

Combination Therapy with Paricalcitol and Enalapril Ameliorates Cardiac Oxidative Injury in Uremic Rats

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Key Words

Paricalcitol · Enalapril · Cardiac oxidative stress · Uremia

Abstract

Aims: This study investigated the protective effect of the angiotensin-converting enzyme inhibitor, enalapril, and the vitamin D analog, paricalcitol, alone or in combination, on cardiac oxidative stress in uremic rats. **Methods:** Rats were made uremic by 5/6 nephrectomy and treated for 4 months as follows: (1) uremic + vehicle (n = 11); (2) uremic + enalapril (30 mg/l in drinking water, n = 13); (3) uremic + paricalcitol (200 ng 3× week, n = 6); (4) uremic + enalapril + paricalcitol (n = 14), and (5) controls (n = 6). **Results:** Cardiac NADPH oxidase activity increased by 300% in uremic rats compared to normal controls. Treatment with enalapril, paricalcitol or the combination of the two protected uremic rats from cardiac oxidative stress by inhibiting enzyme activity. Cardiac malondialdehyde (MDA) levels were significantly increased in uremic rats compared to normal controls. Only the combination therapy inhibited the increase in MDA levels in uremic rats. Cardiac glutathione was significantly reduced in uremic rats compared to normal controls. Enalapril, paricalcitol or the two in combination all protected against this reduction in glutathione. Cardiac copper/zinc superoxide dismutase

(CuZn-SOD) activity decreased whereas manganese (Mn-SOD) activity increased in uremic rats compared to controls. Both mono and combination therapies ameliorated the alterations in cardiac SOD activity seen in uremic rats. **Conclusion:** Enalapril, paricalcitol and their combined therapy afford protection against cardiac oxidative stress in uremia.

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Introduction

The progression of chronic renal disease leads to end-stage renal disease and consequent renal replacement therapy. However the major cause of mortality and morbidity in end-stage renal failure patients is cardiovascular complications [1]. The renin-angiotensin-aldosterone system (RAAS) regulates extracellular volume homeostasis which contributes to blood pressure stability [2]. Activation of RAAS is manifested by glomerular hypertension, fibrosis and proteinuria resulting in renal damage [2]. Since the RAAS has an essential role in both renal as well as cardiovascular pathophysiology, agents that inhibit this system such as angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs), have been shown to have beneficial effects in pa-

tients with both chronic renal and cardiovascular diseases [3–6]. Angiotensin II induces oxidative stress in both the renal and cardiovascular systems by inducing free radical generation via activation of NADPH oxidase [7–9]. These reactive oxygen species (ROS) initiate vascular membrane lipid peroxidation leading to inflammation and generation of inflammatory cytokines (TNF- α) through NF- κ B activation [9–11]. These reactive species oxidize cellular bio-molecules such as proteins and DNA and initiate membrane lipid peroxidation leading to cardiovascular dysfunction [12].

To protect against free radical damage, the cell is endowed with an elaborate antioxidant defense system that includes antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), reduced glutathione (GSH) – the most important endogenous antioxidant and vitamins A, C and E [12]. Antioxidant enzymes act in tandem to scavenge ROS generated in various cellular compartments. Cytosolic copper/zinc superoxide dismutase (CuZn-SOD) and mitochondrial manganese superoxide dismutase (Mn-SOD) enzymes are the first line of cellular antioxidant defense [12]. Most importantly, depletion of a major cellular antioxidant such as GSH has been reported to cause cardiovascular dysfunction in rats [13]. Antioxidant therapy has been shown to ameliorate cardiovascular oxidative stress by scavenging excess ROS and upregulating the antioxidant defense system [12–15]. However, since the protective mechanisms of ACEIs in uremia-induced oxidative stress in the heart are not completely understood, we proposed to investigate this topic in the present study.

