) In the diseased kidney, RAP-011 treatment decreased kidney expression of Dkk1 and decreased circulating Dkk1 levels. These effects are consistent with cooperative effects of activin and the Wnt pathways during development\(^{70-73}\) and kidney disease as shown here. Thus, we have shown that reactivation of two kidney development factors, activin and Wnt in kidney disease produce circulating Wnt inhibitors and activin in CKD, which may interact in causing vascular disease. Our data are consistent but do not prove that renal production of a circulating ActRIIA ligand was the basis for the downregulation of vascular ActRIIA signaling which was stimulated by RAP-011 treatment, and they do not identify the ActRIIA ligand affected by RAP-011 further than indicating that activin A is a candidate for this function. However, the ActRIIA ligand trap, decreased Dkk1 levels linking the findings reported here to our previous report on Dkk1 inhibition.

In conclusion, we report the discovery of disordered ActRIIA signaling during CKD induced atherosclerotic calcification and renal fibrosis. The results demonstrate that a ligand trap for the receptor improved ActRIIA signaling, and smooth muscle specific protein expression in
the aorta. It decreased osteoblastic transition, and decreased atherosclerotic vascular calcification. RAP-011 also increased renal αklotho expression and decreased renal fibrosis. Secondarily, RAP-011 decreased renal Wnt signaling and circulating Dkk1, which contributed to the efficacy of RAP-011 in restoring vascular function during CKD. Since the human ortholog of RAP-011 (sotatercept) is in clinical trials for the anemia of CKD (NCT01146574, NCT01999582), and ACE-011 has been shown to be effective in reducing elevated GDF-11 levels in β-thalassemia, there may be opportunity for further exploring the efficacy of ActRIIA ligand traps in the CKD-MBD. Thus, we have shown that regulation of ActRIIA signaling in CKD may be a new therapeutic target modulating vascular disease and kidney disease progression.

Methods

The studies reported here used methodology previously reported and described in detail in the supplementary materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


