Novel Markers of Left Ventricular Hypertrophy in Uremia

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Key Words

Left ventricular hypertrophy · Hyperphosphatemia · p27 · Cyclin D2

Abstract

Aims: Left ventricular hypertrophy (LVH) is the most frequent cardiac complication in chronic renal disease. Previous studies implicate elevated serum phosphorus as a risk factor for LVH. **Methods:** We treated 5/6 nephrectomized rats with enalapril or enalapril + sevelamer carbonate for 4 months to determine if sevelamer carbonate had an additional beneficial effect on the development of LVH and uremia-induced left ventricle (LV) remodeling. **Results:** Uremia increased LV weight and cardiomyocyte size. Enalapril and enalapril + sevelamer blunted the increase in left ventricular weight. Only enalapril + sevelamer diminished the increase in cardiomyocyte size. Uremia increased cyclin D2 and PCNA and decreased p27 protein expression in the heart. Enalapril + sevelamer diminished the decrease in p27 expression caused by uremia. Uremia increased Ki67-positive and phosphohistone H₃-positive interstitial cells. This was not seen in cardiomyocytes. Multivariable regression analysis showed that increased phosphorus was an independent risk factor for both increased LV weight and cardiomyocyte size. **Conclusions:** These data suggest left ventricular remodeling

consists of cardiomyocyte hypertrophy and interstitial cell proliferation, but not cardiomyocyte proliferation. p27 and cyclin D2 may play important roles in the development of LVH. In addition, phosphorus can be an independent risk factor for the development of LVH.

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Introduction

Left ventricular hypertrophy (LVH) is the most frequent cardiac complication in chronic renal disease and carries a poor prognosis [1, 2]. Nearly 75% of adult patients have LVH at the time of initiation of dialysis for end-stage renal disease [3]. The development of LVH is associated with decreased survival in patients receiving dialysis [2]. The reason for this is that LVH may cause cardiac arrhythmias, diastolic dysfunction, ischemic heart disease and progression to overt heart failure [4]. The high risk for cardiovascular disease results from multiple factors, including hemodynamic overload and several metabolic and endocrine abnormalities more or less specific to uremia [4].

Hyperphosphatemia is a widely recognized risk factor for cardiovascular mortality in the end-stage renal disease population [5, 6]. Recent data suggest that the delete-

rious effects of hyperphosphatemia may be associated with LVH [7–9]. Ayus et al. [9] recently reported that the correction of hyperphosphatemia is associated with reduction in left ventricular mass index. Neves et al. [10] studied parathyroidectomized uremic rats fed either a high or low phosphorus (P) diet with parathyroid hormone (PTH) replaced via a mini-pump. They found that hyperphosphatemia was a strong risk factor for the development of LVH. Thus, recent research again underscores the importance of controlling serum P in chronic kidney disease (CKD) patients.

Cardiac remodeling is clinically manifested by changes in cardiac size, shape and function in response to cardiac injury or increased cardiac load [11]. The cardiac cells involved in the remodeling process are cardiomyocytes and fibroblasts. Fibroblast stimulation increases collagen synthesis and causes fibrosis of both the infarcted and non-infarcted regions of the ventricle [12]. This leads to a loss of cardiomyocytes by apoptosis or necrosis. Eventually these cardiomyocytes are replaced by fibroblasts and extracellular collagen [13]. Previous studies, using experimental models of uremia, have reported the expansion of interstitial tissue [14], interstitial fibrosis [15], capillary/myocyte mismatch [16, 17], and the increase in intramyocardial arteriole wall thickness [18]. Marked remodeling of the heart has also been observed in CKD patients [19].

Hypertrophic growth is a primary mechanism through which the heart normalizes ventricular wall stress. LVH is characterized by enhanced protein synthesis and increases in the size and organization of cardiomyocyte sarcomeres [20, 21]. LVH results mainly from an increase in the size of individual cardiomyocytes (hypertrophy) and from the proliferation of non-muscle cells, mostly notably fibroblasts (hyperplasia). Little information is available on cardiomyocyte proliferation, since cardiomyocytes have long been thought to be terminally differentiated. Quaini et al. [22], however, demonstrated mitotic division in cells adjacent to necrotic areas after myocardial infarction, suggesting that cardiomyocytes may not be terminally differentiated. Whether cardiac cell proliferation is present in uremia-induced LVH has not been fully studied.

