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Comparison of the effects of novel vitamin D receptor analog VS-105 and paricalcitol on chronic kidney disease-mineral bone disorder in an experimental model of chronic kidney disease

Running title: Effects of VS-105 and paricalcitol on CKD-MBD

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Abstract

When using vitamin D, the most important clinical problems are hypercalcemia, hyperphosphatemia, and vascular calcification. VS-105 is a novel vitamin D receptor (VDR) analog. In the present study, we compared the effects of VS-105 and paricalcitol on chronic kidney disease-mineral bone disorder (CKD-MBD) in a CKD rat model. We used male Sprague-Dawley (SD) rats and performed 5/6 nephrectomy at 8-9 weeks. At 10 weeks, the rats were classified into five groups and administered vehicle (V), low-dose paricalcitol (LP, 0.1 μg/kg), high-dose paricalcitol (HP, 0.3 μg/kg), low-dose VS-105 (LV, 0.2 μg/kg), and high-dose VS-105 (HV, 0.6 μg/kg) three times a week for 10 weeks. There were no significant differences in blood pressure or renal function among the five groups. Alhough serum calcium levels were comparable between the LP and LV groups, they were higher in the HP group than in the HV group. Serum phosphate levels were higher in the paricalcitol-treated groups than in the VS-105-treated groups and paticularly higher in the HP group than in the other groups. The urinary excretion of phosphate was greater in the VS-105-treated groups than in the paricalcitol-treated groups. Serum parathyroid hormone (PTH) levels decreased and serum fibroblast growth factor-23 (FGF23) levels were elevated after administering paricalcitol and VS-105; however, serum FGF23 levels were remarkably elevated in the paricalcitol-treated groups. Further biochemical analyses

revealed that the calcium content of the aorta was higher in the paricalcitol-treated groups than in the VS-105-treated group. VDR and Klotho expression in the kidney was significantly higher in the VS-105-treated groups than in the paricalcitol-treated groups although both agents increased these expressions. Our data suggest that VS-105 had a lesser effect on CKD-MBD than paricalcitol except in the case of serum PTH levels. The mechanism appears to be associated with the difference in VDR and Klotho expression.

Keywords: VS-105, vitamin D analog, CKD-MBD, FGF23, Klotho

1. **Introduction**

In patients with chronic kidney disease (CKD), various abnormalities of mineral and bone metabolism frequently emerge, and together, these are known as chronic kidney disease-mineral bone disorder (CKD-MBD) [1]. It has recently become well known that these abnormalities lead not only to osteopenia or fracture but also to cardiovascular disease (CVD) and mortality [2, 3]. Therefore, the control of CKD-MBD is crucial and strategies for managing it are necessary. Among the abnormalities, secondary hyperparathyroidism (SHPT) is frequently observed in the advanced stages of CKD, and most patients need to be treated using active vitamin D or vitamin D receptor analogs (VDRAs) to suppress the elevated serum parathyroid hormone (PTH) levels. However, the treatment of SHPT with vitamin D or VDRAs sometimes causes hypercalcemia or vascular calcification [4, 5]. There is a possibility that vitamin D or VDRAs potentially induce vascular calcification in the presence of hyperphosphatemia or a high phosphate load. Therefore, drug that is less likely to affect serum calcium (Ca) and/or phosphate (P) levels and can effectively suppress serum PTH levels would be ideal. Paricalcitol is commonly used for patients with SHPT worldwide and has been reported to have such favorable properties [6, 7]. However, high-dose administration of paricalcitol leads to hypercalcemia, hyperphosphatemia, and vascular calcification [8]. VS-105 is a novel

VDRA that has been reported to have a lesser effect on serum Ca levels and prevent the progression of cardiovascular complications [9, 10].

In the present study, we compared the effects of VS-105 with those of paricalcitol on CKD-MBD using a CKD rat model.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats were obtained from SLC Japan Inc., Shizuoka, Japan. These rats were housed with food and water available ad libitum in a temperature-controlled room. To create a model of CKD (Nx), the rats were anesthetized with isoflurane and 2/3 right nephrectomy was performed at 8 weeks of age. One week later, the left kidney was resected under anesthesia. At 10 weeks of age, these rats were divided into five groups: Nx + vehicle (control, n = 6), Nx + low-dose VS-105 (LV, n = 6), Nx + low-dose paricalcitol (LP, n = 6), Nx + high-dose VS-105 (HV, n = 6), and Nx + high-dose paricalcitol (HP, n = 6). After classification, the rats were fed a high-protein and high-phosphate diet including 1.0% Ca and 1.2% P and each treatment was started.

Vehicle, VS-105 (Vidasym, Chicago, IL, USA) and paricalcitol (AbbVie Inc. North Chicago, Illinois, USA) were administered intraperitoneouslly three times per week for

10 weeks (LV; 0.1 μg/kg body weight; LP, 0.3 μg/kg body weight; HV, 0.2 μg/kg body weight; HP, 0.6 μg/kg body weight). Based on the results of a preliminary study and a previous study [9, 10], the dose of each drug was chosen so that VS-105 and paricalcitol would have comparable effects on PTH suppression.

Twenty-four-hour urine samples were collected from each rat at baseline and before the rats were killed, using a metabolic cage. At 20 weeks, the rats were killed under ether anesthesia. Blood samples for serum measurements were collected from the left ventricle, and the kidneys and aorta were removed for RNA extraction and histomorphological analysis. The animal care and all experimental procedures were approved by the Institutional Animal Care and Use Committee guidelines (Permit Number: P130103) and were in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.2. Serum and urine measurements

Serum and urine samples were centrifuged for 5 min at 800 × *g* and stored at −80°C until analysis. Serum creatinine, urea nitrogen, albumin (Alb), Ca, and P levels, and urinary Ca and P levels were measured using a Fuji Dri-Chem 3500 (FUJIFILM Japan, Tokyo, Japan). Urinary creatinine and Alb levels were measured using enzyme-linked

immunosorbent assay (ELISA) kits (creatinine: Nephrat; Exocell, Inc., Philadelphia, Pa., USA; albumin: Exocell, Inc.). Serum PTH levels were measured with a rat PTH-ELISA kit (Immutopics, San Clemente, CA, USA) and serum fibroblast growth factor-23 (FGF23) levels were determined with an intact FGF23 ELISA kit (Kainos Laboratories, Inc., Tokyo, Japan). Serum 25-hydroxyvitamin D (25D) and 1,25-dihydroxyvitamin D (1,25D) levels were measured using a 25D ^{125}I radioimmunoassay kit (DiaSorin Inc., Stillwater, USA) and a TFB 1,25D radioimmunoassay kit (Immunodiagnostic Systems Ltd., Boldon, UK), respectively.

