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COUPLED CALCIUM AND ZINC DYSHOMEOSTASIS AND OXIDATIVE STRESS IN CARDIAC MYOCYTES AND MITOCHONDRIA OF RATS WITH CHRONIC ALDOSTERONISM

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Abstract

A dyshomeostasis of extra- and intracellular Ca^{2+} and Zn^{2+} occurs in rats receiving chronic aldosterone/salt treatment (ALDOST). Herein, we hypothesized the dyshomeostasis of intracellular Ca^{2+} and Zn^{2+} is intrinsically coupled that alters the redox state of cardiac myocytes and mitochondria, with Ca^{2+} serving as a prooxidant and Zn^{2+} as an antioxidant. Toward this end, we harvested hearts from rats receiving 4 wks ALDOST alone or cotreatment with either spironolactone (Spiro), an aldosterone receptor antagonist, or amlodipine (Amlod), an L-type Ca^{2+} channel blocker (LTCC), and from age-/gender-matched untreated controls. In each group we monitored cardiomyocyte $[\text{Ca}^{2+}]_i$ and $[\text{Zn}^{2+}]_i$ and mitochondrial $[\text{Ca}^{2+}]_m$ and $[\text{Zn}^{2+}]_m$; biomarkers of oxidative stress and antioxidant defenses; expression of Zn transporters, Zip1 and ZnT-1; metallothionein (MT)-1, a Zn^{2+} -binding protein; and metal-response element transcription factor (MTF)-1, a $[\text{Zn}^{2+}]_i$ sensor and regulator of antioxidant defenses. Compared to controls, at 4 wks ALDOST we found: a) increased $[\text{Ca}^{2+}]_i$ and $[\text{Zn}^{2+}]_i$, together with increased $[\text{Ca}^{2+}]_m$ and $[\text{Zn}^{2+}]_m$, each of which could be prevented by Spiro and attenuated with Amlod; b) increased levels of 3-nitrotyrosine and 4-hydroxy-2-nonenal in cardiomyocytes, together with increased H_2O_2 production, malondialdehyde and oxidized GSSG in mitochondria that were coincident with increased activities of Cu/Zn-superoxide dismutase and glutathione peroxidase; and c) increased expression of MT-1, Zip1 and ZnT-1, and MTF-1, attenuated by Spiro. Thus, an intrinsically coupled dyshomeostasis of intracellular Ca^{2+} and Zn^{2+} occurs in cardiac myocytes and mitochondria in rats receiving ALDOST, where it serves to alter their redox state through a respective induction of oxidative stress and generation of antioxidant defenses. The importance of therapeutic strategies that can uncouple these two divalent cations and modulate their ratio in favor of sustained antioxidant defenses is therefore suggested.

Keywords

cardiac myocytes and mitochondria; calcium; zinc; zinc transporters; metallothionein; metal-response element transcription factor-1

Introduction

Effector hormones of an activated renin-angiotensin-aldosterone system, integral to the appearance of the congestive heart failure syndrome, have been linked to the induction of oxidative stress and its deleterious consequences (1). In rats receiving chronic aldosterone/salt treatment (ALDOST) a dyshomeostasis of extra- and intracellular Ca^{2+} and Zn^{2+} contributes to an imbalance of pro- and antioxidants in favor of prooxidants (2-5). Heightened urinary and fecal Ca^{2+} losses are seen throughout 6 weeks of ALDOST, and account for ionized hypocalcemia with consequent sustained elevations in parathyroid hormone (PTH) (2). The resultant secondary hyperparathyroidism (SHPT) seeks to restore extracellular Ca^{2+} homeostasis through ongoing bone resorption (2,6).

Juxtaposed to this imbalance in extracellular Ca^{2+} is the simultaneous rise in $[\text{Ca}^{2+}]_i$ facilitated by PTH receptor-mediated Ca^{2+} channel entry (7). This Ca^{2+} overloading, referred to as a Ca^{2+} paradox (8), ensues in diverse tissues, including the heart and peripheral blood mononuclear cells (PBMC) (7,9-11). Persistent intracellular Ca^{2+} overloading serves as a prooxidant inducing the generation of reactive oxygen and nitrogen species (ROS/RNS), which ultimately overwhelm antioxidant defenses (12). Concentration-dependent outcomes follow with cytotoxic levels of ROS/RNS leading to cardiomyocyte necrosis and subsequent replacement fibrosis, or myocardial scarring, in both the right and left heart at wk 4 ALDOST (2,13,14).

The altered redox state is also evident in PBMC that have invaded the heart. As demonstrated by immunohistochemistry and *in situ* hybridization, biomarkers indicative of oxi-/nitrosative stress in these lymphocytes and monocytes include 3-nitrotyrosine, an activation of membrane-bound NADPH oxidase and redox-sensitive nuclear transcription factor (NF)- κB , together with the proinflammatory genes it induces, including adhesion molecules, chemokines and cytokines (9,10,13). The relative contribution of membrane-bound NADPH oxidase in these inflammatory cells vis-à-vis cardiac mitochondria to the heart's altered redox state in aldosteronism remains uncertain and was explored herein by examining cardiac myocytes and mitochondria harvested from the heart at wk 4 ALDOST, coincident with the appearance of cardiac pathology.