Myocardial hypertrophy is regulated by multiple signaling pathways, with the D-type cyclins playing a critical role. Indeed, cyclin D expression and cyclin D-dependent kinase (CDK) activity is induced in hypertrophic cardiomyocytes *in vitro*. Hypertrophic signals upregulate cyclin D2 expression and CDK activity in cardiac myocytes [23, 24]. Angelis et al. [25] demonstrated that in

response to pressure overload, cyclin D2 mRNA and protein levels in hypertrophic hearts of wild-type mice are increased while hypertrophy is attenuated in cyclin D2-null mice.

The CDK inhibitor p27 is a potent inhibitor of cell growth and division [26]. Antiproliferative signals lead to the accumulation and stabilization of p27, which can then inhibit CDK2 and cause cell cycle arrest [27–31]. In mice, the deletion of p27 results in multiorgan hyperplasia and tumor development [32–34]. The enhanced organ size was shown to be due to an increase in cell number in those tissues studied, with no significant increase in cell size. Poolman et al. [35] demonstrated that loss of p27 in the mouse resulted in a significant increase in heart size and in the total number of cardiac myocytes by 36 days. However, Hauck et al. [36] recently demonstrated that, p27 knockout mice develop cardiac hypertrophy and a marked increase in cardiomyocyte size by 4 months of age. These reports raise the possibility that manipulation of p27 protein levels could yield therapeutic benefits in the treatment of cardiac hypertrophy.

The present long-term study was conducted in uremic rats fed a high P diet to determine whether blocking the renin-angiotensin system with enalapril inhibits LVH, and if combining sevelamer carbonate with enalapril therapy has an added benefit in preventing LVH over enalapril therapy alone. Furthermore, this study was conducted to answer the following questions related to uremia-induced LV remodeling and the impact of P on LVH: (1) Does uremia induce cardiomyocyte proliferation? (2) Are cell cycle regulatory proteins involved in uremia-induced LVH? (3) Is P an independent risk factor for LVH?

Material and Methods

Experimental Protocol

Our current study is an extension of previous work on the blockade of the renin-angiotensin system attenuating mortality but not vascular calcification in uremic rats [37]. The data in this paper is novel and investigates the effect of P on cardiomyocytes. All studies were approved by the Washington University Animal Studies Committee in accordance with federal regulations.

Renal insufficiency was induced by 5/6 nephrectomy in a group of female Sprague-Dawley rats that weighed 200–225 g. The 5/6 nephrectomy involves the ligation of several branches of the left renal artery and excision of the right kidney. After 7 days of uremia, blood was taken to determine serum creatinine (Cr), calcium (Ca) and phosphorus (P) levels and the rats were divided into three experimental groups with similar serum Cr, Ca and P as follows: (1) uremic control (UC), (2) uremic + enalapril (UE), given the angiotensin-converting enzyme inhibitor, enalapril, in their drinking water (15 mg/l), and (3) uremic + enalapril +

sevelamer carbonate (UES), given enalapril and 3% sevelamer carbonate (Genzyme Corp., Cambridge, Mass., USA) in powdered rodent chow. A group of normal rats treated with vehicle served as control (NC). All animals were fed a powdered rodent chow with 1.0% P, 0.8% Ca, and 20% protein (Purina, St. Louis, Mo., USA). In the last few days of the study, the rats were placed in metabolic cages and urine was collected for 2 × 24-hour periods to determine creatinine clearance. Ionized Ca was determined using a Nova-8 electrolyte analyzer (Nova Biomedical, Waltham, Mass., USA). Plasma Cr and P were determined by autoanalyzer (Cobas Mira Plus, Branchburg, N.J., USA). Intact PTH was measured by an immunoradiometric assay specific for intact rat PTH (Immutopics, San Clemente, Calif., USA).

Systolic blood pressure (SBP) was measured before 5/6 nephrectomy and then monthly using the Non-Invasive Blood Pressure System XBP 1000 (Kent Scientific Corp., Torrington, Conn., USA). After 4 months, rats were killed under anesthesia by exsanguination via the dorsal aorta, and blood was taken for analytical determinations. The heart was removed, and the left atrium, right atrium and right ventricle were separated from the left ventricle (LV). The LV was then blotted dry and weighed on a microbalance (CAHN-31, Orion Instruments, Inc., Boston, Mass., USA). The LV was then divided into four sections perpendicular to the longitudinal axis. Two pieces were rinsed in 1% PBS and fixed in 10% formalin for histological examination. The other two pieces were immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

Western Blot Analysis

Protein expression of cyclin D1, cyclin D2, cyclin D3, p27, and PCNA were determined by Western blot analysis. Briefly, LV tissue was homogenized in RIPA buffer (50 mM Tris-HCl [pH 7.4]; 1% NP-40; 0.25% Na-deoxycholate, 150 mM NaCl; 1 mM Na₃VO₄, and 1 mM NaF) with 40 µl/ml of fresh protease inhibitor cocktail (Roche, Indianapolis, Ind., USA). Samples were centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatants were assayed.