2.3. Blood pressure measurements

Systolic blood pressure was measured in conscious, restrained rats by tail-cuff plethysmography (Model MK-2000; Muromachi Kikai Co. Ltd., Japan). To reduce the possibility of stress artifacts, the rats were allowed to acclimatize to the environment for at least 15 minutes. Blood pressure was determined using multiple readings (at least 10). Blood pressure was measured at baseline and at the end of the study period.

2.4. Immunohistochemical analyses

The remaining kidney was removed and fixed with 10% formaldehyde, dehydrated at

room temperature through an ethanol series, embedded in paraffin and cut into in 3-μm sections. To evaluate the expression of vitamin D receptor (VDR) and Klotho in the kidney, immunohistochemical staining was performed as described previously [11]. In brief, paraffin-embedded sections were stained with the following primary antibodies: anti-VDR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Klotho antibody (Alpha Diagnostic Intl Inc., Texas, USA). The VDR- and klotho-positive intraglomerular and tubular cells in the kidney tissue were counted in 20 random microscopic fields to give VDR- and Klotho-positive cell scores. All evaluations were performed in a blinded manner.

2.5. RNA extraction and renal-time PCR

As previously reported [12], total RNA was extracted from the rat kidneys using an ISOGEN kit (Wako Pure Chemicals Industries, Ltd, Osaka, Japan) according to the manufacturer's instructions. Total RNA from the rat kidneys was used as the template for cDNA synthesis in a 20-μL volume with the SuperScript First-Strand Synthesis System (Invitrogen, CA, USA) using the oligo-dT hexamer as per the manufacturer's instructions. The reaction mixture was incubated at 65°C for 5 min and placed on ice for 1 min. Another reaction mixture was then added and the mixture was incubated at 50°C for 50

min, followed by 85°C for 5 min and then chilled on ice for 1 min. Next, 1 μL of RNase H was added and the mixture was incubated at 37°C for 20 min. The synthesized cDNA was stored at −20°C until polymerase chain reaction (PCR) analysis. Real-time PCR was performed using a LightCycler 350s Real-Time PCR System (Roche, Mannheim, Germany) with the LightCycler FastStart DNA Master SYBR Green I Kit (Roche). The analysis was performed with the second derivative maximum method of the LightCycler software (version. 4.0; Roche). The relative amount of the sample mRNA was normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. For PCR analysis, the following primers were used: rat VDR (5′- ACAGTCTGAGGCCCAAGCTA-3′, 5′-TCCCTGAAGTCAGCGTAGGT-3′), rat Klotho (5′-CGTGAATGAGGCTCTGAAAGC-3′, 5′-GAGCGGTCACTAAGCGAATACG-3′), and rat *GAPDH* (5′-TGGGAAGCTGGTCATCAAC-3, 5′-GCATCACCCCATTTGATGTT-3′).

2.6. Statistical analysis

Data were analyzed with StatView 5.0 (SAS Institute, Cary, NC, USA). Values are presented as the mean ± SEM. The significance of the differences between two groups was analyzed by Mann–Whitney U test. The differences between the control and the other

groups were assessed by Dunnett's test. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Changes in serum Ca and P levels

At baseline (10 weeks), there were no significant differences in characteristics or biochemical data among the groups (data not shown). At 20 weeks, blood pressure, body weight, renal function and serum Alb levels were comparable among the five groups. Although serum Ca levels were comparable between the LV and LP groups, they were higher in the HP group than in the HV group. Serum P levels tended to be higher in the LP group than in the LV group, and were significantly higher in the HP group than in the HV group (Figure 1).

3.2. Changes in urinary excretion of Ca and P

At baseline, urinary excretion of Ca and P did not differ among the five study groups. After the 10-week treatment, although urinary excretion of Ca was comparable between the LP and LV groups, it was greater in the HP group compared to the HV group. On the

other hand, although urinary excretion of P was also comparable between the LP and LV groups, it was greater in the HV group than in the HP group (Figure 2).

3.3. Assessment of CKD-MBD biomarkers

We examined serum PTH levels, FGF23 levels, 1,25D levels, and 25D levels in the five study groups. Serum PTH levels were comparable between the LV and LP groups and between the HP and HV groups, whereas serum FGF23 levels tended to be higher in the LP group than in the LV group and those were significantly and remarkably elevated in the HP group compared to the HV group (Figure 3). There were no significant and statistical differences in serum 25D levels among all the study groups (Figure 4). Serum 1,25D levels were remarkably suppressed to approximately the same levels in the HP and HV groups and tended to be higher in the LV group than in the LP group (Figure 4).

3.4. Ca content of the aorta

To assess the effect of each VDRA on vascular calcification, we measured the Ca contents of the aorta. Although the Ca contents tended to be greater in the paricalcitol-treated groups (the LP and HP groups) than in the VS-105-treated groups (the LV and HV groups), there were no statistically significant differences (Figure 5).

3.5. Assessment of intrarenal expression of VDR and Klotho

We evaluated expression of VDR and Klotho in the kidney tissues. The number of VDR-positive cells was significantly higher in the VS-105-treated groups compared to the paricalcitol-treated groups (Figure 6A). In contrast, although the number of Klotho-positive cells was similar between the LP and LV groups, there were significantly greater numbers in the HV group compared to the HP group (Figure 6B).

VDR and Klotho mRNA levels in the kidney were also determined by real-time PCR. At 20 weeks, the mRNA expression of VDR was significantly higher and the mRNA expression of Klotho tended to be higher in the HV group compared to the HP group (Figure 6C, D).

4. Discussion

We demonstrated that (1) although serum Ca levels showed overall comparable results at low doses at 20 weeks, at higher doses, serum Ca levels were higher in the HP group compared to the HV group; (2) serum P levels were higher and the urinary excretion of P was lower in the paricalcitol-treated groups than in the VS-105-treated groups; (3) despite comparable serum PTH levels, serum FGF23 levels were remarkably elevated in the

paricalcitol-treated groups compared to the VS-105-treated groups; (4) VDR and Klotho expression in the kidney was significantly higher in the VS-105-treated groups compared to the paricalcitol-treated groups.

SHPT is frequently observed especially in patients with advanced-stage CKD, and this abnormality is related to the progression of CKD and CVD [13-15]. Hypercalcemia and hyperphosphatemia induced by the progression of SHPT lead to vascular calcification, which is linked to CVD events and mortality [3, 16-18]. Therefore, it is very important to halt this vicious cycle. VDRA therapy is frequently performed for this purpose in the clinical setting.

