Published in final edited form as:

J Cardiovasc Pharmacol. 2008 September ; 52(3): 245–252. doi:10.1097/FJC.0b013e3181833eb8.

CAUSES AND CONSEQUENCES OF ZINC DYSHOMEOSTASIS IN RATS WITH CHRONIC ALDOSTERONISM

Malay S. Gandhi, MD¹, Prajwal A. Deshmukh, MD², German Kamalov, MD², Tieqiang Zhao, MD, PhD¹, Wenyuan Zhao, MD, PhD¹, Jonathan T. Whaley, Jill R. Tichy, Syamal K. Bhattacharya, PhD^{1,3}, Robert A. Ahokas, PhD⁴, Yao Sun, MD, PhD¹, Ivan C. Gerling, PhD⁵, and Karl T. Weber, MD¹

¹Division of Cardiovascular Diseases, University of Tennessee Health Science Center, Memphis, TN, USA

²Department of Medicine, University of Tennessee Health Science Center, Memphis, TN, USA

³Department of Surgery, University of Tennessee Health Science Center, Memphis, TN, USA

⁴Department of Obstetrics & Gynecology, University of Tennessee Health Science Center, Memphis, TN, USA

⁵Division of Endocrinology, University of Tennessee Health Science Center, Memphis, TN, USA

Abstract

Iterations in Ca²⁺ and Mg²⁺ balance accompany aldosteronism (inappropriate for dietary Na⁺ intake). Increased Zn excretion and Zn translocation to injured tissues, including the heart, also occurs. Several causes and consequences of Zn dyshomeostasis in rats receiving aldosterone/salt treatment (ALDOST) were examined: 1) the role of urinary acidification in promoting hyperzincuria, acetazolamide (75 mg/kg), a carbonic anhydrase inhibitor, was used as cotreatment to raise urinary HCO₃⁻ excretion; 2) assess Zn levels in the heart, including cardiomyocyte cytosolic free [Zn²⁺]_i and mitochondrial Zn, the expression of metallothionein (MT-I), a Zn binding protein, and biomarkers of oxidative stress; and 3) monitor oxidative stress and cardiac pathology in response to ZnSO₄ supplement (40 mg/day). Compared to controls, at 4 wks ALDOST we found: an acidification of urine and metabolic alkalosis associated with increased urinary Zn excretion and hypozincemia, each of which were prevented by acetazolamide; a rise in cardiac Zn including increased [Zn²⁺]_i and mitochondrial Zn, associated with increased tissue MT-I, 8-isoprostane, malondialdehyde, and gp91^{phox}, coupled with oxidative stress in plasma and urine; and ZnSO₄ prevented hypozincemia, but not ionized hypocalcemia, and attenuated oxidative stress and microscopic scarring without preventing the vasculitis and perivascular fibrosis of intramural coronary arteries. Thus, the hyperzincuria seen with ALDOST is due to urinary acidification. The oxidative stress that appears in the heart is accompanied by increased tissue Zn serving as an antioxidant. Cotreatment with ZnSO₄ attenuated cardiomyocyte necrosis, however, polynutrient supplement may be required to counteract the dyshomeostasis of all 3 cations that accompanies aldosteronism and contribute to cardiac pathology.

Keywords

zinc; aldosteronism; oxidative stress; zinc supplement; acetazolamide

Correspondence: Karl T. Weber, M.D., Division of Cardiovascular Diseases, University of Tennessee Health Science Center, 920 Madison Ave., Suite 300, Memphis, TN 38163, Tel: (901) 448-5750, Fax: (901) 448-8084, email: E-mail: KTWeber@utmem.edu.

Authors have no conflicts of interest to disclose.

Introduction

Zinc (Zn), a trace element, is an essential micronutrient because of its myriad of biologic functions (1,2). Zinc deficiency, together with associated impairment in Zn-dependent metalloenzymes, has been reported in the elderly and those with hypertension (3,4). Hyperzincuria coupled with hypozincemia have been found in untreated patients with congestive heart failure (CHF) irrespective of race, ethnicity, or the etiologic origins to the heart's failure as a muscular pump (5–8). The symptoms and signs of CHF are rooted in a salt-avid state mediated by effector hormones of the renin-angiotensin-aldosterone system (RAAS) (9). Urinary Zn losses are also increased in response to pharmacologic agents commonly used in the management of patients with CHF, such as angiotensin-converting enzyme inhibitor and angiotensin receptor blockers (10). A deficiency of Zn in such patients could have far-reaching consequences that contribute to the pathophysiologic expressions and systemic nature of CHF. These would include: the presence of oxidative stress in diverse tissues, where reactive oxygen species overwhelm Zn-dependent and other antioxidant defenses; an immunostimulatory state with activated lymphocytes and monocytes producing proinflammatory cytokines; as well as skeletal muscle and bone wasting (11–14). Pathogenic mechanisms responsible for Zn dyshomeostasis and its adverse effects in CHF are therefore of clinical importance.

To address causes and consequences of Zn dyshomeostasis in aldosteronism, an integral component of RAAS activation, we used a rat model of aldosterone/salt treatment (ALDOST), where plasma ALDO levels are raised to those found in human CHF. In rats ALDOST has proven a model of Zn dyshomeostasis that includes: a fall in plasma Zn due to an early and persistent increase in urinary and fecal Zn excretion and a translocation of Zn from plasma to injured tissues; a decline in plasma Cu/Zn-superoxide dismutase activity; a fall in bone Zn that occurs in response to resorption mediated by secondary hyperparathyroidism; thymic atrophy; activated peripheral blood mononuclear cells; and a failure to develop normally, including compromised gain in body weight based on skeletal muscle atrophy and bone resorption (15–19). Herein, several causes and consequences of Zn dyshomeostasis are addressed in rats receiving 4 wks ALDOST.