Samples were mixed with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer and boiled for 5 min. Samples (40 µg protein per lane) were electrophoresed on 12% SDS polyacrylamide gels (BioRad Laboratories, Hercules, Calif., USA) and transferred to polyvinylidene difluoride (PVDF) membranes (BioRad Laboratories). The membranes were then blocked for 60 min with Tris-buffered saline that contained 3% skim milk and 0.1% Tween-20 (Sigma, St. Louis, Mo., USA), and then incubated with diluted primary antibody overnight at 4°C in Tris-buffered saline that contained 3% skim milk and 0.1% Tween-20. The source and the concentration of each antibody were as follows: anti-cyclin D1 (sc20044; 1:1,000), anti-cyclin D2 (sc593; 1:500), anti-cyclin D3 (sc6283; 1:200), anti-p27 (sc1641; 1:500), all from Santa Cruz Biotechnology, Santa Cruz, Calif., USA and anti-PCNA (Zymed, South San Francisco, Calif., USA; 1:1,000). The membranes were washed and incubated with diluted secondary anti-rabbit IgG-HRP, 1:2,000 or anti-mouse IgG-HRP, 1:2,000 (Santa Cruz Biotechnology). The membranes were again washed and developed using the enhanced chemiluminescence system (Thermo Scientific, Rockford, Ill., USA). Changes in protein expression were normalized by correction for the densitometric intensity of GAPDH (Cell Signaling Technology, Danvers, Mass., USA; 1:5,000).

Immunohistochemistry

Immunohistochemical staining was performed using a mouse anti-rat Ki67 (DakoCytomation, MIB-5, 1:50) or anti-rabbit phosphohistone H₃ (PHH₃) (Cell Signaling Technology; 1:100). The sections were deparaffinized and rehydrated. Antigen retrieval was performed by microwaving the sections in 0.01 M citrate buffer (pH 6.0) for 10 min followed by incubation at room temperature for 60 min. The sections were then treated with 0.06% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase and subsequently incubated with 3% skim milk in PBS for 20 min at room temperature. Sections were then incubated with the primary antibody overnight at 4°C. The sections were incubated with anti-mouse or anti-rabbit IgG (Vector, Torrance, Calif., USA) for 30 min. The immune complexes were visualized with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, Mo., USA). Finally, all sections were counterstained with hematoxylin and mounted in Cytosol XYL (Fisher Scientific, Jessup, Md., USA).

Proliferation/Mitosis

An anti-Ki67 antibody was used to identify cellular proliferation, and an anti-PHH₃ (Ser10) antibody (Cell Signaling Technology, no. 9701) was used to identify mitosis. PHH₃ serves as a mitotic marker. The total number of positive cardiomyocytes or interstitial cells per 10 fields (400× magnification) was determined. Data are presented as the percentage of positive nuclei versus total cardiomyocyte or interstitial cell nuclei.

Histological Measurements

Morphological changes in cardiomyocytes were observed by light microscopy using a Nikon Eclipse 80i microscope. Three images for each sample in the cross section were analyzed at 200× magnification by an observer who was blind to the treatment groups. The circumference of all cardiomyocytes detected with nuclei (about 30–40 cells per field) in the Masson-stained sections was determined and the area of each cell calculated using the number of pixels. Calculations were made using image analysis MetaVue software. A total number of 90–120 cells from each sample were used to obtain the average cardiomyocyte size. Results are expressed in arbitrary units and the value for the NC group was fixed at 1.

Statistical Analysis

All results were expressed as mean ± SEM. Comparisons among the four groups were made using one-way analysis of variance and Bonferroni post-hoc test. Comparisons between two groups were made using Mann-Whitney's U test. Regression analysis was performed using Spearman's rank correlation test to determine which independent variables significantly influenced dependent variables (LV/BW or cardiomyocyte size). All statistical calculations were performed with StatView software (version 5.0) or Prism4. $p < 0.05$ was considered statistically significant.