Vitamin D and its analogs have been shown to have therapeutic potential in attenuating experimentally induced kidney diseases [16–18]. Clinical studies have also demonstrated beneficial effects of vitamin D analogs in patients with kidney diseases [19–21]. Recent studies have demonstrated that cardiovascular disease-related morbidity and mortality in chronic kidney disease patients are related to vitamin D deficiency [22–24]. Paricalcitol, a vitamin D analog, has been demonstrated to decrease cardiovascular calcification but increased perivascular fibrosis in animals [25, 26]. In clinical studies, paricalcitol reduced proteinuria, inflammation and the mortality rate in chronic kidney disease patients [20, 21, 27]. It is not known, however, whether vitamin D analogs can protect uremic rats from cardiac oxidative stress. Our recent study demonstrated that the combination therapy of paricalcitol and enalapril had an added benefit in ameliorating the progression of renal insufficiency by suppressing inflammatory pathways and inhibiting secondary

hyperparathyroidism in a uremic rat model [18]. Therefore, the aim of the present study is to investigate the effect of an ACEI, enalapril, and a vitamin D analog, paricalcitol, alone or in combination, on oxidant/antioxidant balance in the heart using the 5/6 nephrectomy uremic rat model [26].

Methods

Animals

Our current investigation is an extension of previous work on the effects of vitamin D plus ACEI on chronic kidney disease progression [18]. The data in this paper is novel and investigates oxidative stress in the myocardium of normal and uremic rats. The animal studies were approved by The Washington University Animal Studies Committee in accordance with federal regulations. Fifty female Sprague-Dawley rats (225–250 g) were made uremic by 5/6 nephrectomy. All animals were provided a modified AIN-76 rodent diet containing 0.9% phosphorus, 0.6% calcium and a normal vitamin D level (1,000 IU/kg diet) (Dyets Inc., Bethlehem, Pa., USA) and water ad libitum. They were maintained at a room temperature of 25°C on a 12:12 h light/dark cycle to mimic normal circadian rhythm. Blood pressure (BP) was measured prior to surgery and monitored monthly thereafter using the Non-Invasive Blood Pressure System XBP1000 (Kent Scientific Corporation, Torrington, Conn., USA). Immediately after surgery, uremic rats were randomly divided into groups. A group of normal rats served as control. The groups were treated as follows:

- Group I [uremic + vehicle]: rats were administered vehicle [100 μ l of propylene glycol (i.p.)] 3 times a week for 4 months (n = 11).
- Group II [uremic + enalapril]: rats were given enalapril (30 mg/kg, in their drinking water) for 4 months (n = 13).
- Group III [uremic + paricalcitol]: rats were given paricalcitol (200 ng in propylene glycol, i.p.) 3 times a week for 4 months (n = 6).
- Group IV [uremic + enalapril + paricalcitol]: rats were given enalapril plus paricalcitol for 4 months (n = 14).
- Group V [normal control]: normal rats were given vehicle [100 μ l of propylene glycol (i.p.)] 3 times a week for 4 months (n = 6).

After 4 months, rats were euthanized by exsanguinations via the dorsal aorta. The hearts were isolated, rinsed with PBS, immediately immersed in liquid nitrogen and stored at –80°C until analysis could be completed. Enalapril was purchased from Sigma Chemical Company (St. Louis, Mo., USA) and paricalcitol was provided by Abbott Pharmaceuticals (Abbott Park, Ill., USA).

Determination of Glutathione

GSH was determined as previously described [14]. The proteins present in the tissue homogenate (5% in 50 mM phosphate buffer, pH 7.0, containing 0.1 mM EDTA) were removed by deproteination (using metaphosphoric acid and triethanolamine reagents). 50 μ l of standard (different concentrations) or samples each were added to the designated wells of the 96-well microtiter

plate and covered. Fresh Assay Cocktail, a mixture of MES buffer (11.25 ml), reconstituted Cofactor Mixture (0.45 ml), reconstituted Enzyme Mixture (2.1 ml), water (2.3 ml), and reconstituted dithio bis nitro benzoic acid (0.45 ml), was prepared. The plate cover was removed and 150 μ l of Assay Cocktail was added to each of the wells using a multi-channel pipette. The plate was covered and incubated in the dark on an orbital shaker for 10 min. The absorbance was measured at 405 nm at 25 min (end-point method) using a microtiter plate reader (Automated Microplate Reader, Bio-Rad, USA). There was 3–5% CV within and between animals.