Vitamin D agents including VDRAs not only suppress serum PTH levels and halt the progression of SHPT, but also have various organ protective effects and potency to prolong life independent of serum Ca, P and PTH levels. There have been a large number of basic and clinical studies using paricalcitol, in which the mechanisms of these favorable effects have been demonstrated. Hypercalcemia is the most important adverse effect in the use of vitamin D, and paricalcitol is less likely to induce hypercalcemia and vascular calcification than other vitamin agents [6, 7]. It has been reported that administration of high-dose paricalcitol induced vascular calcification, although clinically relevant dosages of paricalcitol could protect against CKD-stimulated vascular

calcification [8].

VS-105 was developed in 2010. Previous reports have demonstrated that it has not only a serum PTH lowering effect, but also organ protective effects for the heart and vascular system, similar to paricalcitol [19, 20]. In addition, a previous study has demonstrated that VS-105 raises serum Ca levels less than other VDRAs because it is less likely to induce the expression of Ca transporters such as transient receptor potential vanilloid Ca channel 6 (TRPV6) and calbindin [9]. Therefore, we performed the present study to compare the clinical efficacy of paricalcitol and the novel vitamin D agent VS-105.

Based on the results of preliminary study, we set the dose of each agent so that they would have comparable effects on serum PTH levels and we configured high and low doses of each agent to gain more detailed data. The results of the present study showed that VS-105 and paricalcitol had comparable effects on the suppression of serum PTH levels at both high and low doses. Although there were no significant differences in serum Ca levels between the low doses of VS-105 and paricalcitol, the high dose of VS-105 affected serum Ca levels less than did paricalcitol. Although we could not demonstrate the detailed mechanisms of this in the present study, from the results of the previous study, we speculate that they are associated with the difference in the expression of Ca transporters such as TRPV6 and calbindin between these two VDRAs [9].

These agents also differed in their influence on serum P levels, which is a great problem with VDRA treatment. Despite comparable serum PTH levels, serum P levels were higher in the paricalcitol-treated groups than in the VS-105-treated groups. To elucidate the mechanisms behind this, we performed a further evaluation. As a result, we found that urinary P excretion was greater in the VS-105-treated groups than in the paricalcitol-treated groups. A further crucial problem in using VDRAs is their unfavourable effects on serum FGF23 levels. In general, although VDRAs has many favorable effects on the body, VDRAs are known to raise serum FGF23 levels [21]. This is a very important issue that remains to be resolved. Although VDRAs raise serum FGF23 levels, we think that the balance between favorable effects and adverse effects is important in using these agents. Surprisingly, the results of our study showed that the serum FGF23 concentration was lower in the VS-105-treated groups compared to the paricalcitol-treated groups. A recent other study has also revealed that VS-105 affects serum FGF23 concentration less [20]. To elucidate the mechanisms underlying the lower serum P levels and greater urinary P excretion despite lower serum FGF23 levels in the VS-105-treated groups compared to the paricalcitol-treated groups, we evaluated several factors associated with the FGF23 signal. We found that especially high doses of VS-105 induced the expression of VDR and Klotho in the kidney. Previous studies have shown that VDRA therapy

increased Klotho expression in CKD independently of changes in serum Ca and PTH levels [22, 23]. Taken together, we suppose that VS-105 is more likely to increase the expression of VDR and Klotho, leading to the improvement of FGF23 sensitivity. Although we could not prove the detailed mechanisms, as shown in a previous study [9], it is suggested that VS-105 is less likely to induce the expression of Ca and P transporters in the intestine. Changes in the expression of sodium-phosphate cotransporter such as NaPi2a or NaPi2c in the kidney may be also involved in this mechanism.

It is well known that vascular calcification is a crucial risk factor associated with CVD events and mortality [3, 16-18]. In the use of vitamin D agents, development of vascular calcification is an important problem. The progression of vascular calcification is thought to be accelerated by hypercalcemia, hyperphosphatemia and elevated FGF23 due to administration of vitamin D agents. It is suggested that paricalcitol potentially has less effects on serum Ca levels and vascular calcification compared to other VDRAs. Although there was no significant difference between the VS-105-treated and paricalcitol-treated groups, the content of Ca in the aorta was lower in the VS-105-treated groups than in the paricalcitol-treated groups. Considering these facts, we speculate that VS-105 has more favorable effects on CKD-MBD compared to other VDRAs including paricalcitol.

There were several limitations in this study. First, we could not ~~perform enough evaluation~~ sufficiently evaluate the changes in the absorption of Ca and P from intestine or the changes in the expression of transporters related to Ca and P handling in the intestine and kidney. Secondly, it was not proven whether the appropriate doses of each drug were used. In the present study, we set the dose based on the suppression of serum PTH levels. If we had established the dose based on the effect on serum Ca levels, the results may have been different. Thirdly, we could not evaluate the influence of VDR gene polymorphisms on CKD-MBD in the use of VDRAs. It has been reported that the presence of polymorphisms of VDR was associated with CKD-MBD [@@-@@]. A previous study in patients with peritoneal dialysis demonstrated that VDR gene polymorphism was associated with increased risk for developing hypercalcemia [@]. Thus, evaluation of VDR gene polymorphisms also seems to be important. Further study is needed to resolve these issues.

5. Conclusions

Our data suggest that VS-105 is less likely to induce hypercalcemia, hyperphosphatemia and vascular calcification than paricalcitol possibly because of the increased expression of VDR and Klotho. In addition, it also has a lesser effect on serum

FGF23 levels. To explore the utility of VS-105 in clinical settings, it will be necessary to clarify the detailed mechanisms of action and collect clinical data in a human study.

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Conflict of interest statement:

All authors declared no conflict of interest.

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Table 1 Animal characteristics at 20 weeks

	C (N = 6)	LV (N = 6)	LP (N = 6)	HV (N = 6)	HP (n = 6)
Body weight (g)	353.5 ± 11.6	355.2 ± 2.8	350.3 ± 6.9	365.6 ± 7.3	361.0 ± 5.5
SBP (mmHg)	100.6 ± 8.1	101.3 ± 7.8 †	111.8 ± 9.1 #	102.0 ± 3.5 †	111.5 ± 3.1 #
Cr (mg/dl)	0.88 ± 0.07	0.90 ± 0.06	0.89 ± 0.06	0.90 ± 0.04	0.86 ± 0.05
BUN (mg/dl)	48.2 ± 4.4	49.1 ± 1.8	47.4 ± 2.6	45.4 ± 1.5	47.8 ± 2.8
Ccr (ml/min/100g)	1.21 ± 0.20	1.30 ± 0.91	1.18 ± 0.57	1.14 ± 0.06	1.18 ± 0.57
Alb (mg/dl)	4.00 ± 0.06	4.11 ± 0.14	4.22 ± 0.19	4.11 ± 0.12	4.12 ± 0.15

†；SHR v.s MI , $p<0.05$, #；SHR v.s AST-120, $p<0.05$

C, control group; LV, low VS-105 group; LP, low paricalcitol group; HV, high VS-105 group; HP, high paricalcitol group; SBP, systolic blood pressure; Cr, creatinine; BUN, blood urea nitrogen; Ccr, creatinine clearance; Ca, calcium; P, phosphate; U-Ca, urinary excretion of calcium; U-P, urinary excretion of phosphate; int-PTH, intact parathyroid hormone; FGF-23, fibroblast growth factor-23.