Results

Body weights and chemistries from the end of the study are shown in table 1. After 4 months, body weights were not different between the four groups of animals.

Table 1. Serum chemistries

	NC (n = 9)	UC (n = 9)	UE (n = 11)	UES (n = 11)
Body weight, g	286.2 ± 6.3	272.2 ± 5.4	268.0 ± 6.2	272.6 ± 5.1
Serum creatinine, mg/dl	0.72 ± 0.02	2.1 ± 0.20 ^a	1.6 ± 0.1 ^a	1.6 ± 0.2 ^a
Creatinine clearance, ml/min	1.18 ± 0.06	0.41 ± 0.05 ^a	0.57 ± 0.06 ^a	0.62 ± 0.08 ^a
Serum phosphorus, mg/dl	3.9 ± 0.3	8.4 ± 0.9 ^a	6.6 ± 0.8 ^a	4.9 ± 0.5 ^c
Ionized calcium, mg/dl	4.75 ± 0.02	4.36 ± 0.15 ^b	4.69 ± 0.17	4.94 ± 0.05 ^{a, d}
Ca × P product, mg ² /dl ²	37 ± 3	82 ± 8 ^a	65 ± 6 ^a	54 ± 7 ^d
Serum PTH, pg/ml	46 ± 7	2,929 ± 701 ^a	1,939 ± 701 ^b	1,052 ± 248 ^a

Uremic rats underwent one of the following treatments for 4 months UC (vehicle), UE (enalapril), UES (enalapril + sevelamer). Results are expressed as mean ± SEM. n = The number of animals in each group.

^a p < 0.01 vs. NC; ^b p < 0.05 vs. NC; ^c p < 0.01 vs. UC; ^d p < 0.05 vs. UC.

Table 2. Time course for SBP in normal and uremic rats

	NC (n = 9)	UC (n = 9)	UE (n = 11)	UES (n = 11)
Baseline	133 ± 4	127 ± 3	131 ± 3	131 ± 6
1 Month	123 ± 3	161 ± 8 ^a	132 ± 6 ^b	137 ± 6 ^b
2 Months	123 ± 7	171 ± 10 ^a	144 ± 6 ^b	142 ± 5 ^b
3 Months	124 ± 2	186 ± 10 ^a	156 ± 6 ^{a, b}	155 ± 5 ^{a, b}
4 Months	134 ± 2	170 ± 4 ^a	163 ± 5 ^a	172 ± 7 ^a

Uremic rats underwent one of the following treatments for 4 months: UC (vehicle), UE (enalapril), UES (enalapril + sevelamer). Results are expressed as mean ± SEM. n = The number of animals in each group.

^a p < 0.01 vs. NC; ^b p < 0.01 vs. UC.

Serum Cr had increased from 0.72 ± 0.02 mg/dl in the normal control group (NC) to 2.1 ± 0.2 in uremic control (UC) rats, 1.6 ± 0.1 in uremic + enalapril (UE) rats, and 1.6 ± 0.2 in the uremic + enalapril + sevelamer carbonate (UES) group. As expected the increase in serum creatinine was accompanied by a decrease in creatinine clearance (UC 0.41 ± 0.05, UE 0.57 ± 0.06, and UES 0.62 ± 0.8 vs. NC 1.18 ± 0.6 ml/min). Serum Cr and Cr clearance were not significantly different among the uremic groups. Serum P was higher in UC rats compared to both the NC rats and to uremic rats treated jointly with both enalapril and sevelamer. While ionized calcium was decreased in UC rats, the Ca × P product was markedly increased (p < 0.01). Although serum PTH levels were not significantly different among the three groups of uremic rats, they decreased by 34% in rats receiving enalapril

alone and by 64% in rats receiving both enalapril and sevelamer. In both groups receiving enalapril, SBP was well controlled for the first 2 months (table 2). At 3 months, the SBP was higher than normal, but still significantly lower than that of the UC rats. By 4 months, however, all uremic rats had developed significant hypertension (UC 170 ± 4, UE 163 ± 5, and UES 172 ± 7 mm Hg vs. NC 134 ± 2).