Figure One.
Schematic of ActRIIA ligand trap and experimental design of its use in the ldlr−/− high fat fed CKD vascular calcification model. A, schematic diagram of the mouse fusion protein of the extracellular domain of ActRIIA and the Fc domain of IgG1 (RAP-011). B, experimental design of RAP-011 effects on the CKD-MBD in ldlr−/− high fat fed mice. Mice in four groups were fed the high fat diet beginning at 12 weeks (wks) of age. At 12 wks either sham operation (SO) or electrocautery cortical injury (EC) was performed. At 14 wks either SO or contralateral nephrectomy (NX) was performed. At 22 wks of life, vehicle treatment or RAP-011, 10mg/kg subcutaneous twice weekly, was instituted. WT, wild type mice on chow diet for normal reference levels. Sham, sham operated ldlr−/− high fat fed mice; CKD, CKD ldlr−/− high fat fed mice studied at 22 wks to establish levels of vascular calcium at the start of therapy; CKD V, CKD ldlr−/− high fat fed mice vehicle treated; CKD R, CKD ldlr−/− high fat fed mice RAP-011 treated; WT, Sham, CKD V and CKD R mice were euthanized at 28 weeks of age.
Figure Two.
Expression of ActRIIA in mouse aortas. A, Westerns for ActRIIA in aortic homogenates and immunoblot quantitation to the right. For the quantitation, n=4, **p<0.01. B, Immunofluorescent detection of ActRIIA (a,b) in the aortas of sham (a), and CKD (b) mice. a,b, ActRIIA (red) was expressed in aortic VSMC, but was not detected in endothelial cells. VSMC ActRIIA levels remained detectable in CKD compared to sham. CD31 (green) (arrowheads) was used as an endothelial cell marker. Nuclei were stained by DAPI. Scale bar 20 µm.
Figure Three.
Effects of CKD and the ActRIIA ligand trap on aortic gene expression and protein levels in ldlr−/− high fat fed mice with CKD. A, CKD causes increased mRNA expression of osteoblastic proteins (Runx2 and alkaline phosphatase (Alpl)), and decreased levels of aortic smooth muscle cell 22α (Tagln) which were all reversed by treatment with the ActRIIA ligand trap, RAP-011. Aortic myocardin (Myocd) levels were decreased by CKD, but not affected by RAP-011. ***p<0.001,. **p<0.01. *p<0.05. B, Westerns for proteins in aortic homogenates and immunoblot quantitation to the right. CKD causes decreased levels of aortic α-smooth muscle cell actin protein and increased levels of osteoblastic Runx2, which were reversed by treatment with RAP-011, but myocardin levels were not changed. For the quantitation, n=4, **p<0.01.
Figure Four.
Effects of CKD and the ActRIIA ligand trap on aortic calcification in ldlr−/- high fat fed mice with CKD. **A**, Alizarin Red stained sections of proximal aortic atherosclerotic plaques from vehicle and RAP-011 treated CKD mice. Arrow head indicates calcium deposition in intima (i); m – media. Scale bar 100 µm. **B**, Aortic Calcium levels in the groups of mice: wild type (WT); sham operated ldlr−/- high fat fed (Sham); CKD euthanasia at 22 weeks, the time of institution of treatment (CKD); CKD treated with vehicle from 22 to 28 weeks (CKD V); CKD treated with RAP-011, 10mg/kg subcutaneous twice weekly from 22 to 28 weeks (CKD R). The boxes represent median (line in box) and interquartile ranges from 25th to 75th percentile. The error bars represent 1.5 fold of the interquartile range. Groups were compared using ANOVA Holm-Sidak method for multiple comparisons with p<0.05 as level for significant difference. *p<0.02; n for each goup 8-12.
Figure Five.
ActRIIA signaling in aorta. A, analysis of ActRIIA signaling by westerns of aortic homogenates from sham, CKD vehicle and CKD RAP-011 treated mice. Immunoblots of homogenates from two aortas of animals in each group. ActRIIA and Activin (inhibin β-A) levels were decreased in aortic homogenates from CKD mice. The Alk4 (AcvR1B) and Alk1 (AcvRL1) type 1 receptors were present in aortic homogenates but not affected by CKD. RAP-011 decreased Smad 2/3 levels, but CKD decreased smad2/3 phosphorylation in aortas which was increased by RAP-011 treatment. CKD decreased phospho-Erk 1/2 levels. Runx2 levels were increased by CKD and normalized by RAP-011 treatment. B, p-Smad2/3 immunoblot quantitation, n=4, **p<0.01. C, When RAP-011 was administered to WT mice, the decrease in Smad 2/3 levels observed in CKD mice was reproduced, but ActRIIA levels and p-Smad 2/3 levels were very low and not affected by RAP-011 treatment.
Figure Six.
Wnt signaling in aorta, and circulating Dkk1 levels. A, immunofluorescence microscopy of beta-catenin expression in the aortas of: a, wild-type mouse; b, CKD mouse. Red – beta catenin, Bright green - CD31, an endothelial cell marker, yellow – co-localization. There was no immunofluorescence for beta-catenin in the vascular smooth muscle cells. There was beta-catenin expression in the endothelium of aortas from CKD mice. Arrow heads, beta-catenin and CD31 colocalization in endothelial cells. Scale bar 20 µm. B, analysis of Wnt signaling as marked by Dkk1 protein expression in westerns of aortic homogenates from sham, CKD V and CKD R treated mice, immunoblot quantitation below. **p<0.01, n=4. C, effect of CKD V and RAP-011 treatment on plasma Dkk1 levels. *p<0.05, **p<0.01
**Figure Seven.** Renal αklotho levels and ActRIIA signaling in kidney. A, effects of RAP-011 treatment on renal αklotho mRNA levels. CKD decreased αklotho gene expression levels in kidney homogenates, and RAP-011 treatment significantly increased them compared to CKD-3V. *p<0.05, **p<0.01, ***p<0.005. B, analysis of ActRIIA signaling by westerns of kidney homogenates from sham, CKD vehicle, and RAP-011 treated mice. The immunoblots are representative of homogenates from 4 kidneys. ActRIIA levels were not affected by CKD or RAP-011. Activin A (inhibin β-A) levels were increased in kidney homogenates of CKD mice (quantitation in Figure 9) and decreased by RAP-011. The Alk4 (AcvR1B) and Alk2 (AcvR1) type 1 receptors were present in kidney homogenates, but CKD V or CKD R did not significantly affect Alk4 phosphorylation. CKD increased renal smad2/3 phosphorylation (p-Smad2/3), and RAP-011 treatment decreased kidney p-Smad2/3 levels. (immunoblot quantitation in 5C). CKD increased Col1A1 and fibronectin levels, while RAP-011 treatment decreased them. C, Quantitation of the p-Smad 2/3, Col1A1 and fibronectin immunoblots. *p<0.01, **p<0.01.
Figure Eight.
Effects of RAP-011 treatment on renal fibrosis. A, Trichrome staining of kidney sections from two CKDV mice (a and b) and two CKD RAP-011 (c and d) treated mice. Areas of interstitial fibrosis marked by arrowheads. Kidneys of CKD RAP-011 treated mice had decreased interstitial fibrosis. Scale bar 50 µm. See supplemental figure 3 for marking of whole kidney coronal sections as to where the photomicrograph sections were taken. B, Quantitation of fibrosis by measuring interstitial volume. Wild type mice were used in this experiment to establish the normal reference. **p<0.01 C, Effects of RAP-011 on urinary protein. There was significant proteinuria in the CKDV mice, which was decreased by RAP-011 treatment, *p<0.05.
Figure Nine.
CKD increases Activin in the circulation and the kidney. A, induction of circulating activin-A by CKD in two disease models, atherosclerotic ldlr−/− high fat fed CKD-3 mice and Alport’s syndrome mice as described in methods; B, inhibin betaA (Inhba) mRNA expression in mouse kidney (activin-A is formed of homodimers of inhibin betaA); C, Westerns for inhibin β-A in kidney homogenates and immunoblot quantitation to the right, n=6 for the immunoblot quantitation; *p<0.05, **p<0.01, ***p<0.005.
Figure Ten.
Kidney InhbA immunostaining in: a, Sham and b, CKD Vehicle mice. In Sham mice occasional peritubular interstitial cells express activin-A, but in CKD mice many more peritubular interstitial cells are positive for activin-A at varying levels of intensity. Scale bar 50 µm.
Table 1