Cotreatment of ALDOST with spironolactone (Spiro), an aldosterone receptor antagonist, attenuates the augmented excretory losses of Ca^{2+} to prevent ionized hypocalcemia and SHPT, and hence $[\text{Ca}^{2+}]_i$ overloading can be averted. Accordingly, Spiro spared the heart from scarring (2,13). Parathyroidectomy was likewise cardioprotective (15,16). Cotreatment with a Ca^{2+} channel blocker does not abrogate the appearance of hypocalcemia or SHPT, however, it does prevent $[\text{Ca}^{2+}]_i$ overloading and oxidative stress and, therefore, is cardioprotective in chronic mineralocorticoidism (11,17).

Hypozincemia likewise accompanies ALDOST. It is due to excretory renal and gastrointestinal Zn^{2+} losses, together with its translocation from plasma to injured tissues, including the heart (3,5). The translocation of Zn^{2+} to the heart is facilitated by the injured tissue-responsive upregulation of metallothionein (MT)-1, a cysteine-rich Zn^{2+} -binding protein (3). The expression of MT-1 represents a mechanism by which cells can regulate Zn^{2+} distribution and protect against oxidative stress (18). Cardiomyocyte Zn^{2+} -sensitive membrane transporters

(both importers and exporters) may also contribute to Zn^{2+} entry; however, their specific response remains unknown and was explored herein.

An increase in cytosolic free $[\text{Zn}^{2+}]_i$ serves as an antioxidant—a negative feedback response to $[\text{Ca}^{2+}]_i$ overloading. Increased $[\text{Zn}^{2+}]_i$ activates a Zn-finger metal-response element transcription factor (MTF)-1, which is central to the transcriptional expression of such antioxidant defenses as MT-1, Cu/Zn-superoxide dismutase (SOD), and glutathione synthase (19). Its capacity to counterbalance the prooxidant $[\text{Ca}^{2+}]_i$ response during ALDOST, however, may be limited given the prevailing excretory Zn^{2+} losses, hypozincemia with consequent reduced plasma Cu/Zn-SOD activity, and declining endogenous Zn^{2+} stores, where a resorption of bone Zn^{2+} accompanies SHPT (4,5). Spiro cotreatment in rats receiving ALDOST prevents excretory Zn^{2+} losses and hypozincemia. It therefore plays a dual salutary role in preserving both Zn^{2+} and Ca^{2+} homeostasis (2,3). On the other hand, cotreatment with a Zn^{2+} supplement only prevents hypozincemia without mitigating hypocalcemia, and therefore is only partially cardioprotective (4). These findings underscore the importance of the coupled dyshomeostasis of Ca^{2+} and Zn^{2+} that accompanies ALDOST, which to date have only been critically examined separately.

Herein, we hypothesized that intracellular dyshomeostasis of Ca^{2+} and Zn^{2+} , specifically involving cardiac myocytes and mitochondria, is intrinsically coupled to alter their redox state and wherein intracellular Ca^{2+} acts as a prooxidant and Zn^{2+} as an antioxidant. Toward this end, we monitored levels of these divalent cations in intact heart, and in cardiomyocytes and mitochondria harvested from the myocardium of rats at 4 wks ALDOST when cardiomyocyte necrosis and scarring are first evident. We monitored: intracellular Ca^{2+} and Zn^{2+} with fluorescent probes; together with biomarkers of oxidative stress and antioxidant defenses; and expression of Zn^{2+} transporters, Zip1, which increases intracellular Zn^{2+} availability, and ZnT-1 which promotes Zn^{2+} efflux (20,21); metallothionein-1 (MT-1), a major intracellular Zn^{2+} -binding protein (18); and MTF-1, the sensor of cytosolic free $[\text{Zn}^{2+}]_i$ (19). We compared untreated age-/sex-matched controls to those receiving 4 wks ALDOST alone or with cotreatment provided by either Spiro or amlodipine (Amlod), an L-type Ca^{2+} channel (LTCC) blocker, interventions which were demonstrated to be cardioprotective in previous studies (13,17,22).

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 and which suppresses plasma renin activity and circulating levels of angiotensin II (2). Drinking water was fortified with 1% NaCl and with 0.4% KCl to prevent hypokalemia. Separate groups of rats receiving ALDOST were given either spironolactone (Spiro; 150 mg/kg/day by twice-daily gavage) or amlodipine (Amlod; 10 mg/kg/day by gavage) as cotreatment. Unoperated, untreated age-/gender-matched rats served as controls. Animals were killed at week 4 of the above regimens.

Rationale and Experimental Protocols

Previously, we reported the appearance of microscopic scarring, a marker of cardiomyocyte necrosis found in both right and left atria and ventricles, to be delayed and not evident until wk 4 ALDOST (13,14,23). We found cardiac pathology to be absent with the following controls: uninephrectomy alone; 1% NaCl in drinking water alone; administration of the

minipump vehicle; or aldosterone treatment together with dietary Na⁺ deprivation (0.4% NaCl) (22-25). Several interventions in this setting of chronic aldosteronism were demonstrated to be cardioprotective. These included cotreatment with Spiro: given in a large depressor dose, which prevented the appearance of arterial hypertension and left ventricular hypertrophy (LVH); and a nondepressor dose, which did not prevent either the rise in arterial pressure or LVH (22).