Figure 1a shows the effect of treatment on left ventricular weight. Uremia increased left ventricular weight (LV/BW) by 81.0% compared to NC rats (NC 2.1 ± 0.1 vs. UC 3.8 ± 0.1 mg/g BW, p < 0.001). Both enalapril and the combination of enalapril and sevelamer significantly inhibited the increase in LV/BW seen in the UC group (UE 3.1 ± 0.2, p < 0.01, and UES 3.0 ± 0.1 mg/g BW, p < 0.01). There was no significant difference, however, in left ventricular weight between the UE and UES groups. In parallel, cardiomyocyte size was increased by 72% in UC rats compared with normal rats (NC 1 vs. UC 1.72 ± 0.13, p < 0.01) (fig. 1b). Enalapril inhibited the increase in cardiomyocyte size (UE 1.57 ± 0.09), while enalapril + sevelamer had a more marked effect (UES 1.25 ± 0.10, p < 0.05) and was not significantly different from normal. Figure 1c shows Masson trichrome-stained sections of the heart comparing cardiomyocyte size in all four groups of rats.

To determine the molecular mechanism underlying the LVH, we examined the expression of the cell-cycle proteins cyclin D1, cyclin D2, cyclin D3, p27 and PCNA in the heart by Western blot analysis. Figure 2a depicts a representative Western blot analysis of cyclin D2 expression in the hearts of the four groups of rats and figure 2b shows the quantification of several analyses. Cyclin D2

Fig. 1. Effect of enalapril and the combination of both enalapril and sevelamer carbonate on (a) left ventricular weight (LV) and (b) cardiomyocyte size. **a** ^a $p < 0.001$ vs. NC, ^b $p < 0.01$ vs. UE, ^c $p < 0.01$ vs. UES, ^d $p < 0.001$ vs. NC, and ^e $p < 0.01$ vs. NC. **b** ^a $p < 0.01$ vs. NC, ^b $p < 0.05$ vs. UES, and ^c $p < 0.05$ vs. NC.

c Masson trichrome staining of cardiomyocytes in NC, UC, UE and UES rat hearts. Results were expressed as mean $\pm$ SEM. p values were obtained by ANOVA followed by Bonferroni post tests. 254 $\times$ 190 mm (96 $\times$ 96 dpi).

expression was significantly increased (75.0%) in the hearts of UC rats compared with the NC animals ($p < 0.05$). While both treatments seemed to attenuate cyclin D2 expression, 20.6% in UE rats and 18.7% in UES rats, the decrease was not statistically significant. No differences were seen in the cardiac expression of cyclin D1 or cyclin D3 between the four groups (data not shown).

The expression of the CDK inhibitor, p27, is shown in figure 3. Figure 3a shows a representative Western blot analysis of cardiac p27 expression in the four groups of rats and figure 3b the quantification of several analyses. The expression of cardiac p27 was significantly decreased by UC group compared with the NC group ($p < 0.05$). Treatment with enalapril alone inhibited the decrease in p27 expression, although not significantly. Treatment with both enalapril and sevelamer, however, prevented the decrease in p27 seen in UC rats ($p < 0.05$).

Figure 4a shows a representative Western blot analysis of cardiac PCNA expression in the four groups of rats and figure 4b is the quantification of several analyses. PCNA expression increased significantly (89.1%) in uremic

hearts compared with those of NC rats ($p < 0.001$). Both treatments attenuated the increase in PCNA expression (UE 11.7% and UES 13.0%) seen in uremic control rats. This decrease, however, was not significantly different.

Figure 5a shows that the percentage of Ki67-positive interstitial cells was dramatically increased in the hearts of UC rats compared with the NC animals (NC $0.045 \pm 0.045\%$ vs. UC $0.975 \pm 0.16\%$, $p < 0.01$). Enalapril treatment alone inhibited this increase ($0.482 \pm 0.18\%$), while treatment with both enalapril and sevelamer did not ($0.844 \pm 0.262\%$). Ki67 expression was then assessed in cardiomyocytes. No Ki67 staining was seen in normal cardiomyocytes and only 1 out of 8 samples from uremic control hearts showed positive staining for Ki67 in cardiomyocytes. Figure 5b shows representative immunohistochemical staining for Ki67 in cardiac tissue from the NC and UC rats.