The first aim of our study was to determine pathophysiologic responses involved in the increased urinary excretion of Zn during ALDOST. We focused on the role of increased urinary H^+ excretion (via ammonia) and urinary acidification in enhancing Zn excretion recognizing the appearance of a metabolic alkalosis with aldosteronism (20). We therefore chose to increase urinary HCO_3^- excretion by cotreating rats receiving ALDOST with acetazolamide, a carbonic anhydrase inhibitor. Another factor contributing to the appearance of hypozincemia in aldosteronism is a translocation of circulating Zn to injured and stress-responsive tissues (16). This occurs with the oxidative stress that accompanies acute tissue injury or with chronic disease (21–27). Sites of increased ^{65}Zn uptake in the ALDOST model include: healing incised skin; the heart, where cardiomyocyte necrosis with replacement scarring appear in both atria and ventricles, together with a vasculitis and fibrosis of intramural coronary arteries (28); and the liver, a stressor-responsive organ (15,16,29). The rise in tissue Zn at these sites is facilitated by upregulated metallothionein (MT)-I, a Zn-binding protein (15). The second aim of our study was to address oxidative stress, the expression of MT-I, and Zn sequestration in the heart and its cardiomyocytes and mitochondria. Our third aim was to address whether cotreatment with a Zn supplement would prove an effective strategy in preventing hypozincemia, oxidative stress, myocardial injury with scarring and coronary vascular remodeling. Zinc supplementation has proven efficacious in preventing a diabetic cardiomyopathy in streptozocin-treated rodents (26).

METHODS

Animal Model

Eight-week-old male Sprague-Dawley rats were used throughout this series of experiments approved by our institution's Animal Care and Use Committee. As reported previously and following uninephrectomy, an osmotic minipump containing ALDO was implanted subcutaneously to raise circulating ALDO levels to those commonly found in human CHF (30). Drinking water was fortified with 1% NaCl and with 0.4% KCl to prevent hypokalemia. Separate groups of rats receiving ALDOST were given either acetazolamide (75 mg/kg body weight by gavage) or a Zn supplement with ZnSO₄ (40 mg/day by gavage) as cotreatment. Unoperated, untreated age-/gender-matched rats served as controls. Animals were killed at week 4 of the above regimens.

Experimental Protocols

Metabolic cages were used to assess 24 h urinary output and urine pH and the urinary excretion of Zn in controls, and at wk 4 of ALDOST alone or when combined with acetazolamide cotreatment. At sacrifice, blood was collected by cardiac puncture to determine serum pH and plasma Zn.

At week 4 of ALDOST we monitored Zn levels in the myocardium, isolated cardiomyocytes, and cardiac mitochondria. We also studied metallothionein (MT)-I protein expression in the heart by Western blot analysis.

We assessed the impact of the Zn supplement on the following parameters found at wk 4 of ALDOST: plasma Zn; plasma ionized [Ca²⁺]_o; oxidative stress, expressed by 8-isoprostane levels in urine, blood, and heart; malondialdehyde in the heart; an activation of NADPH oxidase expressed by its gp91^{phox} subunit in the heart; and the appearance of microscopic scars and vasculitis involving intramural coronary arteries of the right and left ventricles.

Urinary Zn Excretion

As reported previously, metabolic cages were used to monitor 24 h urinary Zn excretion in controls and rats receiving 4 wks ALDOST, using atomic absorption spectrophotometry, and expressed as µg/24 h (30,31).

Plasma and Tissue Zn and Plasma Ionized [Ca²⁺]_o

Microanalysis of Zn in plasma and tissue was performed using atomic absorption spectroscopy as previously reported, and expressed as µg/dL and ng/mg fat-free dry tissue (FFDT), respectively (32).

The concentration of plasma ionized [Ca²⁺]_o was determined by the direct ion-selective electrode with a Nova8 Analyzer (Nova Biomedical), as previously reported (30), and expressed in mmol/L.

Plasma Cu/Zn Superoxide Dismutase (SOD) Activity

Plasma Cu/Zn-SOD activity was measured as previously reported and expressed as U/mL (15).

Cardiac Metallothionein (MT)-I

MT-I protein levels in the myocardium were measured by Western blot analysis as reported previously (15). The amount of protein detected was measured by a computer image analysis system (NIH Image ver. 1.6).

Isolation of Cardiomyocytes

Ventricular myocytes were isolated using the protocol of Lester et al. (33) with slight modifications. In brief, the aorta of excised hearts was cannulated and mounted on a Langendorff apparatus, with height adjusted for a perfusion pressure of 75 mmHg. The resulting cells were suspended in oxygenated Ringer's solution containing 0.1% bovine serum albumin. Final ventricular cell preparations yielded 65–75% rod-shaped viable cardiomyocytes determined by trypan blue dye exclusion.

Cardiomyocyte Cytosolic Free Zinc

Cytosolic free Zn concentration ($[Zn^{2+}]_i$; nmol/L) of viable cardiomyocytes was measured by 2-color flow cytometry (BD FACSCalibur™, Becton, Dickinson & Co., Franklin Lakes, NJ, USA) using the protocol of Haase et al. (34) with modifications.