More recently, we linked the cardiomyocyte necrosis and scarring found at wk 4 ALDOST to the appearance of oxidative stress, where cotreatment with an antioxidant proved to be cardioprotective (13). We next linked the appearance of oxidative stress to PTH-mediated intracellular Ca²⁺ overloading where SHPT could be prevented by: Spiro, which attenuated the augmented excretory losses of Ca²⁺ and Mg²⁺ to prevent hypocalcemia and hypomagnesemia; parathyroidectomy, performed prior to ALDOST; cotreatment with a Ca²⁺ and Mg²⁺ supplement in combination with calcitriol to prevent hypocalcemia and hypomagnesemia; cinacalcet, a calcimimetic, which prevented the heightened release of PTH during hypocalcemia; or Amlod (2,11,16,26,27). Moreover, we found a concomitant rise in [Zn²⁺]_i, facilitated by upregulated expression of MT-1, to have antioxidant properties that increased activity of Cu/Zn-superoxide dismutase; while cotreatment with a ZnSO₄ supplement prevented microscopic scarring (3,4).

Herein, we addressed the coupled dyshomeostasis of Ca²⁺ and Zn²⁺ involving cardiac myocytes and mitochondria, and its relationship to their redox state at wk 4 ALDOST. We further examined cotreatment regimens involving Spiro and Amlod, each of which are cardioprotective because of their respective prevention of intracellular Ca²⁺ overloading; in the case of Spiro by preventing SHPT and Amlod by reducing LTCC entry.

Isolation of Cardiomyocytes and Mitochondria

Cardiomyocytes were harvested by retrograde collagenase perfusion of the crystalloid-perfused heart and mitochondria were isolated by differential centrifugation of whole heart homogenates as we have previously reported (4). The purity of our mitochondrial preparation was assessed by flow cytometry and mitochondrial-specific dye MitoTracker Red (Invitrogen, Eugene, OR, USA). For H₂O₂ release experiments some mitochondria were used within 2 hours of isolation, the remainder were kept at -70°C until further use. In using differential centrifugation to isolate mitochondria from cardiac tissue and its cardiomyocytes, which provide a rich source of these organelles, we recognize the potential contamination by noncardiomyocytic mitochondria. However, we would suggest this may cause very minor contamination, if at all, given the paucity of mitochondria found in endothelial and smooth muscle cells and fibroblasts.

Myocardial Total Ca²⁺ and Zn²⁺ Contents

Myocardial concentrations of Ca²⁺ and Zn²⁺ were determined using atomic absorption spectroscopy as previously reported (3).

Cardiomyocyte Cytosolic Free [Ca²⁺]_i and [Zn²⁺]_i

Cytosolic free Ca²⁺ concentration ([Ca²⁺]_i, nM) was measured ratiometrically using the Ca²⁺-specific fluorophore Fura-2 (Invitrogen) as we have previously reported (10,11). Cytosolic free Zn²⁺ concentration ([Zn²⁺]_i; nM) of viable cardiomyocytes was measured by 2-color flow cytometry (BD FACSCalibur™, Becton, Dickinson & Co., Franklin Lakes, NJ, USA) using zinc-specific dye FluoZin-3 (Invitrogen) and propidium iodide (Sigma, St. Louis, MO, USA) for detection of non-viable cells as we reported previously (4).

Mitochondrial Free $[Ca^{2+}]_m$ and Total $[Zn^{2+}]_m$

In cardiac mitochondria, free $[Ca^{2+}]_i$ was determined by the ratiometric method using Fura-2 and expressed as nM as previously described (10,11).

Mitochondrial total $[Zn^{2+}]_m$ content was determined by atomic absorption spectrophotometry as described elsewhere (16), and expressed as ng/mg mitochondrial protein.

Oxidative Stress. Prooxidants

1) Malondialdehyde (MDA) and 8-Isoprostane—MDA was measured in heart tissue and cardiac mitochondria. Tissue was homogenized (1:10 w:v) in ice-cold PBS (pH 7.4) containing 5 mM butylated hydroxytoluene (BHT), sonicated and centrifuged at $3000\times g$ for 10 minutes. Isolated mitochondria were sonicated in ice-cold PBS (pH 7.4) containing 5 mM BHT and centrifuged at $3000\times g$ for 10 minutes. Supernatant MDA concentration was determined colorimetrically using a commercially available kit (Oxis Research, Portland, OR, USA). Briefly, duplicate aliquots of supernatant were incubated at $45^\circ C$ with 7.7 mM N-methyl-2-phenylindole in acetonitrile:methanol (75:25 v:v) and 15.4 mM methanesulfonic acid. After clarification by centrifugation at $15000\times g$ for 15 minutes, absorbance was measured at 586 nm using 1,1,3,3-tetramethoxypropane as a standard. Cardiac total 8-isoprostane (free and esterified) was measured using a competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2) Mitochondrial H_2O_2 Production—To measure the release of H_2O_2 from isolated cardiac mitochondria, the Amplex Red (Invitrogen) protocol of Mohanty et al. (28) was used with some modification. Briefly, 200 μg /30 μL of a mitochondrial suspension was added to the wells of a microplate and prewarmed to $37^\circ C$ for 10 min. One hundred μL of phosphate buffer containing 50 μM Amplex Red (10 acetyl-3,7-dihydroxyphenoxazine, Invitrogen) and 0.1 U/mL horseradish peroxidase was subsequently added to each well. To measure stimulated H_2O_2 release, 20 μL of 25 mM succinate was added to the reaction mixture. To account for background absorbance in the sample, unstimulated samples were run in parallel for which succinate was substituted with an equal volume of respiratory buffer. Absorbance at 550 nm was measured after 30 min of incubation at $37^\circ C$ and background absorbance was subtracted from sample absorbance. H_2O_2 concentration was determined from a standard curve and expressed as pmol/min/mg protein (mean $\pm$ SEM). Protein concentration was determined by BCA Protein Assay kit (Pierce, Rockford, IL, USA).