As shown in figure 6a, the percentage of PHH₃-positive interstitial cells in cardiac tissue was also increased in uremic hearts compared with tissue from normal animals (NC $0.06 \pm 0.03\%$ vs. UC $0.30 \pm 0.04\%$, $p < 0.05$). This

Fig. 2. Western blot analysis showing the effect of enalapril and the combination of both enalapril and sevelamer carbonate on the expression of cyclin D2 in the LV. **a** Representative Western blot of cyclin D2 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). **b** Quantification of several analyses. Cyclin D2 was normalized to GAPDH. Results were expressed as mean $\pm$ SEM. p values were obtained by ANOVA followed by Bonferroni post test. ^a p < 0.01 vs. NC. 254 $\times$ 190 mm (96 $\times$ 96 dpi).

Fig. 3. Western blot analysis showing the effect of enalapril and the combination of both enalapril and sevelamer carbonate on the expression of p27 in the LV. **a** Representative Western blot of p27 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). **b** Quantification of several analyses. p27 as normalized to GAPDH. Results were expressed as mean $\pm$ SEM. p values were obtained by ANOVA followed by Bonferroni post test. ^a p < 0.01 vs. NC and ^b p < 0.05 vs. UES. 254 $\times$ 190 mm (96 $\times$ 96 dpi).

Table 3. Univariable regression analysis by Spearman's rank correlation test showing the correlation between LV/BW and cardiomyocyte size and serum biochemistries

	SBP	LV/BW	Cr	ICa	P	Ca $\times$ P	PTH
LV/BW	0.5176 p = 0.008		0.7358 p < 0.0001	-0.7318 p < 0.0001	0.7070 p < 0.0001	0.6980 p < 0.0001	0.7711 p < 0.0001
Cardiomyocyte size	0.1631 p = 0.4572	0.6073 p = 0.0021	0.7257 p < 0.0001	-0.4936 p = 0.0167	0.5958 p = 0.0027	0.5939 p = 0.0028	0.6696 p = 0.0005

Table 4. Multivariable regression analysis showing the correlation between LV/BW and cardiomyocyte size and BP and P

	Regression coefficient	Standard error	Standardized regression coefficient	p value
<i>LV/BW</i>				
Blood pressure	0.012	0.004	0.399	0.0028
Phosphorus	0.137	0.023	0.715	<0.0001
<i>Cardiomyocyte size</i>				
Blood pressure	4.751	21.146	0.041	0.8246
Phosphorus	434.008	128.185	0.613	0.0031

Fig. 4. Western blot analysis showing the effect of enalapril and the combination of both enalapril and sevelamer carbonate on the expression of PCNA in the LV. **a** Representative Western blot of PCNA and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). **b** Quantification of several analyses. PCNA was normalized to GAPDH. Results were expressed as mean $\pm$ SEM. *p* values were obtained by ANOVA followed by Bonferroni post tests. ^a *p* < 0.01 vs. NC and ^b *p* < 0.05 vs. NC. 254 $\times$ 190 mm (96 $\times$ 96 dpi).

increase was attenuated by both treatment with enalapril ($0.13 \pm 0.06\%$) and enalapril + sevelamer ($0.20 \pm 0.08\%$). This inhibition did not reach significance. No PHH₃-positive staining was seen in normal cardiomyocytes and only a small amount was detected in cardiomyocytes from untreated-uremic rats (UC $0.16 \pm 0.10\%$). Figure 6b shows representative immunohistochemical staining for PHH₃ in cardiac tissue from the NC and UC rats.

Univariable analysis (table 3) comparing LV weight and various biochemical factors showed that LV/BW was correlated with SBP ($r = 0.52$, $p = 0.008$), Cr ($r = 0.74$, $p < 0.0001$), ICa ($r = -0.73$, $p < 0.0001$), P ($r = 0.71$, $p < 0.0001$), Ca $\times$ P ($r = 0.70$, $p < 0.0001$) and PTH ($r = 0.77$, $p < 0.0001$). A correlation was also shown between LV/BW and cardiomyocyte size ($r = 0.61$, $p = 0.0021$). Cardiomyocyte size was also correlated with Cr ($r = 0.73$, $p < 0.0001$), ICa ($r = -0.49$, $p = 0.0167$), P ($r = 0.60$, $p = 0.0027$), and PTH ($r = 0.67$, $p = 0.0005$).