Mitochondrial Total Zinc

Myocardium was first homogenized with a motorized tissue homogenizer in ice-cold Tris buffer (20 mM, pH 7.4) containing 210 mM mannitol, 70 mM sucrose, 5 mM EGTA and 1 mg/mL Type II collagenase. The resulting suspension was then further homogenized with a glass mortar and pestle and centrifuged at 700×g for 10 minutes at 4°C. The supernatant containing mitochondria was then centrifuged at 10,000×g at 4°C for 10 minutes and the supernatant discarded. The mitochondrial pellet was then washed 2 times by resuspending the pellet in fresh buffer solution without collagenase and centrifuged at 10,000×g at 4°C for 10 minutes. After the final wash, mitochondria were suspended in buffer solution and diluted to a concentration of 60–80 mg of mitochondrial protein/mL. The supernatant was used to determine the mitochondrial Zn content by atomic absorption spectrophotometry as described elsewhere (31), and expressed as ng/mg mitochondrial protein.

Ventricular gp91^{phox} Expression

Tissue gp91^{phox} expression was assessed by immunohistochemistry in coronal cryostat sections (6 µm) as reported previously (29). Negative control sections were incubated with secondary antibody alone, stained with hematoxylin, dehydrated, mounted, and viewed by light microscopy.

8-Isoprostane and Malondialdehyde (MDA) Determination

Twenty-four hour urinary excretion of 8-isoprostane was measured directly in diluted urine samples by a competitive enzyme-linked immunoassay according to kit instructions (Oxford Biomedical Research, Oxford, MI, USA). Plasma and cardiac total 8-isoprostane (free and esterified) were measured using a competitive enzyme immunoassay (EIA) kit provided by Cayman Chemical Co. (Ann Arbor, MI, USA) according to the manufacturer's protocol.

MDA was measured in aliquots of cardiac homogenate using a kit provided by Oxis Research (Foster City, CA, USA). Briefly, 200 µL aliquots of homogenate were incubated at 45°C for 1 h with 650 µL 7.7 mM N-methyl-2-phenylindole in acetonitrile:methanol (75:25, v:v) and 150 µL 15.4 mM methanesulfonic acid. After clarification by centrifugation at 15,000×g for 10 minutes absorbance was measured at 586 nm using 1,1,3,3-tetramethoxypropane as a standard.

Cardiac and Skeletal Muscle Pathology

The presence of cardiac pathology, based on the presence of fibrosis, was assessed by collagen-specific picrosirius red staining in coronal sections (6 µm) of the ventricles and observed by light microscopy, as previously reported (29). Collagen volume fraction for microscopic scars

and for perivascular fibrosis found in each section was determined using a computer image analysis system (NIH image, 1.60) as previously reported (35).

Statistical Analysis

Values are presented as mean $\pm$ SEM. Data were analyzed by Mann-Whitney rank sum test using SigmaStat statistical software (version 2.0; Systat Software, Inc., Point Richmond, CA, USA). Significant differences between individual means were assigned when p values were <0.05 .

RESULTS

Urinary zinc excretion, plasma Zn concentration and Cu/Zn-SOD activity during ALDOST alone or with acetazolamide cotreatment

Twenty-four hour urine volume, Zn excretion, and urine pH were monitored in unoperated, untreated control rats and those receiving 4 wks ALDOST alone, or in combination with acetazolamide. Daily urine output in controls was 10.1 ± 1.2 mL/24 h which rose to 94.9 ± 12.3 mL/24 h ($p<0.05$) during ALDOST and fell to 64.0 ± 1.17 mL/24 h with acetazolamide cotreatment, but remained greater ($p<0.05$) than controls.

In contrast to the alkaline urinary pH found in controls (8.10 ± 0.24), an acidification of urine occurred during ALDOST with a decline in pH to 6.54 ± 0.13 ($p<0.05$), which was attenuated by acetazolamide (7.15 ± 0.27). Serum pH in controls was 7.56 ± 0.02 and rose to 7.69 ± 0.01 at 4 wks ALDOST ($p<0.05$); however, this metabolic alkalosis was also prevented by acetazolamide (7.52 ± 0.02).

Twenty-four hour urinary Zn excretion in controls was 1.86 ± 0.16 μ g/24 h and rose to 8.61 ± 1.04 μ g/24 h ($p<0.05$) at 4 wks ALDOST, whereas acetazolamide cotreatment regulated it at control levels (1.89 ± 0.44 μ g/24 h).

Given the significantly elevated Zn excretion that occurs during aldosteronism while dietary Zn intake was held constant, plasma Zn levels at 4 wks ALDOST fell (60 ± 1 μ g/dL; $p<0.05$) compared to control values (95 ± 5 μ g/dL). Acetazolamide cotreatment normalized urinary Zn excretion during ALDOST, and in so doing attenuated the fall in plasma Zn (76 ± 3 μ g/dL). A preferential translocation of Zn to injured tissues is another factor contributing to reductions in plasma Zn during ALDOST.

In keeping with Zn deficiency, plasma Cu/Zn-SOD activity seen at 4 wks ALDOST (2.12 ± 0.08 U/mL) was significantly ($p<0.05$) diminished compared to controls (2.51 ± 0.07 U/mL), and was rescued by acetazolamide cotreatment (2.52 ± 0.17 U/mL).