Serum Biochemical Parameters in the Various Groups of Animals

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1 Wild Type</th>
<th>Group 2 Sham</th>
<th>Group 3 CKD-3 V</th>
<th>Group 4 CKD-3 R</th>
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<tbody>
<tr>
<td>Mouse Strain</td>
<td>C57/BJ6</td>
<td>ldlr−/−</td>
<td>ldlr−/−</td>
<td>ldlr−/−</td>
</tr>
<tr>
<td>Diet</td>
<td>Chow</td>
<td>High fat</td>
<td>High fat</td>
<td>High fat</td>
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<tr>
<td>Surgery</td>
<td>NONE</td>
<td>sham</td>
<td>CKD</td>
<td>CKD</td>
</tr>
<tr>
<td>Weeks postnatal</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>Treatment</td>
<td>NONE</td>
<td>NONE</td>
<td>Vehicle</td>
<td>RAP-011</td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td>15</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>24.0 ± 4.6</td>
<td>20.6 ± 3.7</td>
<td>37.7 ± 7.6 *</td>
<td>36.5 ± 5.8 *</td>
</tr>
<tr>
<td>Ca (mg/dl)</td>
<td>8.3 ± 1.8</td>
<td>8.9 ± 0.9</td>
<td>9.4 ± 0.8</td>
<td>8.8 ± 0.3</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>8.9 ± 0.2</td>
<td>7.9 ± 2.3</td>
<td>11.0 ± 1.6 *</td>
<td>11.8 ± 1.2 *</td>
</tr>
</tbody>
</table>

* P<0.05, groups 3 & 4 compared to group 2