In the present study, two independent variables, SBP and P, were selected for multiple regression analysis since previous studies have shown that both P and SBP influ-

ence LVH [7–10]. The independent variables included in the multivariate analyses were those that had proved to be significant and not to be a correlation within each factor in the univariable analysis. Multiple regression analysis showed that LV/BW correlated with SBP ($p = 0.0028$) and P ($p < 0.0001$), and that cardiomyocyte size correlated with P ($p = 0.0031$), not with SBP. These results indicate that LV/BW was more dependent on serum P than on SBP, since the standardized regression coefficient for P was higher than that for SBP (P 0.715 vs. SBP 0.399). Cardiomyocyte size was dependent on serum P, but not on SBP (P 0.613 vs. SBP 0.041) as shown in table 4.

Discussion

This study provides a long-term, experimental rat model of chronic renal failure with which to study the influence of uremia on LV remodeling including alterations of cell cycle regulatory proteins, cardiac cell morphology, cell proliferation, and the effect of ACEI given with or without sevelamer carbonate. Long-term 5/6 nephrectomized rats develop LVH consisting of cardiomyocyte hypertrophy, interstitial cell proliferation and alterations in cell cycle proteins, specifically a decrease in p27, and increases in cyclin D2 and PCNA. Treatment with enalapril attenuated LV/BW and treatment with enalapril + sevelamer carbonate attenuated not only LV/BW but also cardiomyocyte hypertrophy.

Although there was no significant difference in LV/BW or cardiomyocyte size between UE and UES animals, the addition of sevelamer was slightly more effective in reducing both parameters. These results and the following four findings raise the possibility that sevelamer carbonate has an additional benefit over the angiotensin II blockade alone in repressing uremia induced-LVH: (1) enalapril + sevelamer decreased cardiomyocyte size, while E alone did not; (2) the attenuation of cardiomyocyte size was accompanied by a significant reduction in P in the UES group; (3) univariable analysis showed cardiomyocyte size positively correlated with serum P, and (4) multivariable regression analysis revealed that LV/BW was more dependent on P than on SBP and that cardiomyocyte size was dependant on P, not on SBP. These results support recent reports that hyperphosphatemia is associated with left ventricular mass index or LVH [9–10].

We next explored G1 phase proteins of the cell cycle because numerous studies have shown that D-type cyclins and p27 play important roles in regulating cardiac

Fig. 5. Effect of enalapril and the combination of both enalapril and sevelamer carbonate on Ki67 expression in the LV. **a** Quantification of Ki67-positive interstitial cells. ^a $p < 0.01$ vs. NC and ^b $p < 0.05$ vs. NC. **b** Immunohistochemical staining for Ki67 (brown in the online version) in the LVs of NC and UC rats. 254 × 190 mm (96 × 96 dpi).

Fig. 6. Effect of enalapril and the combination of both enalapril and sevelamer carbonate on PHH₃ expression in the LV. **a** Quantification of PHH₃-positive interstitial cells. ^a $p < 0.05$ vs. NC. **b** Immunohistochemical staining for PHH₃ (brown in the online version) in NC and UC LVs. 254 × 190 mm (96 × 96 dpi).

hypertrophy. However, the involvement of these proteins has not been fully studied in patients with CKD or in uremic rat model. We demonstrated that the expression of p27 was significantly decreased in the uremic heart while cyclin D2 and PCNA were increased. These findings imply that re-entry of cardiomyocytes into the cell cycle is involved in the development of LVH in uremic rats. Indeed, in normal cells, p27 levels are high during G0 phase but decrease rapidly when cells re-enter G1 phase [38, 39]. Moreover, the decrease in p27 is compatible with reports that expression of p27 was drastically decreased in both acute and end-stage heart failure in humans [40] and in pressure-overload cardiac hypertrophy [41]. Moreover, a recent report demonstrated that p27 inhibits hypertrophic growth in adult cardiomyocytes exposed to angio-

tensin II [36]. p27 is regulated by the protein kinase CK2 α , which inhibits p27 activity in response to stimulation by angiotensin II, thereby promoting the development of hypertrophy [36]. Therefore, uremia-induced hypertrophy and its suppression by the angiotensin II blockade suggest that the interaction of angiotensin II and p27 plays an important role in the pathogenesis of LVH in this model.

Cyclin D2 expression was significantly increased in UC hearts. The association of the upregulation of cyclin D2 and the downregulation of p27 is also apparent in other cell types [42–44]. These observations suggest the possibility that cyclin D2 may play an important role in the cytoplasmic translocation and downregulation of p27 at the G0-G1 transition [45].