Figure Legends

Figure 1 Serum calcium and phosphate levels in the C, LV, LP, HV and HP groups at 20 weeks

#; VS-105 v.s Paricalcitol, $p<0.05$, *; v.s Control, $p<0.05$

Figure 2 24-hour urinary excretion of calcium and phosphate in the C, LV, LP, HV and HP groups at 20 weeks

#; VS-105 v.s Paricalcitol, $p<0.05$, *; v.s Control, $p<0.05$

Figure 3 Serum parathyroid hormone and fibroblast growth factor-23 levels in the C, LV, LP, HV and HP groups at 20 weeks

#; VS-105 v.s Paricalcitol, $p<0.05$, *; v.s Control, $p<0.05$

Figure 4 Serum 25 (OH) vitamin D and 1, 25 (OH) vitamin D levels in the C, LV, LP, HV and HP groups at 20 weeks

#; VS-105 v.s Paricalcitol, $p<0.05$, *; v.s Control, $p<0.05$

Figure 5 Calcium contents of aorta in the C, LV, LP, HV and HP groups at 20 weeks

#; VS-105 v.s Paricalcitol, $p<0.05$, *; v.s Control, $p<0.05$

Figure 6 Expression of vitamin D receptor and klotho in the C, LV, LP, HV and HP groups at 20 weeks