3) 3-Nitrotyrosine and 4-Hydroxy-2-nonenal Levels (4-HNE)—3-Nitrotyrosine and 4-HNE content in cardiac myocytes and mitochondria was assessed by slot-blot analysis (29). 4-HNE is generated from the oxidation of polyunsaturated fatty acids by ROS and is a biomarker of lipid peroxidation. Cardiomyocyte or mitochondrial homogenate was resuspended in TBS buffer (100 mM Tris, 150 mM NaCl, pH 7.4). Homogenate solution containing 10 μg /well was applied to a vacuum-assisted slot-blot apparatus (48-well template, Bio-Rad Laboratories, Hercules, CA, USA; nitrocellulose membrane) and all samples were analyzed in duplicate. To adjust for unequal protein transfer following protein application, the membrane was stained with Ponceau S (Sigma) and scanned for optical density. Nitration was detected using a monoclonal anti-3-nitrotyrosine antibody (1:1000; Cayman Chemical) and 4-HNE protein adducts-polyclonal rabbit antibody (Calbiochem, San Diego, CA, USA). After incubation with peroxidase-conjugated secondary antibody (Sigma), protein bands were visualized using an ECL system (Pierce, Rockford, IL, USA). Films were digitally scanned with an HP ScanJet 5200c (Hewlett Packard, Palo Alto, CA, USA). The optical density and intensity of 3-nitrotyrosine and 4-HNE bands were measured using ImageJ software (NIH, Bethesda, MD, USA). The values were calculated as a ratio of 3-nitrotyrosine or 4-HNE band

intensity divided by corresponding Ponceau S band intensity to normalize for equivalent protein loading, and data expressed as mean $\pm$ SEM.

Oxidative Stress. Antioxidants

1) Glutathione Peroxidase (GSHPx) Activity—GSHPx activity was measured in cardiac myocytes and isolated mitochondria utilizing a commercially available kit (Oxis Research). Suspensions of myocytes or mitochondria were lysed by sonication. Oxidized glutathione (GSSG) produced by the reduction of organic peroxide by GSHPx was recycled to its reduced form (GSH) by glutathione reductase and NADPH. With this method, GSHPx activity was measured indirectly by the oxidation of NADPH to NADP⁺, which was assessed by the rate of decrease in absorbance at 340 nm. GSHPx activity was expressed as mU/minute/mg protein.

2) Cu/Zn-SOD and Mn-SOD Activity—Total SOD activity in mitochondria and cardiac myocytes was measured spectrophotometrically using a commercial kit (Cayman Chemical), which utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Mn-SOD activity was determined after inhibition of Cu/Zn-SOD activity with 3 mM KCN. Cu/Zn-SOD was calculated as the difference between total and Mn-SOD activities. Activities are expressed as U/mg protein.

3) Reduced and Oxidized Glutathione—To measure reduced and oxidized glutathione in cardiomyocytes and isolated mitochondria, the GSH/GSSG-412 assay kit from Oxis Research was used. To minimize GSH oxidation during sample storage, a thiol-scavenging reagent, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate, was added to each sample immediately after isolation according to manufacturer's recommendations. The method for quantitative determination of total (reduced and oxidized or GSht) glutathione employs Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid or DTNB), which reacts with GSH to form a spectrophotometrically detectable product at 412 nm. GSSG is determined by the reduction of GSSG to GSH, which is then measured by the reaction with Ellman's reagent. GSH and GSSG levels were expressed as nM/mg protein; the GSH/GSSG ratio was also calculated.

Immunoblotting for MTF-1 and NADPH Oxidase Subunit gp91^{phox}

For immunoblotting, cardiomyocytes were lysed with SDS-urea buffer (40 mM Hepes, 4 M urea, 1% SDS, 75 mM Tris, pH 7.4). From each sample, 20 μ g of total protein was separated on 10% SDS-polyacrylamide gels, and then transferred to a nitrocellulose membrane in accordance with standard procedures. The membrane was blocked in 5% non-fat milk in TBST (0.05% Tween 20) for 1 hour. Incubation with the primary antibodies to rabbit polyclonal anti-MTF-1 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal antibody for gp91^{phox} (1:1000) (BD Biosciences, San Jose, CA, USA), and mouse monoclonal anti-GADPH (1:10000) (Chemicon, Temecula, CA, USA) was carried out overnight at 4°C. Immunodetection was performed using the horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG (1:10000) (Sigma), and bands were visualized with the ECL system. To quantitate MTF-1 expression, optical density of the protein bands was measured using ImageJ software (NIH). Protein loading was normalized using GADPH as housekeeping protein. Immunoblotting data were presented as fold change relative to control cardiomyocytes (30).

