ORIGINAL ARTICLE

Upregulation of ppET-1/ETBR/eNOS mRNA Expression After Calcitriol Treatment in Chronic Kidney Diseases Model in Rats

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ABSTRACT

Introduction: Myofibroblast formation in the interstitial area is the hallmark of chronic kidney disease (CKD). Endothelin signalling has been known to play role in physiology and pathophysiology in the kidney. Vitamin D has a reno-protective effect through inhibiting inflammation and fibrosis. However, the interaction between vitamin D and endothelin signalling in the CKD model has not been elucidated yet. Therefore, we aimed to check the difference impact of endothelin (ET) receptor in CKD. **Methods:** Sprague Dawley rats (3-months-old, 150-250grams) underwent 5/6 subtotal nephrectomy (SN) to induce CKD. Then, it was divided into 4 groups (each contains 6 rats): sham operation (SO), 5/6 subtotal nephrectomy (SN), calcitriol groups (0.01µg/100grBW/day (SN-D1), and 0.05µg/100grBW/day (SN-D2). Calcitriol was administered for 14 days after the surgery. The Sham Operation (SO) group was injected with NaCl. At the specified date, the rats were sacrificed and the kidneys were harvested. Fibrosis was quantified based on Sirius Red staining. Immunostaining was done for localizing fibroblast (PDGFRβ). The mRNA expressions of prepro-ET-1, endothelin receptor A (ETAR), endothelin receptor B (ETBR), and endothelial nitrite oxide synthase (eNOS) were quantified using reverse-transcriptase PCR (RT-PCR). **Results:** The CKD promotes an elevation of prepro-ET-1, ETBR, and eNOS, and reduction of ETAR ($p<0.05$) mRNA expression compared to the SO group. Administration of calcitriol (SN-D1 and SN-D2) showed the vice versa effects. However, only SN-D2 group consistently showed statistically significant differences whenever compared to either SO or SN groups. **Conclusion:** Calcitriol might attenuate interstitial fibrosis in CKD model via ET-1/eNOS signalling.
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Keywords: Chronic kidney disease, Calcitriol, Endothelin-1, eNOS, Endothelin receptors

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INTRODUCTION

Chronic kidney disease (CKD) is a global socio-economic burden that its incidence and prevalence continues to increase along the aging human population (1). This disease is characterised by the occurrence of fibrosis resulting in the inability of the kidneys to carry out its functions of producing urine, maintaining fluid balance in the body, maintaining the acid base balance of the body, metabolizing vitamin D, and stimulating bone marrow function by producing the erythropoietin hormone which is important in the production of red blood cells. Disruption of these functions causes accumulation of metabolic waste substances that are toxic and result in death (2).

There are 3 forms of renal fibrosis, namely glomerulosclerosis, tubulointerstitial fibrosis, and alteration in the glomerular and peritubular capillary structures. Tubulointerstitial fibrosis is the most developed to end stage renal disease (3). Interstitial fibrosis begins with tissue injury resulting in the inflammatory response and activation of myofibroblasts, mainly from interstitial fibroblasts and pericytes that produce extracellular matrix. The accumulation of matrix in the interstitial space and in the glomerular capillary is believed to accelerate the death of nephrons (4).

Interstitial cells such as fibroblasts and pericytes can be identified by using platelet-derived growth factor-β (PDGFRβ) staining, although it is not specific (5). Myofibroblasts are activated fibroblasts that have special contraction properties and express α-smooth muscle actin (α-SMA). The levels of α-SMA and PDGFRβ are related to the severity of renal fibrosis and can predict a decrease in kidney function (6,7). The process of fibrosis

formation is characterised by increased levels of marker fibrosis, such as transforming growth factors beta-1 (TGF-β1), and Endothelin-1 (ET-1). It has been known that ET-1 has 2 different receptor subtypes, ETA receptor (ETAR) and ETB receptor (ETBR) (8). ETAR activation results in renal vasoconstriction, glomerular damage, and hypertension (9). While activation of ETBR raises the vasodilation effect by activating endothelial nitric oxide synthase (eNOS) (10).

Replication of chronic human renal failure conditions in experimental animals can be done using the 5/6 Subtotal Nephrectomy (SN) method that has been shown to be able to produce chronic renal failure and show all important structural and functional changes found as in humans (11). Based on a review conducted by Nogueira regarding animal models to induce renal fibrosis, male Sprague-Dawley (SD) rats have good success rates (11). Reduction of renal mass and renal nephron cause renal hypertrophy in the remnant kidney. It is characterised by unequal dilatation in the afferent arterioles which then contributes to hyper-perfusion and hyperfiltration. The morphology of glomerular and tubules alter dramatically starting from sclerosis, mesangial cell expansion, tubular atrophy, and interstitial fibrosis (12–14). Right after diminished renal mass, the macrophage infiltrates and causes an immunologic reaction that resembles the pathophysiology of chronic kidney disease (15).