Oxidative stress, metallothionein (MT)-I, and cardiac Zn during ALDOST

In keeping with the appearance of oxidative stress at a systemic level and as seen in Figure 1, plasma 8-isoprostane at 4 wks ALDOST was increased ($p<0.05$) compared to controls (7.57 ± 0.52 vs. 2.53 ± 0.50 ng/mL) as was urinary 8-isoprostane level increased above controls levels (54.48 ± 5.57 vs. 25.03 ± 2.18 ng/24h). In the heart, the level of 8-isoprostane was increased at 4 wks ALDOST (1182.7 ± 488.5 pg/mg protein; $p<0.05$) compared to controls (494.2 ± 97.8 pg/mg protein) as was the case for MDA level in the heart (0.39 ± 0.06 nmol/mg protein; $p<0.05$) compared to controls (0.29 ± 0.08 nmol/mg protein). An activation of gp91^{phox} subunit of NADPH oxidase was not seen in normal hearts (panel A, Figure 2) but appeared in inflammatory cells found at the site of microscopic scarring that followed previous cardiomyocyte necrosis and within the perivascular space of intramural coronary arteries (see panels B and C, Figure 2).

Coincident with the appearance of oxidative stress found at 4 wks ALDOST, the expression of MT-I protein in the heart was increased (1.32 ± 0.07 OD; $p < 0.05$) compared to controls (1.00 ± 0.08 OD).

The level of total Zn found in the rat heart (see Figure 3) at 4 wks ALDOST (90.8 ± 2.2 ng/mg FFDT) was significantly ($p < 0.001$) greater than that found in control hearts (74.6 ± 1.4 ng/mg FFDT). Cytosolic free $[Zn^{2+}]_i$ present in cardiomyocytes isolated from control rat hearts (29.0 ± 4.0 nmol/L) was also increased to 80.2 ± 4.5 nmol/L ($p < 0.001$) at 4 wks ALDOST. Likewise, mitochondrial total Zn was increased ($p < 0.05$) at 4 wks ALDOST (41.60 ± 2.69 ng/mg mitochondrial protein) compared to control hearts (26.25 ± 2.75 ng/mg protein).

Responses to Zn supplement during ALDOST

The plasma Zn level in control rats was 103 ± 3 μ g/dL (see top panel, Figure 4). After 4 wks ALDOST it declined to 72 ± 5 μ g/dL ($p < 0.05$) and was essentially rescued by cotreatment with $ZnSO_4$ (99 ± 6 μ g/dL).

Plasma ionized $[Ca^{2+}]_o$ (1.12 ± 0.03 mmol/L) was less at 4 wks ALDOST ($p < 0.05$) compared to controls (1.19 ± 0.01 mmol/L) in keeping with the well-known excessive urinary and fecal excretion of Ca^{2+} when dietary Ca was held constant (see bottom panel, Figure 4). However, the Zn supplement did not prevent ionized hypocalcemia (1.09 ± 0.02 mmol/L).

Plasma 8-isoprostane remained at control levels when ALDOST was accompanied by Zn supplement (2.61 ± 0.36 ng/mL) (see Figure 1, top panel) while the rise in urinary 8-isoprostane was attenuated ($p < 0.05$) by $ZnSO_4$ cotreatment (33.35 ± 6.23 ng/24h) (see Figure 1, bottom panel and the preceding Results section). The rise in cardiac 8-isoprostane seen with 4 wks ALDOST (1182.7 ± 488.5 pg/mg protein) was prevented ($p < 0.05$) by Zn supplement cotreatment (251.5 ± 68.7 pg/mg protein), as was cardiac levels of MDA (0.39 ± 0.06 vs. 0.28 ± 0.04 nmol/mg protein). The appearance of gp91^{phox} immunolabeling found in inflammatory cells invading the myocardium in response to cardiomyocyte necrosis and present at sites of repair at 4 wks ALDOST was attenuated but not eliminated by Zn supplement cotreatment. The gp91^{phox} labeling in the case of circulating inflammatory cells that invaded the perivascular space of intramural coronary arteries remained unchanged (see panels D and E, Figure 2).

In hearts harvested from control rats, there were no microscopic scars while interstitial collagen accounted for a collagen volume fraction of $2.22 \pm 0.04\%$. Microscopic scars that replaced cardiomyocytes lost to necrotic cell death appeared in the right and left ventricles at 4 wks ALDOST raising collagen volume fraction to $4.79 \pm 0.87\%$ ($p < 0.05$). Furthermore, a perivascular fibrosis involving intramural coronary arteries of the right and left ventricles appeared at wk 4 ALDOST raising collagen volume fraction at these sites to $4.29 \pm 0.61\%$ ($p < 0.05$) compared to controls ($2.96 \pm 0.02\%$). The Zn supplement cotreatment attenuated microscopic scarring ($3.89 \pm 0.34\%$), whereas the perivascular fibrosis ($4.20 \pm 0.31\%$) remained unaltered from 4 wks ALDOST.