RT-PCR for Zinc Transporter Proteins (ZnT-1, Zip1), Metallothionein-1 (MT-1), and MTF-1

Total RNA was isolated from cardiac myocytes using the Trizol method and manufacturer's protocol (Invitrogen). The RNA pellet was dissolved in 100 μ L of RNase-free water and then purified with a DNA-freeTM kit (Ambion, Austin, TX, USA). RNA integrity and concentration were determined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesized using a SuperScriptTM First-Strand Synthesis System for RT-PCR

(Invitrogen). Sense and antisense primers were constructed for rat ZnT-1, Zip1, MTF1, MT-1, and a housekeeping gene, β -tubulin, using Universal Probe Library Assay Design Center (Roche Applied Sciences, Indianapolis, IN, USA). ZnT-1: 5'-AACACCAGCAATTCCAACG (sense), 5'-CCACTGGATCATCACTTCTCAA (antisense); MT-1: 5'-CACCAGATCTCGGAATGGAC (sense), 5'-CTGGAGCAGGTGCAGGAG (antisense); α -tubulin: 5'-AAGAAGCAACACCTCCTCCTC (sense), 5'-TTGCCGATCTGGACACC (antisense); Zip1: 5'-GTCCCTGCGGCTTCTACAG (sense), 5'-CAGGAGAAGAGGATCCCA (antisense); and MTF-1: 5'-ATCTGCGGAAGCACATTC (sense), 5'-GCAAATGCTTTTCCACAG (antisense). Quantitative real-time PCR was carried out on an LC480 thermocycler using FastStart TaqMan Probe Master (Roche) according to the manufacturer's instructions. Amplification efficiencies of the target and the housekeeping gene were established using template dilution, and appeared to be approximately equal. Expression of ZnT-1, Zip1, MTF-1 and MT-1 in treatment groups relative to controls was calculated using the delta-delta Ct method $2^{-[\Delta\Delta C_t]}$, where $[\Delta\Delta C_t] = [\Delta C_t]_{\text{treatment}} - [\Delta C_t]_{\text{control}}$ (<http://www.ambion.com/techlib/basics/rtPCR/index.html>). Here, $[\Delta C_t]$ is the C_t value for any sample normalized to β -tubulin gene. To exclude potential bias owing to data averaging that have been transformed through the $2^{-[\Delta\Delta C_t]}$ equation, comparisons between groups were performed between delta Ct values, but not fold changes (31).

Statistical Analysis

Group data 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 group means were assigned when p values were <0.05.

RESULTS

Myocardial Total Ca^{2+} and Zn^{2+} Contents

Total Ca^{2+} found in myocardial tissue at 4 wks ALDOST (5.42 ± 0.31 nEq/mg FFDT) was increased by 43% ($p < 0.05$) compared to controls (3.78 ± 0.16 nEq/mg FFDT).

Total Zn^{2+} present in the myocardium at 4 wks ALDOST (93 ± 3 ng/mg FFDT) was increased by 18% ($p < 0.05$) compared to control tissue (79 ± 5 ng/mg FFDT).

Cardiomyocyte Cytosolic Free $[\text{Ca}^{2+}]_i$ and $[\text{Zn}^{2+}]_i$

Given our focus on oxidative stress and the synergistic regulatory roles of Ca^{2+} and Zn^{2+} , we monitored cytosolic free Ca^{2+} and Zn^{2+} in cardiac myocytes and mitochondria. In cardiomyocytes harvested from the hearts of untreated, age-/gender-matched controls, cytosolic free $[\text{Ca}^{2+}]_i$ and $[\text{Zn}^{2+}]_i$ were found to be 29 ± 4 nM and 0.76 ± 0.12 nM, respectively, with intracellular $[\text{Zn}^{2+}]_i$ approximately 2.6% of intracellular $[\text{Ca}^{2+}]_i$. At wk 4 ALDOST, and as shown in Figure 1, cardiomyocyte $[\text{Ca}^{2+}]_i$ had risen 2.8-fold to 80 ± 5 nM and $[\text{Zn}^{2+}]_i$ had risen 2.2-fold to 1.64 ± 0.08 nM, both significantly greater ($p < 0.05$) than controls.

At wk 4 ALDOST, the rise in $[\text{Ca}^{2+}]_i$ and $[\text{Zn}^{2+}]_i$ could be prevented by Spiro cotreatment. $[\text{Ca}^{2+}]_i$ loading was prevented by Amlod, while the increment in $[\text{Zn}^{2+}]_i$ was attenuated by approximately 30% with this Ca^{2+} channel blocker (see Figure 1). Thus, the intracellular $[\text{Ca}^{2+}]_i$ overloading that accompanies the SHPT of aldosteronism occurs through LTCC and could be prevented by either Spiro, which prevents SHPT, or by Amlod, a LTCC blocker. Taken together, our findings implicate cardiomyocyte $[\text{Ca}^{2+}]_i$ overloading, which is accompanied by a coupled increase in cytosolic free $[\text{Zn}^{2+}]_i$.