Lipid Peroxidation Assay

A lipid peroxidation assay was performed to determine malondialdehyde (MDA) levels using the method described by Ohkawa et al. [28]. 1,1,3,3-Tetraethoxypropane was used as the standard.

Antioxidant Enzyme Assays

SOD activity was determined at room temperature according to the method of Misra and Fridovich [29]. Activity was expressed in units with 1 unit equal to the amount of enzyme that inhibits the oxidation of epinephrine by 50%. 20 mM NaCN was used to inhibit CuZn-SOD activity to determine the Mn-SOD activity. CuZn-SOD activity was calculated by subtracting Mn-SOD activity from total SOD activity.

CAT activity was determined by a slightly modified version of Aebi [30]. A molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$ was used to determine CAT activity. One unit of activity is equal to the moles of H_2O_2 degraded $min^{-1}mg\ protein^{-1}$.

GSH-Px activity was determined at 37°C using a modified version of the method of Flohe and Gunzler [31]. A molar extinction coefficient of $6.22 \times 10^3 M^{-1}cm^{-1}$ was used to determine activity. One unit of activity is equal to the mM of NADPH oxidized $min^{-1}mg\ protein^{-1}$.

NADPH Oxidase Assay

The enzyme activity was assayed based on superoxide-induced lucigenin photoemission as previously described [14]. The enzyme assay was carried out in the dark in a total volume of 1 ml in a tube containing 50 mM phosphate buffer pH 7.0, 1 mM EGTA, 150 mM sucrose, 0.1 mM NADPH, 0.5 mM lucigenin and 0.5 mg tissue protein. The enzymatic reaction was started by the addition of lucigenin. Photoemission was measured for 3 at 20-min intervals.

Protein Assay

Protein concentrations in tissue homogenates and extracts were estimated according to the method of Read and Northcole [32] using Coomassie protein assay dye and bovine serum albumin as the standard.

Statistical Analysis

The data were expressed as mean \pm SEM. The data were analyzed statistically using one-way analysis of variance (ANOVA). Two-way ANOVA was followed by a post-hoc Scheffe test for comparison between different treatment groups. The relationship of changes in systolic blood pressure (SBP) and biochemical variables was assessed by multiple linear regression analyses. Statistical significance was set at $p < 0.05$.

Table 1. Effect of mono and combination therapies on systolic blood pressure in uremic rats (mean \pm SEM)

Groups	Systolic BP, mm Hg
Control (n = 6)	125 \pm 12
Uremic (n = 11)	176 \pm 8 ^a
Uremic + enalapril (n = 13)	104 \pm 3 ^b
Uremic + paricalcitol (n = 6)	171 \pm 25 ^a
Uremic + enalapril+ paricalcitol (n = 14)	114 \pm 4 ^b

The normal range of SBP in rats has been reported to be 110–129 mm of Hg [13, 14, 18].

^a $p < 0.02$ as compared to group 1; ^b $p < 0.01$ as compared to group 2.

Results

As depicted in table 1, uremic rats receiving vehicle alone developed significant hypertension (increased systolic BP) compared to normal control rats ($p < 0.02$). Treatment with enalapril or enalapril in combination with paricalcitol prevented the increase in systolic BP ($p < 0.01$). However, paricalcitol alone did not lower the systolic BP ($p < 0.02$).

Cardiac NADPH oxidase activity was significantly increased (300%) in uremic rats compared to normal controls ($p < 0.001$) indicating an excess production of superoxide in uremia. Enalapril, paricalcitol and the combination of the two protected against cardiac oxidative stress by significantly ($p < 0.01$) inhibiting NADPH oxidase activity (50%), thus lowering the superoxide production in the heart (fig. 1a). The increase in SBP was correlated with an increase in NADPH oxidase activity ($r = 0.69$).

Figure 1b shows that the levels of the cardiac lipid peroxidation end product, MDA, increased by 133% ($p < 0.001$) in uremic rats compared to normal controls, indicating the level of cardiac oxidative stress in uremia. Enalapril did not significantly inhibit this increase in MDA levels, while paricalcitol actually enhanced the levels. Combining these two drugs, however, decreased MDA levels by 15% ($p < 0.05$).