DISCUSSION

Our study in rats receiving 4 wks ALDOST led to several major findings. First, an increment in urinary Zn excretion was one factor that contributed to a reduction in plasma Zn and plasma Cu/Zn-SOD activity. In epithelial cells of the kidneys, aldosterone receptor-ligand binding leads to upregulation of a Na^+/H^+ exchanger involved in transepithelial H^+ secretion (36). This increased activity of the Na^+/H^+ exchanger during aldosteronism leads to loss of protons via excessive excretion of ammonia with a resultant metabolic alkalosis (20,37). Herein we found an acidification of urine coupled with hyperzincuria and a metabolic alkalosis in rats receiving

4 wks ALDOST. We previously reported that cotreatment with an aldosterone receptor antagonist, spironolactone, would prevent the increased loss of Zn in both urine and feces (15,30). We now have shown that cotreatment with acetazolamide attenuates urinary acidification to prevent hyperzincuria and fall in plasma Zn. We cautiously suggest that the acidification of urine that appears during ALDOST involving the Na^+/H^+ exchanger contributes to the augmented urinary losses of Zn and the appearance of hypozincemia. Similar excessive excretory losses of Ca^{2+} and Mg^{2+} contribute to the ionized hypocalcemia and hypomagnesemia that accompany chronic mineralocorticoid excess in rats, each of which could be prevented by spironolactone (30,37,38). Similar findings have been reported for human volunteers (39) and in patients with either primary or secondary aldosteronism (40, 41). In patients having primary aldosteronism treatment with spironolactone or adrenal surgery abrogates the heightened excretory losses of Ca^{2+} and Mg^{2+} (40,42).

Our second major finding centers around the appearance of oxidative stress in the heart expressed as increased levels of 8-isoprostane and MDA within the myocardium and the gp91^{phox} subunit of NADPH oxidase found in inflammatory cells that invaded sites of injury in both ventricles. We previously reported on the increased expression of a redox-sensitive nuclear transcription factor (NF)- κB and the presence of 3-nitrotyrosine at sites of myocardial injury (29). The presence of oxi-/nitrosative stress was coupled to upregulated MT-I expression. Oxidative stress induces MT expression in diverse tissues, which leads to increased Zn accumulation at these sites (43). We found increased Zn concentration within the myocardium at 4 wks ALDOST, including its intracellular reservoirs in cardiomyocytes and mitochondria. Increased tissue levels of Zn, another factor contributing to hypozincemia, serve to augment endogenous antioxidant defenses, such as Cu/Zn-SOD activity, and where its catalytic and structural properties contribute to tissue repair (44).

In aldosteronism, however, the rise in intracellular Zn^{2+} acting as antioxidant does not occur in isolation. Intracellular Ca^{2+} overloading is also present. It acts as a prooxidant and mediator of oxidative stress. Ca^{2+} -induced oxidative stress, such as occurs in response to ischemia/reperfusion injury, can be attenuated by Zn^{2+} ionophore (45). The intracellular Ca^{2+} overloading seen with ALDOST is parathyroid hormone (PTH)-mediated. Secondary hyperparathyroidism (SHPT) is a parathyroid response to the ionized hypocalcemia that accompanies marked excretory losses of Ca^{2+} (30). Intracellular Ca^{2+} overloading occurs in such diverse tissues as heart, skeletal muscle, and peripheral blood mononuclear cells (30). Massry et al. have shown a similar Ca^{2+} overloading of diverse tissues with the SHPT that accompanies chronic renal failure (46). Further evidence in support of the presence of SHPT in rats receiving ALDOST has been provided by a reduction in bone mineral density (17). We have used various interventions to confirm the importance of PTH-mediated intracellular Ca^{2+} overloading in providing the induction of oxidative stress during ALDOST (reviewed in 47).

Our third finding relates to the Zn supplement in attenuating the appearance of oxidative stress, both at systemic and cardiac levels. In the case of the heart, however, results with the Zn supplement were mixed. Oxidative stress-induced necrosis of cardiomyocytes with scarring, a footprint of prior myocyte necrosis, was attenuated by ZnSO_4 cotreatment. Therefore the invasion of gp91^{phox}-expressing inflammatory cells found in the myocardium at sites of tissue repair was reduced. Hence, ZnSO_4 was partially cardioprotective. However, it did not prevent vasculitis, involving the invasion of the perivascular space by circulating ED1+ monocytes/macrophages and CD4+ lymphocytes (29) nor the appearance of perivascular fibrosis of intramural coronary arteries. These peripheral blood mononuclear cells are activated in response to PTH-mediated Ca^{2+} overloading and by the induction of oxidative stress (18,19, 31,48). Of importance, the Zn supplement did not prevent the ionized hypocalcemia that

accompanied ALDOST and, therefore, we would infer it did not prevent SHPT and which would include Ca^{2+} overloading of lymphocytes and monocytes (18).

Figure 5 summarizes the sequence of pathophysiologic events leading to causes and consequences of Zn dyshomeostasis in rats with aldosteronism, and where iterations in Zn balance are inextricably linked to those involving extra- and intracellular Ca^{2+} . Intracellular Ca^{2+} overloading represents a prooxidant stimulus, whereas Zn^{2+} mobilized from extracellular space to intracellular compartments contributes to antioxidant defenses. The magnitude of each of these responses corresponds to the overall redox state of the myocardium and the appearance and extent of tissue injury. Supplemental Zn offers an adjuvant as a cardioprotective strategy in aldosteronism. However, and as the paradigm in Figure 5 implicates, it is difficult to discern the contributions of Zn and Ca^{2+} dyshomeostasis individually, without the synergistic influence of the other. Cellular Zn^{2+} entry, moreover, includes Ca^{2+} channels. In like fashion, the coupling of Ca^{2+} and Mg^{2+} during aldosteronism is quite relevant, as evidenced by excessive urinary and fecal Mg^{2+} losses which are comparable to those for Ca^{2+} , and notwithstanding, intracellular Mg^{2+} is known to serve as a physiologic antagonist to Ca^{2+} entry. A dietary deficiency of Mg^{2+} is accompanied by oxidative stress, a proinflammatory phenotype, and cardiac pathology (49). Finally, recent evidence links Se^{2+} to the release and/or binding of intracellular Zn^{2+} (50). Hence, a polynutrient supplement that includes Zn^{2+} , Se^{2+} , Ca^{2+} , and Mg^{2+} may represent a more effective cardioprotective strategy in aldosteronism and will be addressed in future studies.