Current studies are beginning to be directed towards risk factors for chronic kidney failure that have not been widely understood, such as vitamin D deficiency (16). Calcifediol and calcitriol deficiencies that occur in the early stages of kidney disease can interfere with kidney repair and even speed up worsening of the disease. In the reperfusion ischemia model, vitamin D deficiency increases damage to renal capillaries, inflammation and renal fibrosis (17). The supplementation of vitamin D improves renal fibrosis in mice of the unilateral ureteral obstruction (UUO) model which is characterised by a decrease in myofibroblasts formation, inflammation and tubular cell apoptosis (18). The pro-fibrosis effect of TGF-β can also be suppressed by vitamin D by decreasing TGF-β expression (19), as well as the modulation of the involved signalling pathways (20). In addition, administration of vitamin D is also able to increase the expression of ET-1 and NO in endothelial cell cultures (21). Activation of the signal pathway ET-1/ETBR which increases NO production in the collecting duct is reno-protective by regulating arterial pressure and sodium excretion (22). The effect of vitamin D administration on the expression of ET-1 receptors, ETAR and ETBR, has not been established yet. The mechanism of protection by vitamin D for renal fibrosis has been investigated but the exact mechanism of action of vitamin D against protection of renal fibrosis has not been fully explained. This study elucidated effects of Vitamin D treatment in CKD model in relation with ET-1/eNOS signalling. This study is continuation of our previous study which demonstrate renoprotective effect of Vitamin D in CKD model through attenuation of fibrosis, inflammation and glomerulosclerosis (19). We would like to relate the Vitamin D treatment with ET-1/ET receptors activation in CKD condition. Inhibition of dual ET blocker can attenuate proteinuria in diabetic nephropathy patients, however it may induce pulmonary oedema due to inhibition of ETBR (20,21). Based on the results, it may need to elucidate different impact of ET receptor in CKD, and elaborate other substance which may regulate the ET-1/ET receptor signalling.

MATERIALS AND METHODS

5/6 Subtotal Nephrectomy model

This study had been approved by the Institutional Review Board of the Medical and Health Research Ethics Committee (MHREC) of Faculty of Medicine, Universitas Gadjah Mada – Dr. Sardjito General Hospital (Forum for Ethical Review Committees in Asia and Western Pacific / FERCAP) for research involving animal Faculty of Medicine Gadjah Mada University with the ethical expediency number: KE/FK/0351/EC/2018.

The Sprague Dawley strain male rats (3-4 months-old and 150-250 grams of body weight (BW)) were obtained from the UGM Integrated Research and Testing Laboratory (LPPT) and were divided into 4 groups: sham operation (n=6; SO), 5/6 subtotal nephrectomy (n=6; SN), 5/6 subtotal nephrectomy + Calcitriol 0.01μg/100grBW/day (n=6; SN-D1), and 5/6 subtotal nephrectomy + Calcitriol 0.05μg/100grBW/day (n=6; SN-D2). Rats in 5/6 SN, SN-D1, and SN-D2 groups were anesthetized with ketamine at a dose of 60-100mg/kgBW by intramuscular injection. The disinfection procedure using povidone iodine was performed in the shaved area. The layers of skin, peritoneum, and muscles were opened layer by layer until the kidneys exposed. Once the kidney was identified, then the right renal artery was ligated and cut, accompanied by bleeding control. The kidney that have been cut were immediately weighed as a guide for normal kidney weight. After 2 days, the superior and inferior poles of the left kidney were removed. To ensure that kidney size have reached 2/3 parts, each cut part was weighed and compared with the total weight of the right kidney that was cut in the previous operation.

Vitamin D administration

Vitamin D (Cayman®) was dissolved using 0.2% ethanol. Then, it was intraperitoneally injected daily (0.01μg/mL/100gramBW, and 0.05μg/mL/100gramBW) for 14 days after the surgery.

RNA extraction and cDNA making

The anterior part of the left kidney which was previously stored in RNA preservation solution (Ambion, AM7021) was homogenized using GENEzol™ (Geneaid, GZR100) according to the guidelines. Then, the total RNA was measured using a spectrophotometer prior to cDNA

synthesis. Afterward, 3000ng of RNA was used for the downstream reaction of cDNA synthesis (SMOBio, RP1400) according to the manufacturer.