Mitochondrial Free $[Ca^{2+}]_m$ and Total $[Zn^{2+}]_m$

We found the Ca^{2+} overloading of heart tissue to include both cytosolic and mitochondrial compartments of cardiomyocytes (see Figures 1 and 2, upper panels). The 1.7-fold ALDOST-induced elevation in $[Ca^{2+}]_m$ was prevented by Spiro, while Amlod cotreatment did not alter the rise in $[Ca^{2+}]_m$. Furthermore, the rise in $[Zn^{2+}]_i$ was accompanied by a 1.6-fold increase in total $[Zn^{2+}]_m$ (see Figure 2, lower panel). This coupled response in $[Zn^{2+}]_m$ and $[Ca^{2+}]_m$ was prevented by Spiro cotreatment, while Amlod did not influence the rise in $[Ca^{2+}]_m$ or $[Zn^{2+}]_m$ (see Figure 2).

Zn^{2+} Transporters

Quantitative real-time PCR was used to assess the mRNA expressions of Zip1 and ZnT-1 (and a housekeeping gene tubulin), and were compared to controls derived from the delta-delta Ct method; although their relative variability was not calculated. Increased expressions of Zip1 and ZnT-1 were seen in cardiomyocytes at 4 wks (see Figure 3) with ALDOST. We cautiously interpret these findings to suggest that the rise in cardiomyocyte $[Zn^{2+}]_i$ is facilitated by increased expression of the importer Zip1, while increased expression of the exporter ZnT-1 serves to prevent intracellular Zn^{2+} overloading and cytotoxicity. Cotreatment with Spiro prevented the increased expression of Zip1 and ZnT-1, while Amlod cotreatment did not alter their expression.

Expression of MT-1 and MTF-1

Expression of major intracellular Zn binding protein MT-1 mRNA followed a pattern similar to the Zn^{2+} transporters. ALDOST led to a 2.8-fold upregulation of MT-1 mRNA ($p<0.05$). We previously reported on the increased protein expression of MT-1 found in the heart at 4 wks ALDOST (3). Spiro completely prevented and Amlod attenuated the augmented mRNA expression of MT-1.

The protein expression of the intracellular $[Zn^{2+}]_i$ sensor and transcription factor, MTF-1, is shown in Figure 4. In order to quantitate MTF-1 expression, protein band density was normalized using GADPH as a housekeeping protein. Immunoblotting data are presented in fold changes relative to control cardiomyocytes and, as such, variability was not calculated. The increased expression of MTF-1, which accompanied ALDOST, was abrogated by Spiro cotreatment and attenuated by amlodipine. These responses mirror those found for the increase in cytosolic free $[Zn^{2+}]_i$ seen at wk 4 ALDOST, which was prevented by Spiro and only attenuated by Amlod (see Figure 1, lower panel). The increase in MTF-1 protein paralleled the rise in MTF-1 mRNA expression (control, 1; ALDOST, 4.28; Spiro, 0.85; and Amlod, 2.2; $p<0.05$ control vs. ALDOST).

Oxidative Stress. Prooxidants

The level of MDA found in the heart at 4 wks ALDOST was increased ($p<0.05$) above control values (1.83 ± 0.27 vs. 1.23 ± 0.17 nM/mg protein). Similarly, the level of 8-isoprostane in the heart was increased ($p<0.05$) compared to tissue harvested from untreated controls (318.8 ± 40.0 vs. 25.6 ± 3.37 pg/mg protein). Cotreatment with Spiro or Amlod prevented ($p<0.05$) the rise in heart tissue MDA levels (1.14 ± 0.32 and 1.16 ± 0.17 nM/mg protein, respectively) and 8-isoprostane (17.5 ± 3.53 and 19.9 ± 2.6 pg/mg protein, respectively) seen at 4 wks ALDOST.

An altered redox state in cardiomyocytes was seen during ALDOST and was evidenced by increased ($p<0.05$) 3-nitrotyrosine optical density in cardiomyocytes (2.95 ± 0.29) compared to controls (2.26 ± 0.20), which was attenuated ($p<0.05$) by Spiro or by Amlod cotreatment (2.51 ± 0.30 and 2.60 ± 0.27), respectively. A similar response in optical density was seen with 4-HNE levels in cardiomyocytes at 4 wks ALDOST compared to controls (1.53 ± 0.08 vs. 1.33 ± 0.04),

and with Spiro or Amlod cotreatments (1.45 ± 0.07 and 1.43 ± 0.07), respectively. We were unable to detect NADPH oxidase activation in cardiomyocytes by Western blot.

Oxidative stress in mitochondria was assessed by H_2O_2 production and MDA levels. Compared to controls, H_2O_2 production by these organelles was increased ($p < 0.05$) at 4 wks ALDOST (148.2 ± 13.2 vs. 95.7 ± 13.5 pmol/mg/min). Mitochondrial MDA (see upper panel, Figure 5) concentration was increased ($p < 0.05$) with ALDOST vs. controls (0.84 ± 0.06 vs. 0.39 ± 0.05 nM/mg mitochondrial protein), which was prevented by Spiro (0.40 ± 0.03) and Amlod (0.39 ± 0.02) cotreatment.

4-HNE levels were significantly increased in mitochondria following ALDOST (2.30 ± 0.10 in control vs. 2.96 ± 0.14 in ALDOST, $p < 0.05$). These changes were attenuated with Spiro (2.59 ± 0.18) or Amlod (2.64 ± 0.06) treatments. In contrast to cardiomyocytes, no significant differences in nitrosative stress were found in cardiac mitochondria with ALDOST (data not shown).