There was a marked decrease in cardiac reduced GSH (36%, $p < 0.001$) in vehicle-treated uremic rats compared to normal animals (fig. 2a). All three therapies significantly protected against the reduction in GSH ($p < 0.01$), indicating an upregulation of the cardiac antioxidant system.

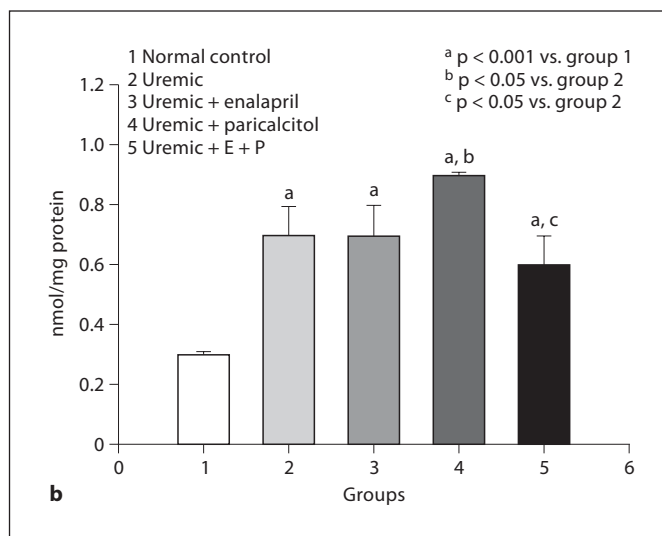
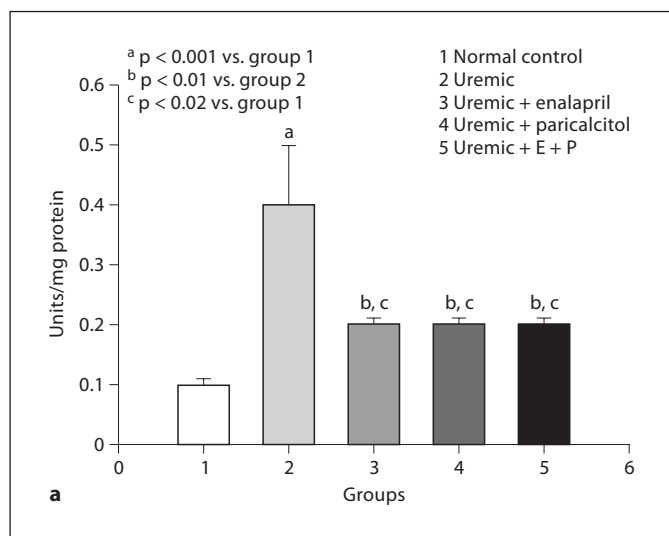


Fig. 1. a Effect of mono and combination therapies on cardiac NADPH oxidase activity in uremic rats. Enalapril (n = 13), paricalcitol (n = 6) and combination of the two (n = 14) significantly (p < 0.01) ameliorated the oxidative stress in uremic rats (n = 11) by inhibiting NADPH oxidase activity by 50%. **b** Effect of mono

and combination therapies on cardiac MDA levels in uremic rats. Enalapril (n = 13) and paricalcitol (n = 6) alone did not reduce cardiac MDA levels but the combination of the two (n = 14) significantly (p < 0.05) decreased cardiac MDA levels (15%) in uremic rats (n = 11).

There was a marked increase in cardiac mitochondrial Mn-SOD activity (335%, p < 0.001) whereas cytosolic CuZn-SOD activity decreased by 30% (p < 0.05) in uremic rats compared to their normal counterparts (fig. 2b, c). Enalapril and paricalcitol alone or in combination significantly (p < 0.02) decreased the Mn-SOD activation. Both enalapril and the combined therapy protected against cardiac oxidative stress by significantly (p < 0.05) enhancing CuZn-SOD activity (27 and 34%, respectively), whereas paricalcitol alone did not significantly increase CuZn-SOD activity. Cardiac CAT and GPX activities were not significantly altered by mono and combination therapies.