Acknowledgments

This work was supported, in part, by NIH/NHLBI grant R01-HL73043 (KTW) and NIH training grant T32-HL07641 (MSG).

References

1. Hambidge M. Human zinc deficiency. *J Nutr* 2000;130(5S Suppl):1344S–1349S. [PubMed: 10801941]
2. Maret W, Krezel A. Cellular zinc and redox buffering capacity of metallothionein/thionein in health and disease. *Mol Med* 2007;13:371–375. [PubMed: 17622324]
3. Prasad AS, Fitzgerald JT, Hess JW, Kaplan J, Pelen F, Dardenne M. Zinc deficiency in elderly patients. *Nutrition* 1993;9:218–224. [PubMed: 8353362]
4. Tubek S. Role of zinc in regulation of arterial blood pressure and in the etiopathogenesis of arterial hypertension. *Biol Trace Elem Res* 2007;117:39–51. [PubMed: 17873391]
5. Kosar F, Sahin I, Acikgoz N, Aksoy Y, Kucukbay Z, Cehreli S. Significance of serum trace element status in patients with rheumatic heart disease: a prospective study. *Biol Trace Elem Res* 2005;107:1–10. [PubMed: 16170217]
6. Ripa S, Ripa R, Giustiniani S. Are failed cardiomyopathies a zinc-deficit related disease? A study on Zn and Cu in patients with chronic failed dilated and hypertrophic cardiomyopathies. *Minerva Med* 1998;89:397–403. [PubMed: 10212663]
7. Oster O. Trace element concentrations (Cu, Zn, Fe) in sera from patients with dilated cardiomyopathy. *Clin Chim Acta* 1993;214:209–218. [PubMed: 8472386]
8. Topuzoglu G, Erbay AR, Karul AB, Yensel N. Concentrations of copper, zinc, and magnesium in sera from patients with idiopathic dilated cardiomyopathy. *Biol Trace Elem Res* 2003;95:11–17. [PubMed: 14555795]
9. Weber KT. Aldosterone in congestive heart failure. *N Engl J Med* 2001;345:1689–1697. [PubMed: 11759649]
10. Koren-Michowitz M, Dishy V, Zaidenstein R, Yona O, Berman S, Weissgarten J, Golik A. The effect of losartan and losartan/hydrochlorothiazide fixed-combination on magnesium, zinc, and nitric oxide metabolism in hypertensive patients: a prospective open-label study. *Am J Hypertens* 2005;18:358–363. [PubMed: 15797654]