Reverse-Transcriptase PCR and Electrophoresis

The amplification of ET-1, ETAR, and eNOS used the primer sequences as shown in Table I. Amplification condition was performed in initial denaturation 94°C for 2 min, 35 cycles with 94°C for 10 second, 60°C for 30 second and 72°C for 1minute, final extension phase 72°C for 10 minutes. The PCR products were subjected into gel electrophoresis and detected by gel stain (SMOBio, NS1000) staining. The amplification gene in PCR product was quantified using densitometry analysis by imageJ software.

Table I: List of primers

Gene		Sequences	Target genes (bp)
prepro-ET-1	F:	5′-GTCGTCCCGTATGGACTAGG-3′	100
	R:	5′-ACTGGCATCTGTTCCCTTGG-3′	
ETAR	F:	5′-GCAACAGAGGCATGACTGAAAA-3′	201
	R:	5′-TTCCTTCTTCACTTAAGCCGAA-3′	
ETBR	F:	5′-TCTCAGCCTTTTGTCCGAGC-3′	176
	R:	5′-CGCCGTTTTCAGTCTCGCA-3′	
eNOS	F:	5′-CCGGCGCTACGAAGAATG-3′	78
	R:	5′-AGTGCCACGGATGGAAATT-3′	
GAPDH	F:	5′-TCCCGTTGATGACCAGCTTC-3′	163
	R:	5′-GTTACCAGGGCTGCCTTCTC-3′	

Immunohistochemical (IHC) staining of fibroblast (PDGFRβ positive cells)

The kidney paraffin section was deparaffinized using xylene, and rehydrated. Then, the slides underwent antigen retrieval for 20 minutes using citrate buffer pH 6 followed by blocking endogenous peroxidase using 3% H2O2 for 5 minutes. Next, the slides were incubated with blocking serum and primary antibody of PDGFRβ (1:200, Abcam, ab32570) overnight. In the following days, the slides were incubated with the secondary antibody and probed with diaminobenzidine (DAB). Examination of protein was then carried out by observing using a light microscope (Olympus CX22) which was connected to a computer with 400x magnification.

Sirius red staining

The four-micrometre thickness of the paraffin section was deparaffinized using xylene followed by rehydrated using graded-ethanol. Then, it was washed under tap water, and phosphate buffer saline (PBS) prior to incubation with pico-Sirius red for 1 hour at room temperature. Finally, the slides were washed with absolute ethanol and xylene before the mounting step. The slides were observed under the light microscope (Olympus CX22) with 100x magnification.

Statistical Analysis

Data were analysed with the IBM® SPSS® Statistics Version 23 program, with a probability value (p) <0.05 was considered as statistically significant. The variables were analysed using one-way ANOVA followed by post-hoc LSD.

RESULTS

SN group demonstrated fibroblast expansion

In PDGFRβ immunostaining, brown-stained cells can be found but very few in the SO group, which represented resident fibroblast (Figure 1). The SN group shows cells in the interstitial brown tissue spread evenly and appear more which demonstrated fibroblast proliferation. In the SN-D1 group, cells that expressed the PDGFRβ protein seemed to be less than the SN group. The SN-D2 group showed that there were not many brown-stained interstitial cells. This result was in accordance with our previous study with increasing myofibroblast formation and upregulation of TGF-β1expression in SN group, and reducing in Vitamin D treated group (19).

Administration of Vitamin D improved vasodilator and vasoconstrictor agents

5/6 SN contributed to the significant lower vasodilator agent, eNOS mRNA expression, compared to the control group (p=0.004). Daily administration of Vitamin D was able to regain eNOS mRNA expression that obviously seen in SN-D2 compared to the SN (p=0.009) group. Interestingly, the mRNA expression of vasoconstrictor agent, prepro-ET-1, gradually elevated in the SN (p=0.049), SN-D1 (p=0.034), and SN-D2 (p=0.006) compared to the control groups (Figure 2).