Oxidative Stress. Antioxidants

To assess antioxidant defenses of cardiac myocytes and mitochondria in response to 4 wks ALDOST, we evaluated the activity of Cu/Zn-SOD, Mn-SOD, and GSHPx, as well as oxidized glutathione (GSSG) and the ratio of reduced GSH to GSSG (GSH/GSSG). Cytosolic Cu/Zn-SOD activity was increased ($p < 0.05$) with ALDOST (22.9 ± 1.1 vs. 34.8 ± 3.9 U/mg protein), and this rise was attenuated ($p < 0.05$) by cotreatment with either Spiro or Amlod (27.2 ± 4.1 and 25.3 ± 4.7 U/mg protein, respectively). In contrast, the activity of mitochondrial Mn-SOD remained unchanged with ALDOST (20.9 ± 2.64 U/mg protein) compared to controls (18.7 ± 3.2 U/mg protein). No change in Mn-SOD activity was observed with Spiro (21.3 ± 3.12 U/mg protein) or Amlod (18.9 ± 3.0 U/mg protein). Activity of GSHPx was elevated in cardiomyocytes (0.8 ± 0.08 vs. 1.21 ± 0.05 U/mg protein; $p < 0.05$) and normalized with Spiro (0.78 ± 0.02 U/mg protein) or Amlod (0.96 ± 0.07 U/mg protein) cotreatment. As seen in Figure 5, lower panel, a rise in GSHPx activity with ALDOST was observed in cardiac mitochondria (1.5 ± 0.15 vs. 2.7 ± 0.25 U/mg protein; $p < 0.05$), and was prevented with Spiro (1.3 ± 0.11 U/mg protein; $p < 0.05$) or Amlod (1.4 ± 0.18 U/mg protein; $p < 0.05$).

ALDOST markedly increased the levels of oxidized GSSG in mitochondria (2.89 ± 0.52 vs. 1.25 ± 0.12 nM/mg protein; $p < 0.05$) compared to controls. A similar trend was observed in cytosolic GSSG in cardiomyocytes (2.04 ± 0.31 vs. 1.57 ± 0.12 nM/mg protein). Cotreatment with either Spiro and Amlod attenuated the rise in GSSG in mitochondria (2.0 ± 0.13 nM/mg protein and 1.7 ± 0.21 nM/mg protein), respectively, but not in cardiomyocytes (1.97 ± 0.042 nM/mg protein and 1.91 ± 0.22 nM/mg protein, respectively). The GSH/GSSG ratio significantly decreased in mitochondria with ALDOST (16.3 ± 1.89 vs. 27.8 ± 1.72 ; $p < 0.05$) and was attenuated by Spiro and Amlod (20.1 ± 1.26 and 23.2 ± 0.85), respectively. However, as opposed to mitochondria, the GSH/GSSG ratio in cardiomyocytes did not change significantly with ALDOST, or cotreatment with Spiro or Amlod (data not shown).

DISCUSSION

We previously found a dyshomeostasis of extra- and intracellular Ca^{2+} in rats receiving ALDOST to be accompanied by the induction of oxidative stress in the heart (2,16,27). In yet other studies, we reported on the dyshomeostasis of extra- and intracellular Zn^{2+} , which is associated with an upregulation of antioxidant defenses in the heart (3-5). Herein, we hypothesized and provided *in vivo* experimental evidence supporting that intracellular dyshomeostasis of Ca^{2+} and Zn^{2+} involving cardiac myocytes and mitochondria in rats receiving ALDOST are intrinsically coupled that alter their redox state, wherein an elevation in intracellular Ca^{2+} acts as a prooxidant and Zn^{2+} as an antioxidant. To our knowledge, this

represents the first study to address whether iterations in cardiac myocyte and mitochondrial Ca^{2+} and Zn^{2+} are intrinsically coupled. Our study led to several novel findings.

Cardiomyocyte cytosolic free $[\text{Ca}^{2+}]_i$ found at wk 4 ALDOST was increased beyond the values seen in control cells. We interpret this finding to reflect a $[\text{Ca}^{2+}]_i$ overloading in keeping with the persistent elevations in plasma PTH we found throughout chronic ALDOST, and coincident with ongoing bone resorption (2,16). This well-established PTH-mediated $[\text{Ca}^{2+}]_i$ overloading, or Ca^{2+} paradox (7,8), that accompanies the hypocalcemia and SHPT of ALDOST can be averted by various interventions (2,16,26,27). Amlodipine abrogated the escalation in $[\text{Ca}^{2+}]_i$ that appeared with ALDOST, suggesting Ca^{2+} was derived from extracellular sources and that its influx occurred through LTCC. In adult cardiomyocytes, harvested from transgenic mice with renal salt wasting, where plasma ALDO is chronically elevated, Benitah et al. found an increased amplitude of the LTCC current, which correlated with plasma levels of this mineralocorticoid (32). We therefore cannot discount an increased LTCC density may contribute to PTH-mediated intracellular $[\text{Ca}^{2+}]_i$ overloading of cardiomyocytes in rats receiving ALDOST. ALDO selectively increases LTCC current amplitude in rat ventricular myocytes (33). The possibility that a certain population of cardiomyocytes is selectively vulnerable to $[\text{Ca}^{2+}]_i$ overloading and thereby necrosis, must be called into question based on their likelihood of having a higher density of LTCC. Increased $[\text{Ca}^{2+}]_i$ could also be explained by ROS-mediated plasmalemmal injury and consequent hyperpermeability (34). Also contributory to cardiomyocyte vulnerability would be selectively limited antioxidant defenses. It is tempting to speculate that such heterogeneity could account for the patchy scarring found throughout the right and left ventricles during ALDOST (13). Moreover, if the ascent in intracellular Ca^{2+} overloading is gradual over 4 wks ALDOST and involves a continual consumption of antioxidant defenses, cardiomyocyte necrosis and scarring would be delayed. Future studies will address each of these issues.