11. Singal PK, Khaper N, Farahmand F, Bello-Klein A. Oxidative stress in congestive heart failure. *Curr Cardiol Rep* 2000;2:206–211. [PubMed: 10980894]
12. Yndestad A, Holm AM, Müller F, Simonsen S, Frøland SS, Gullestad L, Aukrust P. Enhanced expression of inflammatory cytokines and activation markers in T-cells from patients with chronic heart failure. *Cardiovasc Res* 2003;60:141–146. [PubMed: 14522416]
13. Anker SD, Chua TP, Ponikowski P, Harrington D, Swan JW, Kox WJ, Poole-Wilson PA, Coats AJS. Hormonal changes and catabolic/anabolic imbalance in chronic heart failure and their importance for cardiac cachexia. *Circulation* 1997;96:526–534. [PubMed: 9244221]
14. Anker SD, Clark AL, Teixeira MM, Hellewell PG, Coats AJ. Loss of bone mineral in patients with cachexia due to chronic heart failure. *Am J Cardiol* 1999;83:612–615. [PubMed: 10073875]
15. Thomas M, Vidal A, Bhattacharya SK, Ahokas RA, Sun Y, Gerling IC, Weber KT. Zinc dyshomeostasis in rats with aldosteronism. Response to spironolactone. *Am J Physiol Heart Circ Physiol* 2007;293:H2361–H2366. [PubMed: 17616752]
16. Selektor Y, Parker RB, Sun Y, Zhao W, Bhattacharya SK, Weber KT. Tissue ⁶⁵zinc translocation in a rat model of chronic aldosteronism. *J Cardiovasc Pharmacol* 2008;51:359–364. [PubMed: 18427278]
17. Chhokar VS, Sun Y, Bhattacharya SK, Ahokas RA, Myers LK, Xing Z, Smith RA, Gerling IC, Weber KT. Loss of bone minerals and strength in rats with aldosteronism. *Am J Physiol Heart Circ Physiol* 2004;287:H2023–H2026. [PubMed: 15475529]
18. Ahokas RA, Warrington KJ, Gerling IC, Sun Y, Wodi LA, Herring PA, Lu L, Bhattacharya SK, Postlethwaite AE, Weber KT. Aldosteronism and peripheral blood mononuclear cell activation. A neuroendocrine-immune interface. *Circ Res* 2003;93:e124–e135. [PubMed: 14576195]
19. Gerling IC, Sun Y, Ahokas RA, Wodi LA, Bhattacharya SK, Warrington KJ, Postlethwaite AE, Weber KT. Aldosteronism: an immunostimulatory state precedes the proinflammatory/fibrogenic cardiac phenotype. *Am J Physiol Heart Circ Physiol* 2003;285:H813–H821. [PubMed: 12860567]
20. Welbourne TC, Francoeur D. Influence of aldosterone on renal ammonia production. *Am J Physiol* 1977;233:E56–E60. [PubMed: 18022]
21. Ali MM, Frei E, Straub J, Breuer A, Wiessler M. Induction of metallothionein by zinc protects from daunorubicin toxicity in rats. *Toxicology* 2002;179:85–93. [PubMed: 12204545]
22. Antila H, Salo M, Nanto V, Irjala K, Brenner R, Vapaavuori M. Serum iron, zinc, copper, selenium, and bromide concentrations after coronary bypass operation. *J Parenter Enteral Nutr* 1990;14:85–89.
23. Bobillier-Chaumont S, Maupoil V, Berthelot A. Metallothionein induction in the liver, kidney, heart and aorta of cadmium and isoproterenol treated rats. *J Appl Toxicol* 2006;26:47–55. [PubMed: 16158394]
24. Song Y, Wang J, Li Y, Du Y, Arteel GE, Saari JT, Kang YJ, Cai L. Cardiac metallothionein synthesis in streptozotocin-induced diabetic mice, and its protection against diabetes-induced cardiac injury. *Am J Pathol* 2005;167:17–26. [PubMed: 15972948]
25. Feng W, Benz FW, Cai J, Pierce WM, Kang YJ. Metallothionein disulfides are present in metallothionein-overexpressing transgenic mouse heart and increase under conditions of oxidative stress. *J Biol Chem* 2006;281:681–687. [PubMed: 16243847]
26. Wang J, Song Y, Elsherif L, Song Z, Zhou G, Prabhu SD, Saari JT, Cai L. Cardiac metallothionein induction plays the major role in the prevention of diabetic cardiomyopathy by zinc supplementation. *Circulation* 2006;113:544–554. [PubMed: 16432057]
27. Crawford AJ, Bhattacharya SK. Excessive intracellular zinc accumulation in cardiac and skeletal muscles of dystrophic hamsters. *Exp Neurol* 1987;95:265–276. [PubMed: 3803514]
28. Sun Y, Ramirez FJA, Weber KT. Fibrosis of atria and great vessels in response to angiotensin II or aldosterone infusion. *Cardiovasc Res* 1997;35:138–147. [PubMed: 9302358]
29. Sun Y, Zhang J, Lu L, Chen SS, Quinn MT, Weber KT. Aldosterone-induced inflammation in the rat heart. Role of oxidative stress. *Am J Pathol* 2002;161:1773–1781. [PubMed: 12414524]
30. Chhokar VS, Sun Y, Bhattacharya SK, Ahokas RA, Myers LK, Xing Z, Smith RA, Gerling IC, Weber KT. Hyperparathyroidism and the calcium paradox of aldosteronism. *Circulation* 2005;111:871–878. [PubMed: 15710759]