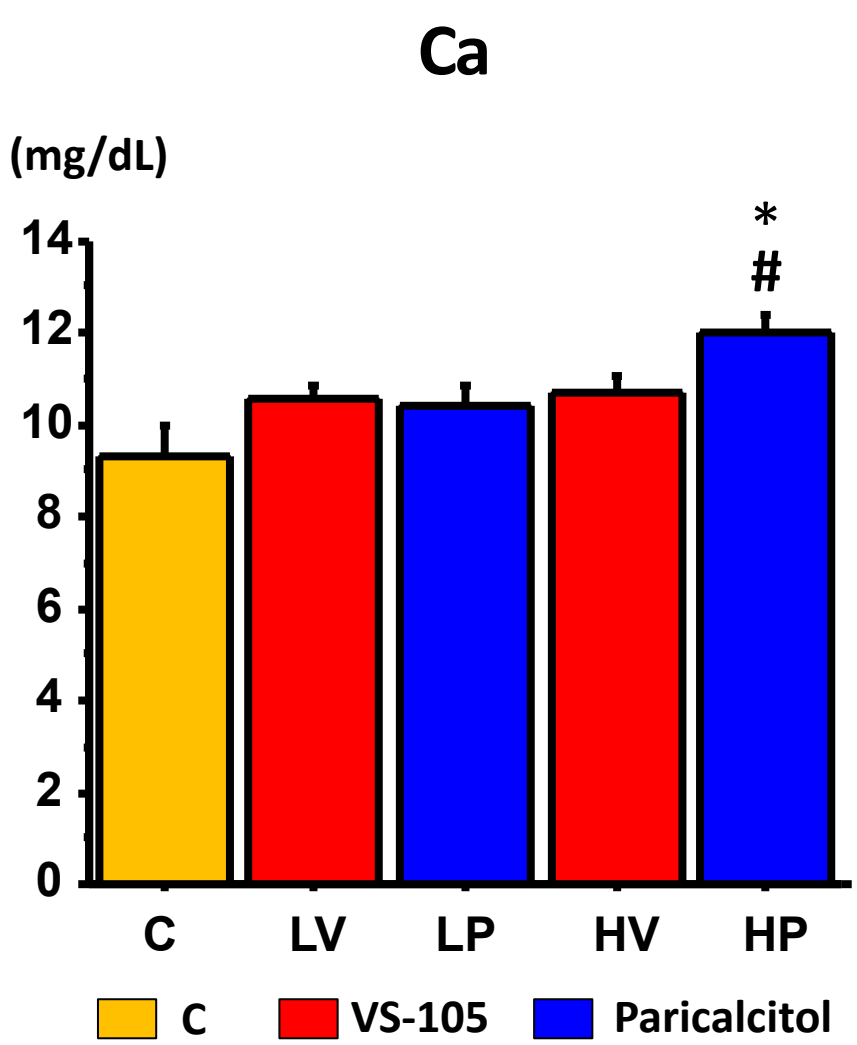
(A) VDR positive cell score

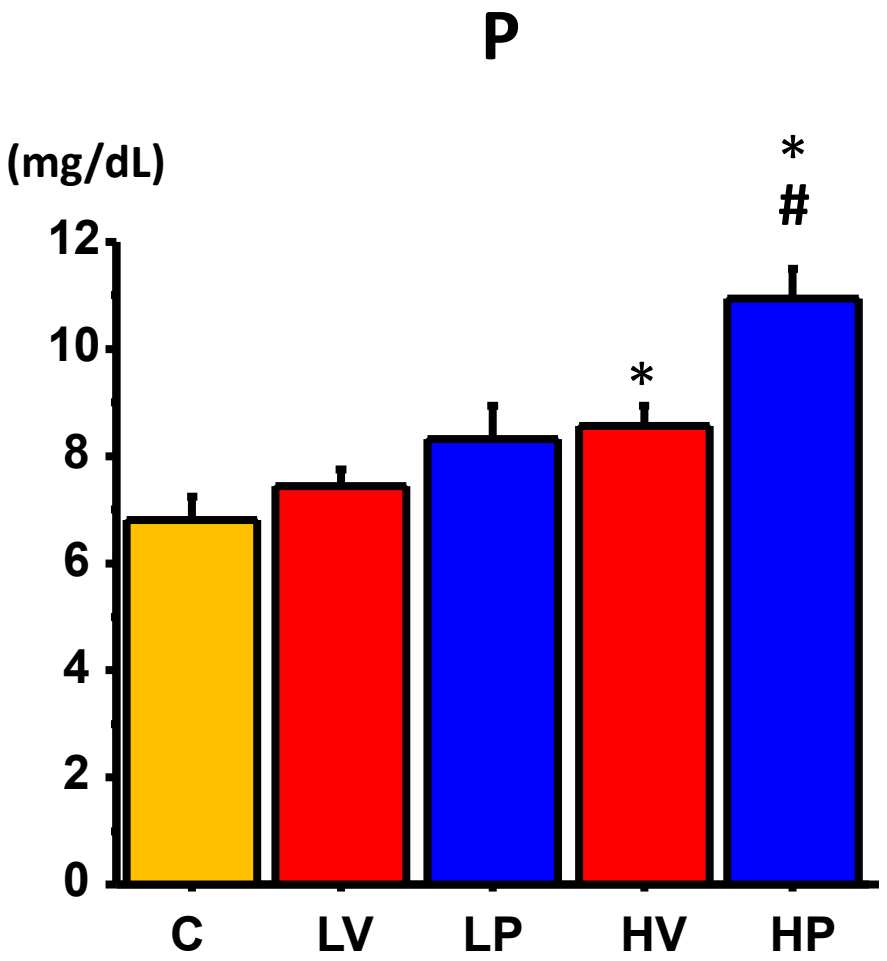
(B) Klotho positive cell score

(C) mRNA expression of *VDR*

(D) mRNA expression of *Klotho*

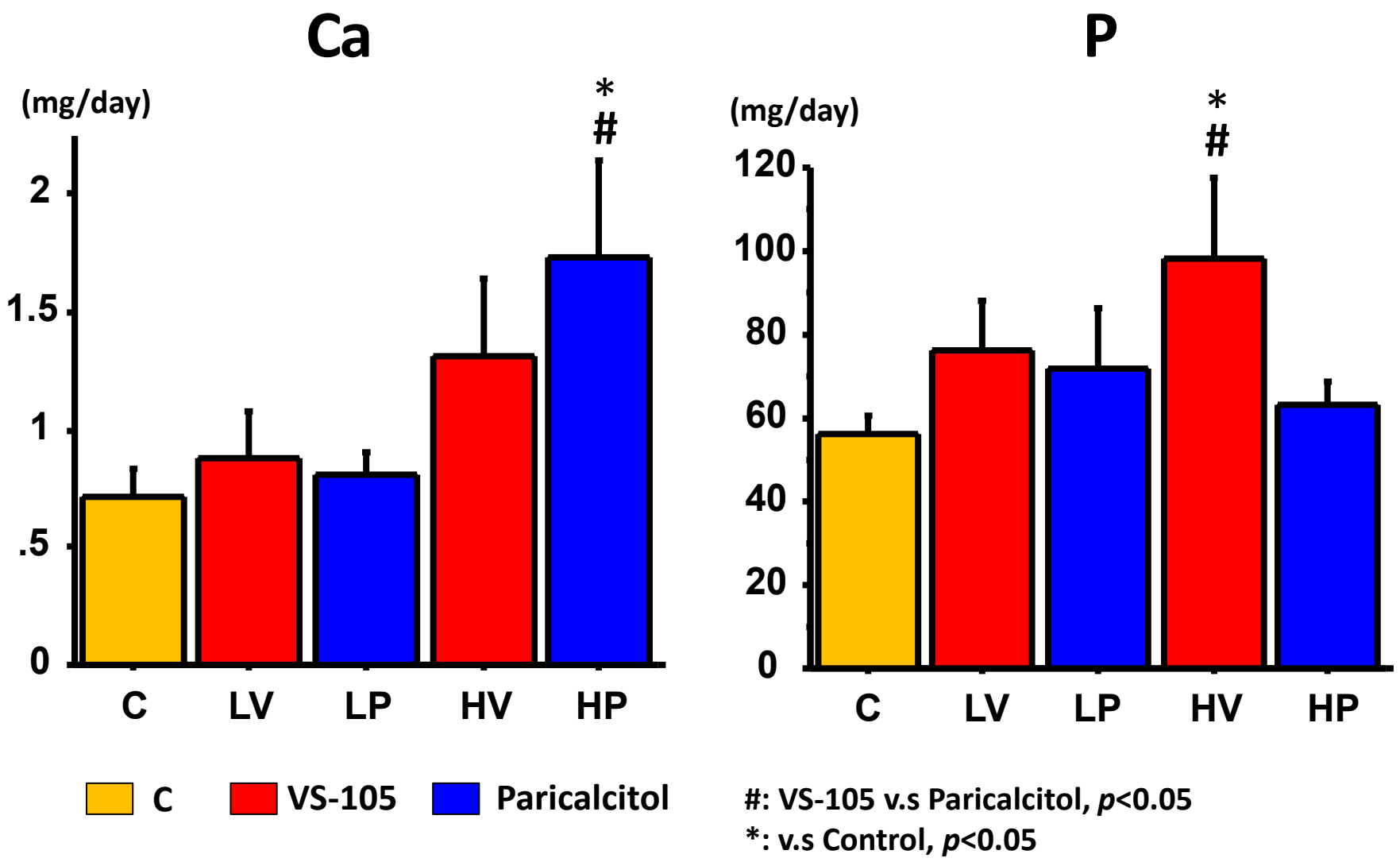
#; VS-105 v.s Paricalcitol, $p<0.05$, *; v.s Control, $p<0.05$