Discussion

This study addresses the protective efficacy of the ACEI, enalapril, and the vitamin D analog, paricalcitol, either alone or in combination, on cardiac oxidative stress in uremic rats. Several clinical and experimental studies have demonstrated the beneficial effects of ACEI in renal as well as cardiovascular diseases [3–6, 33–35]. However, the mechanisms by which ACEI provide protection against cardiorenal diseases are not fully under-

stood. It is well established that oxidative stress and inflammation are implicated in both renal diseases, such as uremia, and cardiovascular diseases, such as cardiac failure, atherosclerosis and hypertension [7, 9, 11]. Chronically increased angiotensin II activity has been implicated in promoting vascular inflammation, atherosclerosis, and nephropathy [2, 36]. While we chose to use the ACEI, enalapril, in the present study, presumably other ACEI, such as benazepril or trandolapril, given alone or in combination with paricalcitol would provide similar results. This could also be true for ARBs. The oxidative stress response in uremic rats can stimulate the cellular production of free radicals/ROS, deplete the cellular antioxidant defense system or do both [15, 37]. Angiotensin II has been shown to induce vascular oxidative stress through the activation of NADPH oxidase, which leads to the generation of superoxides [7–9]. The present data in a rat model of uremia show that a profound increase in cardiac NADPH oxidase activity corresponds to elevated systolic BP (r = 0.69). The inhibition of cardiac NADPH oxidase activity by enalapril, paricalcitol or the two in combination, clearly suggests a role for RAAS in the progression of cardiac oxidative stress. Interestingly, the vitamin D analog, paricalcitol, which has been shown to be efficacious in renal diseases [16–21], has an anti-

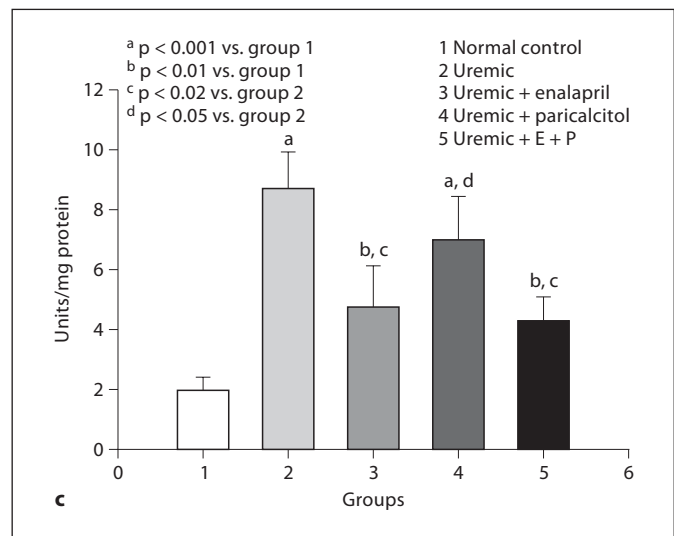
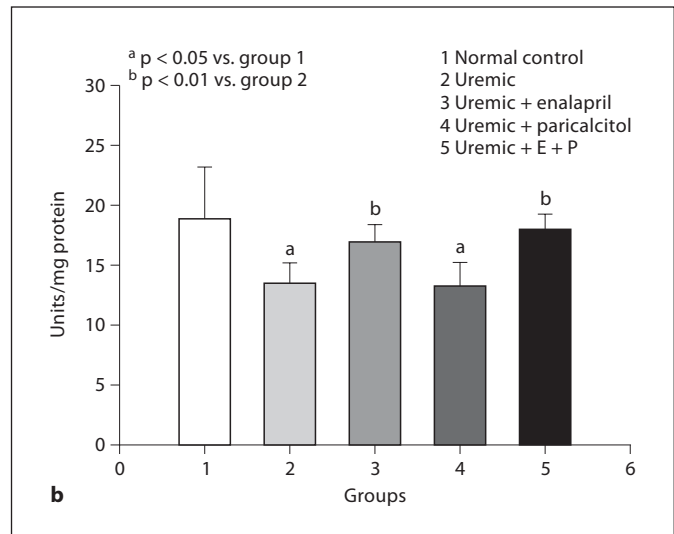
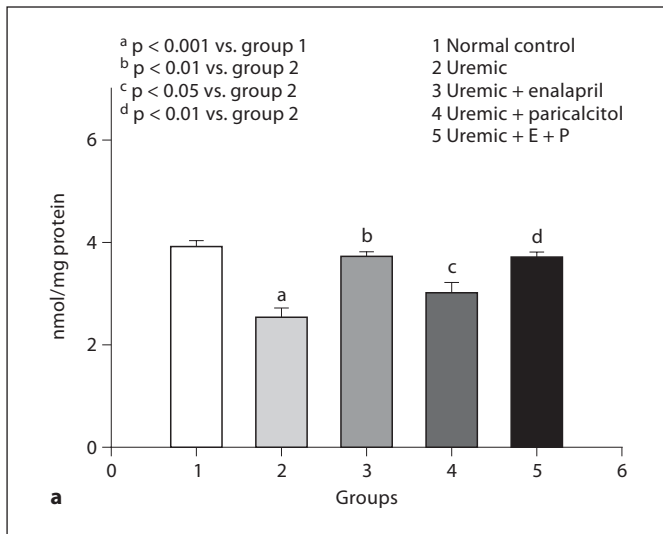