31. Vidal A, Sun Y, Bhattacharya SK, Ahokas RA, Gerling IC, Weber KT. The calcium paradox of aldosteronism and the role of the parathyroid glands. *Am J Physiol Heart Circ Physiol* 2006;290:H286–H294. [PubMed: 16373592]
32. Arroyo M, LaGuardia SP, Bhattacharya SK, Nelson MD, Johnson PL, Carbone LD, Newman KP, Weber KT. Micronutrients in African-Americans with decompensated and compensated heart failure. *Transl Res* 2006;148:301–308. [PubMed: 17162251]
33. Lester JW, Gannaway KF, Reardon RA, Koon LD, Hofmann PA. Effects of adenosine and protein kinase C stimulation on mechanical properties of rat cardiac myocytes. *Am J Physiol* 1996;271:H1778–H1785. [PubMed: 8945891]
34. Haase H, Hebel S, Engelhardt G, Rink L. Flow cytometric measurement of labile zinc in peripheral blood mononuclear cells. *Anal Biochem* 2006;352:222–230. [PubMed: 16545333]
35. Sun Y, Ratajska A, Weber KT. Inhibition of angiotensin-converting enzyme and attenuation of myocardial fibrosis by lisinopril in rats receiving angiotensin II. *J Lab Clin Med* 1995;126:95–101. [PubMed: 7602241]
36. Gekle M, Silbernagl S, Oberleithner H. The mineralocorticoid aldosterone activates a proton conductance in cultured kidney cells. *Am J Physiol* 1997;273:C1673–C1678. [PubMed: 9374654]
37. Lemann J Jr, Gray RW, Maierhofer WJ, Cheung HS. The importance of renal net acid excretion as a determinant of fasting urinary calcium excretion. *Kidney Int* 1986;29:743–746. [PubMed: 3702225]
38. Massry SG, Coburn JW, Chapman LW, Kleeman CR. The effect of long-term desoxycorticosterone acetate administration on the renal excretion of calcium and magnesium. *J Lab Clin Med* 1968;71:212–219.
39. Cappuccio FP, Markandu ND, MacGregor GA. Renal handling of calcium and phosphate during mineralocorticoid administration in normal subjects. *Nephron* 1988;48:280–283. [PubMed: 3362272]
40. Horton R, Biglieri EG. Effect of aldosterone on the metabolism of magnesium. *J Clin Endocrinol Metab* 1962;22:1187–1192. [PubMed: 13964048]
41. LaGuardia SP, Dockery BK, Bhattacharya SK, Nelson MD, Carbone LD, Weber KT. Secondary hyperparathyroidism and hypovitaminosis D in African-Americans with decompensated heart failure. *Am J Med Sci* 2006;332:112–118. [PubMed: 16969139]
42. Rossi E, Sani C, Perazzoli F, Casoli MC, Negro A, Dotti C. Alterations of calcium metabolism and of parathyroid function in primary aldosteronism, and their reversal by spironolactone or by surgical removal of aldosterone-producing adenomas. *Am J Hypertens* 1995;8:884–893. [PubMed: 8541003]
43. Andrews GK. Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochem Pharmacol* 2000;59:95–104. [PubMed: 10605938]
44. Agay D, Anderson RA, Sandre C, Bryden NA, Alonso A, Roussel AM, Chancerelle Y. Alterations of antioxidant trace elements (Zn, Se, Cu) and related metallo-enzymes in plasma and tissues following burn injury in rats. *Burns* 2005;31:366–371. [PubMed: 15774296]
45. Karagulova G, Yue Y, Moreyra A, Boutjdir M, Korichneva I. Protective role of intracellular zinc in myocardial ischemia/reperfusion is associated with preservation of protein kinase C isoforms. *J Pharmacol Exp Ther* 2007;321:517–525. [PubMed: 17322024]
46. Massry SG, Smogorzewski M. Mechanisms through which parathyroid hormone mediates its deleterious effects on organ function in uremia. *Semin Nephrol* 1994;14:219–231. [PubMed: 8036356]
47. Sun Y, Ahokas RA, Bhattacharya SK, Gerling IC, Carbone LD, Weber KT. Oxidative stress in aldosteronism. *Cardiovasc Res* 2006;71:300–309. [PubMed: 16631148]
48. Ahokas RA, Sun Y, Bhattacharya SK, Gerling IC, Weber KT. Aldosteronism and a proinflammatory vascular phenotype. Role of Mg^{2+} , Ca^{2+} and H_2O_2 in peripheral blood mononuclear cells. *Circulation* 2005;111:51–57. [PubMed: 15611366]
49. Weglicki WB, Mak IT, Stafford RE, Dickens BF, Cassidy MM, Phillips TM. Neurogenic peptides and the cardiomyopathy of magnesium-deficiency: effects of substance P-receptor inhibition. *Mol Cell Biochem* 1994;130:103–109. [PubMed: 8028589]
50. Jacob C, Maret W, Vallee BL. Ebselen, a selenium-containing redox drug, releases zinc from metallothionein. *Biochem Biophys Res Commun* 1998;248:569–573. [PubMed: 9703967]

Figure 1.

Plasma and urinary 8-isoprostane levels found in age-/gender-matched control rats, at 4 wks aldosterone/salt treatment (ALDOST) alone, or in combination with ZnSO₄ as Zn supplement. See text.

Figure 2.

Immunohistochemical detection of gp91^{phox} in the rat myocardium. Panel A, controls; panels B and C, 4 wks ALDOST alone; and panels D and E, ALDOST in combination with ZnSO₄ cotreatment. (A) control rat myocardium with intramural coronary artery surrounded by a normal perivascular space. (B) myocardium at 4 wks ALDOST with expanded perivascular space containing invading inflammatory cells (arrows) expressing gp91^{phox} subunit of NADPH oxidase (brown staining). (C) myocardium at 4 wks ALDOST reveals multiple microscopic areas of injury (arrow) with invading inflammatory cells expressing gp91^{phox}. (D) intramural coronary artery with expanded perivascular space containing gp91^{phox}-positive inflammatory cells (arrow) found in rats receiving 4 wks ALDOST plus ZnSO₄. (E)

cotreatment with ZnSO_4 attenuated, but did not completely prevent microscopic areas of injury with invading inflammatory cells expressing gp91^{phox} (arrow). See text.

Figure 3.
Cardiac Zn levels found in control rats and at 4 wks ALDOST alone or with Zn supplement as cotreatment. See text.

Figure 4. Plasma Zn and ionized [Ca²⁺]_o in control rats and at 4 wks ALDOST without or with ZnSO₄ cotreatment. See text.

Figure 5.

ALDOST leads to increased urinary and fecal excretion of both Zn^{2+} and Ca^{2+} with an accompanying fall in their plasma concentrations. In the case of ionized hypocalcemia with reduced plasma $[\text{Ca}^{2+}]_o$, parathyroid glands secrete PTH to account for an increase in cardiac $[\text{Ca}^{2+}]_i$ and consequent appearance of oxidative stress likely originating from mitochondria. Oxidative stress provokes an increased expression of MT-I that contributes to a sequestration of Zn^{2+} within the myocardium and a further fall in plasma Zn, together with redox-mediated release of Zn^{2+} bound to MT. As we have shown previously (15), the rise in $[\text{Zn}^{2+}]_i$ contributes to increased activity of Cu/Zn-SOD activity in the heart.