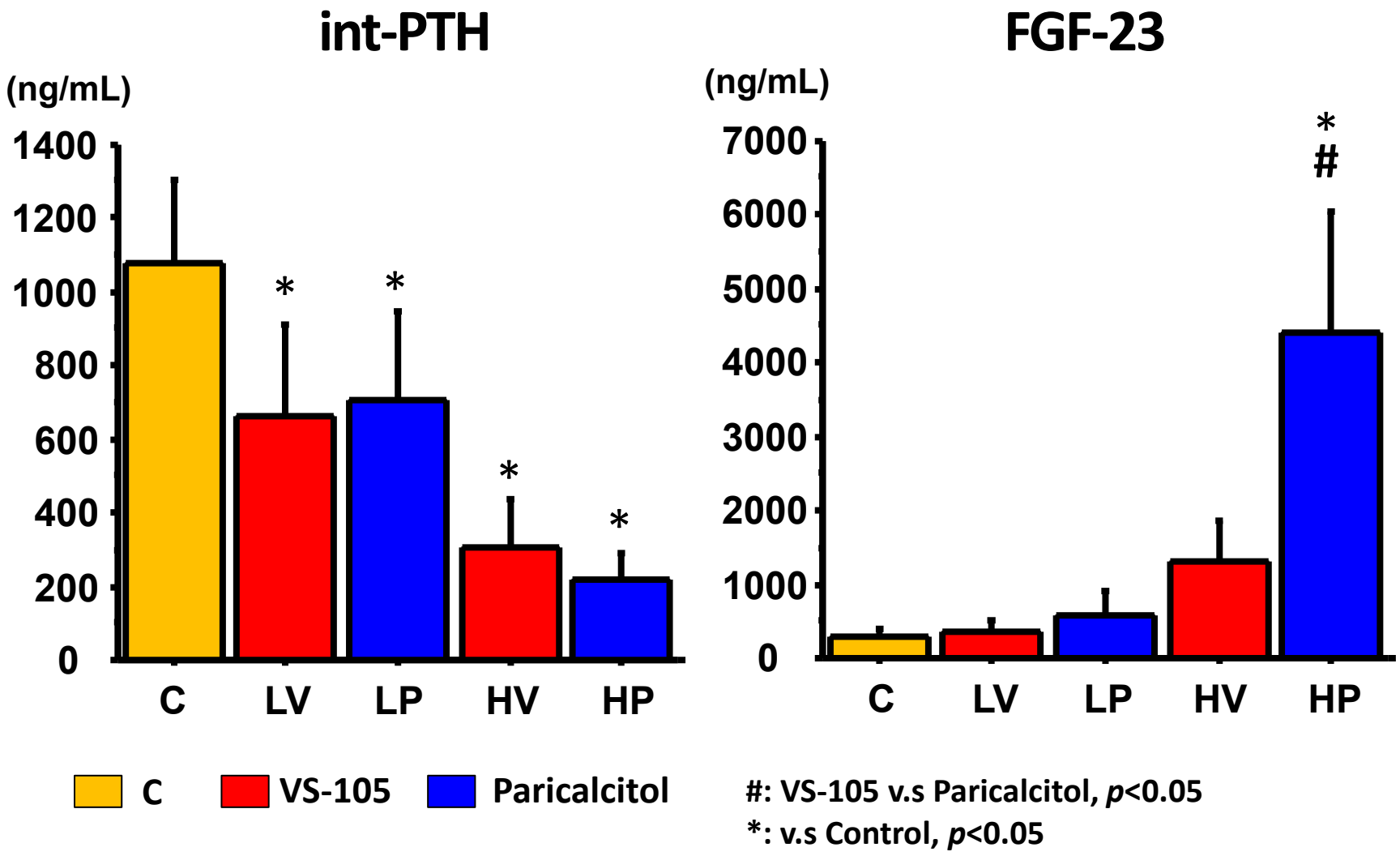




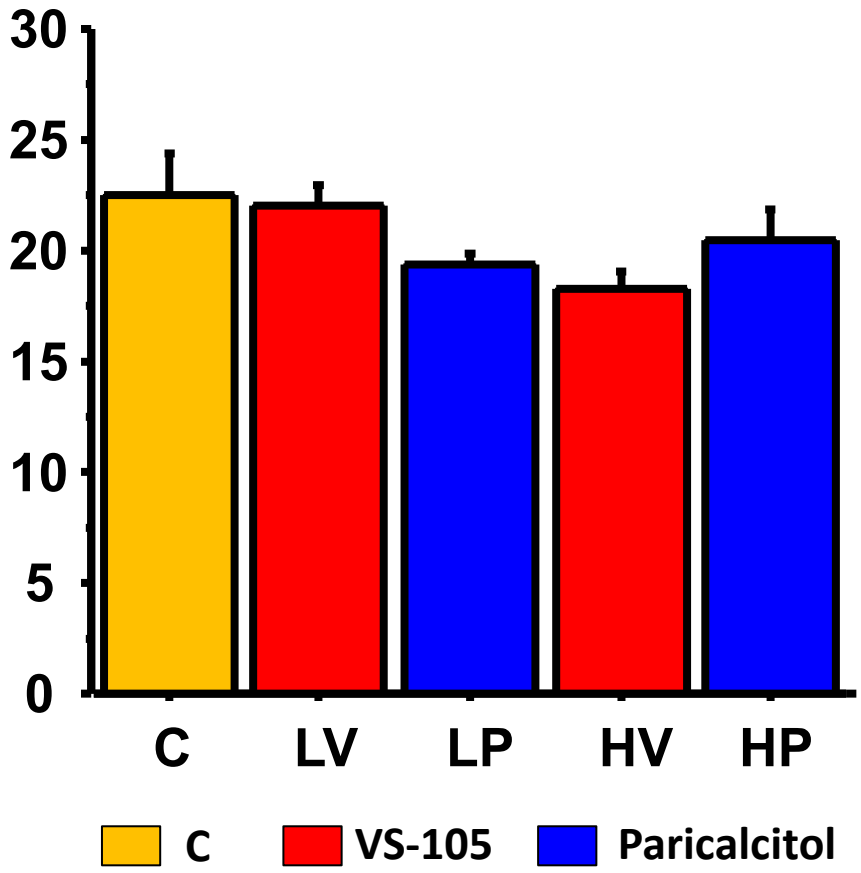
#: VS-105 v.s Paricalcitol, $p<0.05$

*: v.s Control, $p<0.05$

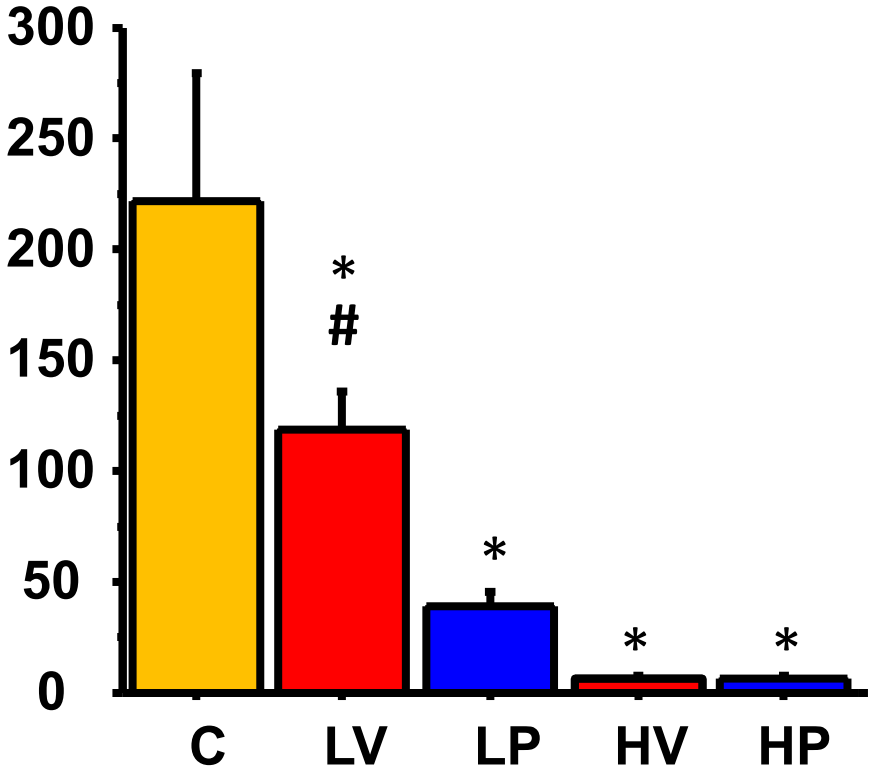

Ca
(mg/day)
2
1.5
1
.5
0
*
#
C
LV
LP
HV
HP
P
(mg/day)
120
100
80
60
40
20
0
*
#
C
LV
LP
HV
HP
C
VS-105
Paricalcitol
#: VS-105 v.s Paricalcitol, p<0.05
*: v.s Control, p<0.05


int-PTH
(ng/mL)
1400
1200
1000
800
600
400
200
0
*
*
*
*
C
LV
LP
HV
HP
FGF-23
(ng/mL)
7000
6000
5000
4000
3000
2000
1000
0
*
#
C
LV
LP
HV
HP
C
VS-105
Paricalcitol
#: VS-105 v.s Paricalcitol, p<0.05
*: v.s Control, p<0.05