Fig. 2. a Effect of mono and combination therapies on cardiac GSH levels in uremic rats. Enalapril (n = 13) and paricalcitol (n = 6) alone and in combination (n = 14) significantly (p < 0.01) increased cardiac GSH levels (48%) in uremic rats (n = 11). **b** Effect of mono and combination therapies on cardiac CuZn-SOD activity in uremic rats. Enalapril (n = 13) and the combination of enalapril and paricalcitol (n = 14) significantly (p < 0.01) increased cardiac CuZn-SOD activity in uremic rats (n = 11). **c** Effect of mono and combination therapies on cardiac Mn-SOD activity in uremic rats. Enalapril (n = 13) and paricalcitol (n = 6) alone and in combination (n = 14) significantly (p < 0.02) decreased the Mn-SOD activity in uremic rats (n = 11).

oxidant effect by inhibiting the superoxide generating enzyme (NADPH oxidase activity) in the heart. This action may be related to the negative regulation of RAAS by vitamin D analogs [38, 39]. We know from previous work that excess superoxide generation due to NADPH oxidase activation causes inflammation and further generation of inflammatory cytokines (TNF- α) through NF- κ B activation [9–11]. The role of NF- κ B activation is also demonstrated in renal disease [40]. A recent report demonstrated that 1,25-dihydroxyvitamin D₃ blocks hyperglycemia-induced renal injury by blunting NF- κ B activation [41]. Our recent study demonstrates that renal expression of inflammatory mediators such as monocyte

chemoattractant protein-1 (MCP-1) and macrophage infiltration are profoundly depressed when paricalcitol was combined with the ACEI, enalapril, in uremic rats [18]. Therefore, the current study, together with previous data, suggests that the cardiovascular protection provided by mono or combination therapy is most likely due to their anti-inflammatory actions. While no other vitamin D analogs were tested in the current study, it is quite possible that calcitriol and other vitamin D analogs in combination with ACEI would also induce beneficial effects on cardiac oxidative stress.

On the other hand, excess superoxide generation in uremic rats further initiates cardiovascular membrane

lipid peroxidation, which is evidenced by increased cardiac MDA levels (an end product of the lipid peroxidation reaction of unsaturated lipids). Clinical studies have also demonstrated enhanced lipid peroxidation end products in the tissues of cardiac and renal disease patients [33–36]. The data in the present study show that the administration of one drug alone does not significantly deplete cardiac MDA levels. There are different cellular sources of superoxide apart from NADPH oxidase. ROS other than superoxide may also initiate membrane lipid peroxidation as well as different reaction products from lipid peroxidation [12, 14, 42]. Enalapril had no effect on MDA levels while paricalcitol increased them indicating that the actions of the two drugs are mediated via different pathways of ROS generation and lipid peroxidation. Furthermore, the increased cardiac MDA levels seen after paricalcitol treatment may likely be due to a further reduction in endogenous calcitriol or the conversion of 25-hydroxyvitamin D (25-OHD) to calcitriol [22, 26]. The extrarenal conversion of 25-OHD to 1,25-dihydroxyvitamin D (1,25-(OH)₂D) may have played a role in the results seen in the current study. However, since intracellular levels of 25-OHD were not measured, we cannot answer this possibility. The combination of the two drugs, however, resulted in a significant reduction in cardiac MDA levels suggesting that the activation of the vitamin D receptor enhances the mechanism by which enalapril likely alters the lipid peroxidation reaction cascade.