Vitamin D upregulated ETBR mRNA expression and downregulated ETAR mRNA expression

We also examined the mRNA expression of prepro-ET-1 receptors, ETAR and ETBR (Figure 3). We demonstrated

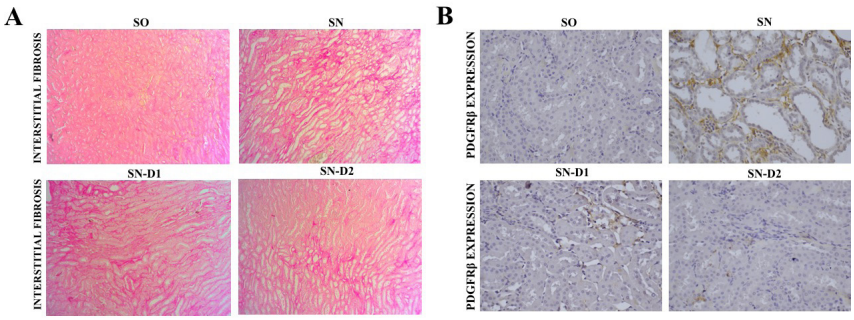

Fig. 1: Vitamin D improved interstitial fibrosis and PDGFRβ protein expression. A. The representative picture of interstitial fibrosis using Sirius Red staining. The image was taken in 100X magnification B. The image shows the expression of PDGFRβ protein displaying brown staining and represent the fibrosis area. The staining areas were more extensive in the SN group and reduced in the SN-D1 and SND2 groups. The image was taken in 400X magnification. Scale bar 100μm

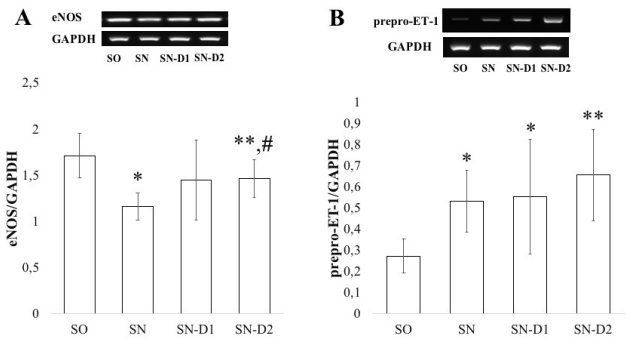

Fig. 2: Vitamin D promoted an upregulation of eNOS, and prepro-ET-1. A. Semi-quantitative analysis of eNOS mRNA expression. B. Semi-quantitative analysis of prepro-ET-1 mRNA expression. *: p<0.01 vs SO; **: p<0.05 vs SO; #: p<0.01 vs SN.

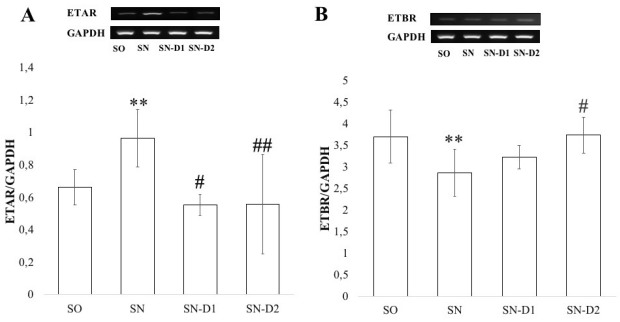

Fig. 3: Vitamin D reduced ETAR followed by upregulation of ETBR mRNA expression. A. Semi-quantitative analysis of ETAR mRNA expression. B. Semi-quantitative analysis of ETBR mRNA expression. *: p<0.01 vs SO; **: p<0.05 vs SO; #: p<0.01 vs SN; ##: p<0.05 vs SN.

that 5/6 SN was significantly higher ETAR mRNA expression compared to the SO group (p=0.038). Daily administration of Vitamin D, SN-D1 (p=0.006) and SN-D2 (p=0.045), was sharply lower mRNA expression of ETAR compared to the SN group. In addition, the mRNA expression of ETBR was significantly lower in SN group compared to the SO group (p=0.011). The SN-D2 showed higher expression compared to the SN group (p=0.005).

DISCUSSION

Our previous study demonstrated reno-protective effect of Calcitriol in 5/6-Subtotal Nephrectomy (SN) which represented CKD through reducing podocytopathy, inflammation and fibrosis (19). Continuing the study, in this study, Vitamin D treatment induces upregulation of ppET-1/ETBR/eNOS. SN is a model of chronic renal disease that has been proven successful and is often used to study the process of kidney adaptation along with changes in genotypes and phenotypes that occur in it. In general, the state of renal fibrosis will cause a decrease in number and function of healthy nephrons due to connective tissue deposition, thus causing tissue remodelling and decreased physiological function of the kidney (23). Immunostaining of PDGFRβ demonstrated an increase in the number of fibroblast in interstitial tissue in the SN group compared to the SO group (5). These findings indicate an increase in activation fibroblasts known as one of the origins of myofibroblasts. In accordance with the findings on anti-PDGFRβ staining, the anti-α-SMA staining also showed a positive increase in brown-stained cells with more numbers in the SN group than SO group (19). Both of these findings corroborate the theory that chronic inflammation will cause excessive activation of fibroblasts, resulting in forming myofibroblasts formation; cells that have contractile abilities and are able to produce extracellular matrices. Those abilities cause scar tissue formation which then damages the anatomy and physiology of the surrounding healthy tissue.