25(OH) Vit.D

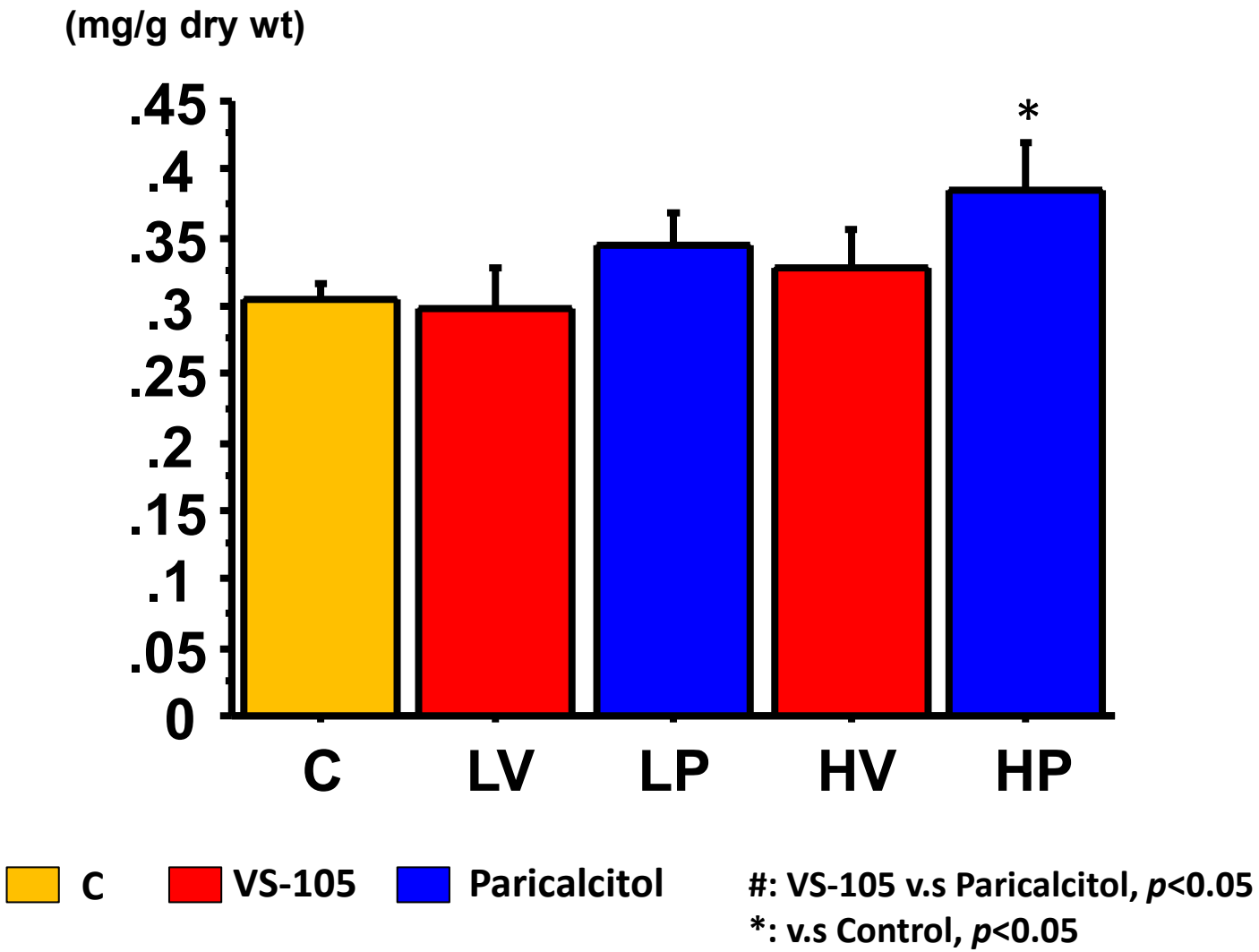


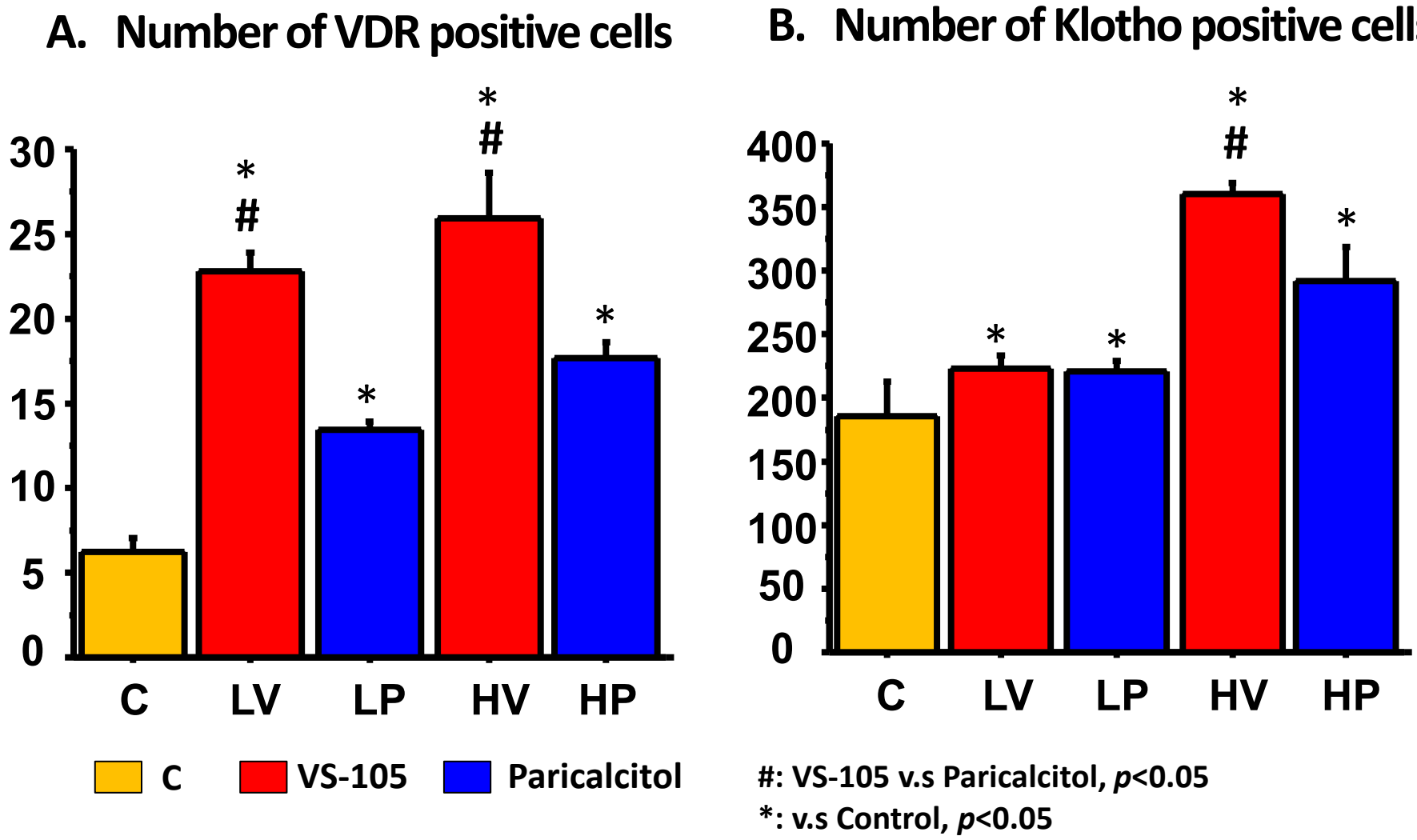
1, 25(OH)$_2$ Vit.D

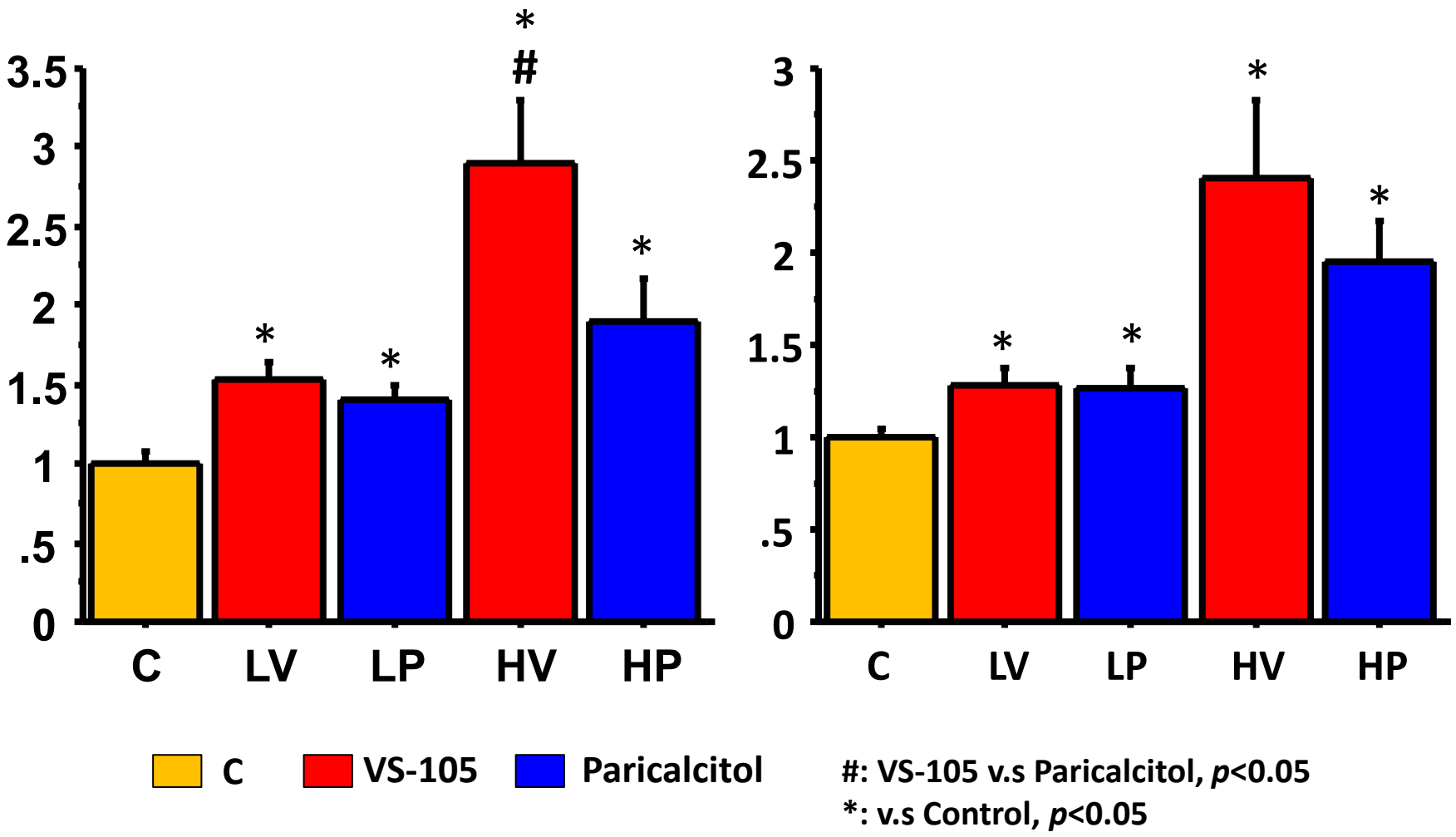


#: VS-105 v.s Paricalcitol, $p<0.05$

*: v.s Control, $p<0.05$


(mg/g dry wt)
.45
.4
.35
.3
.25
.2
.15
.1
.05
0
*
C
LV
LP
HV
HP
C
VS-105
Paricalcitol
#: VS-105 v.s Paricalcitol, p<0.05
*: v.s Control, p<0.05


A. Number of VDR positive cells
30
25
20
15
10
5
0
C
LV
LP
HV
HP
C
VS-105
Paricalcitol
B. Number of Klotho positive cells
400
350
300
250
200
150
100
50
0
C
LV
LP
HV
HP
#: VS-105 v.s Paricalcitol, p<0.05
*: v.s Control, p<0.05


C. mRNA expression of VDR
D. mRNA expression of Klotho
3.5
3
2.5
2
1.5
1
.5
0
C
LV
LP
HV
HP
*
#
C
VS-105
Paricalcitol
#: VS-105 v.s Paricalcitol, p<0.05
*: v.s Control, p<0.05