GSH, a major non-protein thiol antioxidant, plays an important role in maintaining the integrity of cell components [12]. It directly scavenges excess ROS and also regenerates cellular antioxidant vitamins [12]. The results of the present study indicate that cardiac GSH levels are significantly decreased in uremic rats, an indicator of oxidative stress [42]. A decrease in GSH can lead to an impairment of the cellular defense against ROS and may result in peroxidative injury [12, 14, 42]. The reduction in tissue GSH by buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, has been shown to cause hypertension and cardiovascular oxidative stress in rats [13]. Therefore, it is suggested that maintenance of high GSH levels is an important intracellular mechanism for the prevention of oxidative stress. Clinical studies have also demonstrated a lower level of antioxidants in the tissues of patients with cardiorenal diseases [33–36]. The data further show that both enalapril and the combination therapy significantly elevate cardiac GSH levels but that paricalcitol has a lesser effect on GSH, suggesting that the antioxidant actions of the two drugs in the regulation of

GSH synthesis and/or degradation are mediated by different pathways.

Oxidative stress in uremic rats may also be related to an alteration in antioxidant enzymes in the tissues. The present data demonstrate that cardiac mitochondrial Mn-SOD activity is significantly increased suggesting a compensatory response for the disposal of excess superoxides generated by NADPH oxidase activation. SOD is considered to be the first line of defense against the deleterious effects of oxygen radicals in cells. It scavenges superoxide by catalyzing the dismutation of superoxide to H₂O₂ and O₂ [12, 29]. Cardiac Mn-SOD activity significantly decreased in response to both enalapril and combination therapy but paricalcitol had a lesser effect on decreasing Mn-SOD, suggesting that the depression of superoxide generation by these 2 drugs is by different mechanisms. Moreover, the fluxes in SOD activity (either up or down) may be related to the presence of excess ROS [43]. Mitochondrial Mn-SOD is induced by inflammatory cytokines such as TNF- α [44] and both drugs have anti-inflammatory actions [10, 18, 33]. The cytosolic CuZn-SOD activity is susceptible to inactivation by ROS [45]. The data further show that cardiac CuZn-SOD activity increases in response to enalapril or combination therapy but not to paricalcitol, suggesting that ROS generation and scavenging is also mediated via different mechanisms by these two drugs. In the present study, cardiac CAT and GPX activities did not significantly change in uremic rats indicating that these antioxidant enzymes are not upregulated in the uremic condition [15, 37]. CAT and GPX are responsible for detoxification of hydrogen peroxide and lipid hydroperoxides [12, 30, 31]. In conclusion, in uremic rats, mono and combination therapies were unable to alter the activities of CAT and GPX in cardiac tissue further suggesting that these enzymes are not upregulated in response to the protective actions of these drugs.

In summary, we demonstrated that cardiac oxidative stress in uremic rats is due to enhanced NADPH oxidase activity and MDA levels and to a reduction in GSH levels and CuZn-SOD activity. Treatment with enalapril and paricalcitol alone, or in combination ameliorates oxidative stress by decreasing NADPH oxidase and Mn-SOD activities and MDA levels and by restoring GSH levels and CuZn-SOD activity. In conclusion, both mono and combination therapies are efficacious in ameliorating cardiac oxidative stress in uremia. Future studies will be directed at investigating the possible molecular mechanism(s) of the interaction of these two drugs, as well as other vitamin D analogs, in the uremic condition using genomic and proteomic technologies.

Acknowledgements

This study was supported in part by Abbott Pharmaceutical and Washington University. We thank Abbott Pharmaceutical for providing the paricalcitol in this study.

Disclosures

E.S. is a consultant/speaker for both Genzyme and Abbott Lab. E.S. and Washington University may receive income based on a license of related technology by the University of Wisconsin.

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