Forced expression of cyclin D2 in cardiac myocytes provokes cell division and not hypertrophy [46, 47]. Overexpression of cyclin D2 in cardiac myocytes in culture induces cell division and inhibits cellular hypertrophy [46]. While these reports seem to go against our results, it is possible that cyclin D2 is overexpressed at a developmental time point where myocytes still have capacity to divide in these other experimental models. On the other hand, in our animal model, cardiomyocytes have already lost their capacity to proliferate as demonstrated by the fact that uremia did not induce either Ki67 or PHH₃ in cardiomyocytes as detected by immunohistochemical staining. The hypertrophic response to the presence of differentiation inducing factor (DIF-1) can be rescued by overexpression of cyclin D2, suggesting that cyclin D2 is necessary for the development of cardiac hypertrophy [48]. As shown in the present study, cyclin D2 expression is elevated in animals with renal failure suggesting that upregulation of cyclin D2 is also necessary for uremia-induced hypertrophy as seen in the aortic banding model [48].

Proliferating cell nuclear antigen (PCNA), a DNA polymerase- δ auxiliary protein [49, 50], is a nuclear protein necessary for DNA synthesis and for cell cycle progression. When cells are in G₀, PCNA mRNA levels are low but rapidly increase in the presence of growth factors, stimulating cells to divide [51, 52]. The induction of PCNA in myocytes may cause these cells to enter the cell cycle and undergo DNA synthesis and nuclear mitotic division [22]. Since PCNA is expressed in the late G₁ phase and in the S-phase of the cell cycle, positive PCNA staining indicates the activation of cells, but not necessarily cell division [49, 53, 54]. In contrast, Ki67 protein is a cell cycle-associated nuclear antigen and is expressed in all phases of the cell cycle except G₀ and the early G₁ phase when cells re-enter the cell cycle; therefore, it is a sensitive biomarker for cell proliferation [55]. In this present study, immunohistochemical staining showed that Ki67 expression was increased in uremic hearts compared with normal hearts. Immunohistochemical staining for Ki67 and PHH₃, another mitotic marker, revealed that the number of neither Ki67-positive cardiomyocytes nor PHH₃-positive cardiomyocytes was increased in UC hearts, suggesting that uremia caused cardiomyocyte activation rather than interstitial cell replication, causing cardiomyocytes to re-enter into the cell cycle, but not to proliferate, since cardiomyocytes are post-mitotic cells. This could result from reparative processes. As cardiac myocytes occupy over 80% of the volume of ventricular wall [56] and probably contribute over 90% of protein in

whole tissue extract as used here, we propose that the expression pattern of proteins observed in the LV reflects the level of expression in the cardiomyocytes, and not in interstitial cells. We demonstrated that uremia-induced LVH consists of individual cardiomyocyte hypertrophy and proliferation of interstitial cells, mostly fibroblasts. The present paper supports the idea that the downregulation of p27, a potent inhibitor of cell growth, induces both re-entry into the cell cycle and cell enlargement, but not proliferation, leading to LVH.

Using univariate regression analysis, we also show a correlation between PTH and LV weight. While serum PTH levels were not significantly different among the three groups of uremic rats, they were decreased by 34% in rats receiving enalapril alone and by 64% in rats receiving both enalapril and sevelamer. PTH is known to play a role in the development of myocardial hypertrophy. It has been shown that chronic renal failure is associated with increases in the calcium content of the heart and that this is attributed to the increase in PTH seen in chronic renal failure, which augments the entry of calcium into cardiac myocytes and inhibits the extrusion of calcium from these cells [57]. PTH has been shown to induce a hypertrophic effect on cardiomyocytes that is characterized by both an increase in protein synthesis and a selective induction of creatine kinase [58]. Recently, Liu et al. [59] have shown that this induction of cardiac hypertrophy is via the MEK/ERK pathway. Thus, the decreases in PTH in the UE and UES rats may have contributed to the less severe LVH seen in these rats.

In conclusion, uremia-induced left ventricular remodeling consists of cardiomyocyte hypertrophy and interstitial cell proliferation, but not cardiomyocyte proliferation. Downregulation of p27 and upregulation of cyclin D2 may play an important role in the development of LVH induced by uremia. P appears to be an independent factor for the development of LVH in this model.

Acknowledgments

This study was supported in part by grants from Research in Renal Disease – Washington University (310304), Genzyme Corporation and the WUCKDR O'Brien Center (P30DK079333). H. Nakamura received financial support from Showa University Japan.

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