Vitamin D is known to ameliorate kidney injury by suppressing fibrosis, inflammation and apoptosis, through inhibition of several pathways that play an important role in kidney injury, such as RAAS, NF-κB, TGF-β/Smad, and Wnt/B-catenin (24). Vitamin D is known to have 2 different pathways in reducing kidney fibrosis, a classic pathway that decreases TGF-β expression, and non-classical pathways that do not reduce TGF-β expression (21). The 5/6 subtotal nephrectomy that we did in the SN group caused a significant increase in prepro-ET-1 mRNA expression compared to the SO group. This increase is in line with the increase in TGF-β1 mRNA expression which can lead to increased synthesis of ET-1 (19). The effect of increasing ET-1 was also in accordance with the fibrosis area fraction, α-SMA and PDGFRβ expressions examination. With calcitriol administration, we found that prepro-ET-1 mRNA expression continued to increase in the SN-D1 or SN-D2 group compared to SN. However, the increase was not significantly different. A similar result was found by a study previously conducted, that rats receiving calcitriol experienced an increase in synthesis of ET-1. This increase was caused by an increase in the synthesis of AP-1 and ECE-1 mediated by calcitriol bonds with Vitamin D receptors (21). Along with those findings, the ETAR expression in this study also showed a significant increase in SN group compared to SO, then decrease significantly in the calcitriol treated groups in comparison to SN. In contrast to the ETAR expression, ETBR expression that once decline in SN group, showed significant upregulation in both of SN-D1 and SN-D2 groups compared to SN. These phenomenons are assumed to be the mechanism of vitamin D to regulate the endothelin system. Endothelin-1 and ETAR activation induces kidney injury progression in AKI model using bilateral renal clamping with increasing vascular remodelling and inflammation (25). Deletion of ET-1 from endothelial cells and Endothelin Converting Enzyme-1, an enzyme for ET-1 activation attenuated kidney fibrosis in unilateral ureteral obstruction model (26). Furthermore, inhibition of ET-1 attenuate vascular remodelling in diabetic model of mice (27). Dual inhibition of ETAR and ETBR showed attenuation of

proteinuria in diabetic nephropathy patients, however has impact in inducing edema condition due to ETBR inhibition in kidney collecting ducts (28). This finding showed importance of ETBR in the kidney for its natriuretic and diuretic effects. Production of NO as vasodilator is regulated by ET-1 and ETBR activation, then inducing eNOS (29). Vitamin D also contribute to NO availability in association with VDR and eNOS upregulation (30). Our previous study showed association between Vitamin D treatment with upregulation of ppET-1/ETBR/eNOS signaling in kidney fibrosis model and in human umbilical vein endothelial cell (HUVEC) culture (31).
Based on the results of several gene expressions in this study, we assume that vitamin D supplementation can reduce renal fibrosis through activation of the ET-1/ETBR pathway thereby increasing eNOS production. These results correspond to findings that show an increase in the expression of ET-1, ETBR, and eNOS pathways, CD31, VEGF expression, and vascular remodeling improvement with administration of calcitriol in mice with UUO (31). Even so, vitamin D can also increase eNOS expression directly. Calcitriol-induced eNOS stimulation is directly dependent on activation of the vitamin D receptor (VDR), with the VDR-calcitriol complex being a transcription factor that functions as a mediator of this effect (24).

The calcitriol dose given in this study was 10ng/100grBB/day (physiological dose) and 50ng/100grBB/day (high dose). Based on the examination of histological preparations and various gene expressions in this study, it was seen that both doses were able to provide similar effects. Based on previous research, it is known that the physiological dose of calcitriol in mice is <25ng/100grBB/day (32). At this dose, vitamin D does not cause side effects such as hypercalciuria hypercalcemia (32), renal dysfunction (33), and vessel calcification (34). Future studies may be needed to assess those safety parameters, as this study did not address them in specific.

CONCLUSION

Based on the results of this study, it can be concluded Calcitriol treatment induces upregulation of ET-1/ETBR/eNOS signalling which may associate with its renoprotective effect. Further research is needed to determine the more precise effects of calcitriol on the signalling pathway using ET blockers or Vitamin D Receptor blocker.

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