Another novel finding of our study was that the increase in $[\text{Ca}^{2+}]_i$ was accompanied by a concomitant rise in cardiomyocyte $[\text{Zn}^{2+}]_i$. This was only partially prevented by Amlod, which implicates other routes of Zn^{2+} entry must be operative, including Zn^{2+} -sensitive membrane transporters, such as Zip1 whose expression was upregulated at 4 wks ALDOST. Intracellular sources of Zn^{2+} may also be contributory, including Zn^{2+} bound to MT and Zn^{2+} released from mitochondria. In cultured noncardiomyocyte cells, ROS and RNS species promote nitric oxide generation from inducible nitric oxide synthase, which, in turn, provokes the release of Zn^{2+} from the Zn/MT complex (35-38). These *in vitro* studies suggest that nitric oxide regulates intracellular $[\text{Zn}^{2+}]_i$ homeostasis where the release and rise in $[\text{Zn}^{2+}]_i$ activates its sensor, MTF-1, leading to consequent MT gene expression. We cautiously interpret our previous finding of 3-nitrotyrosine in the heart at 4 wks ALDOST, together with our present findings, to reflect a coordinated expression amongst Zn^{2+} transporters, nitric oxide, and MT, which is orchestrated by the activation of MTF-1. This activation occurs in response to increased $[\text{Zn}^{2+}]_i$, where upon its translocation to the nucleus MTF-1 induces the transcriptional activation of ZnT-1 and MT genes. This will be addressed in future studies.

We found the rise in cardiomyocyte $[\text{Ca}^{2+}]_i$ and $[\text{Zn}^{2+}]_i$ at 4 wks ALDOST to be accompanied by increased $[\text{Ca}^{2+}]_m$. Mitochondria are an active intracellular storage site for Ca^{2+} ; large quantities of $[\text{Ca}^{2+}]_m$ can accumulate to regulate $[\text{Ca}^{2+}]_i$ homeostasis during the increased Ca^{2+} influx and cytosolic $[\text{Ca}^{2+}]_i$ overloading that occurs with SHPT, catecholamine excess, and ischemia/reperfusion injury (39). Uptake of Ca^{2+} by cardiac mitochondria likely involves the activation of an anion channel uniporter, located in the organelle's inner membrane, and the proteinaceous mitochondrial permeability transition pore (mPTP) whose Ca^{2+} conductance is increased by Ca^{2+} overloading and ROS (12,40-42). In future studies cotreatment with either 4-chlorodiazepam or cyclosporine A, specific inner membrane and pore inhibitors, respectively, may specifically address the role of each site in raising $[\text{Ca}^{2+}]_m$. The increase in

$[Ca^{2+}]_m$ during ALDOST was accompanied by a simultaneous rise in $[Zn^{2+}]_m$. Given the low cytosolic concentrations of Zn^{2+} (nanomolar to picomolar range) in cardiomyocytes, its mitochondrial uptake is likely to be facilitated. Metallothionein acts as a chaperone protein and donor of Zn^{2+} delivery and uptake to the inner mitochondrial membrane (43-45). In cardiac mitochondria the protein, aconitase, accepts Zn^{2+} directly from the Zn-MT complex (46). Chanoit et al. have shown that a Zn^{2+} supplement can prevent oxidant-induced mPTP opening, and thereby reduce the rate of cell death (47).

Taken together, our findings indicate the dyshomeostasis of intracellular Ca^{2+} and Zn^{2+} seen in cardiac myocytes and mitochondria at 4 wks ALDOST to be intrinsically coupled and linked to a concomitant induction of oxidative stress and generation of antioxidant defenses. In the heart, we previously reported $[Ca^{2+}]_i$ overloading and increased tissue levels of 8-isoprostane and MDA to be associated with increased activity of Cu/Zn-SOD and protein expression of MT-1 (3,4). In the present study, we broaden our findings to indicate increased $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ were coupled to the induction of oxidative stress, while the rise in $[Zn^{2+}]_i$ and $[Zn^{2+}]_m$ was accompanied by a simultaneous activation of MTF-1 and its induction of such antioxidants as MT-1 and GSHPx. The intrinsically coupled unidirectional entry of Ca^{2+} and Zn^{2+} synergistically alters the redox state of cardiac myocytes and mitochondria, and raises the prospect of whether an optimal $[Zn^{2+}]_i:[Ca^{2+}]_i$ ratio in cardiac myocytes and mitochondria must be preserved to combat oxidative stress. Based on their findings in isolated mitochondria and intact heart with ischemia/reperfusion injury, Aon et al. (48,49) have raised the prospect of a critical threshold beyond which mitochondria become dysfunctional and damaged, and which relates to ROS exceeding depleted antioxidant defenses in these organelles. Mitochondria are the primary source of ROS in cardiomyocytes vis-à-vis PBMC invading the myocardium at wk 4 ALDOST, where membrane-bound NADPH oxidase appears to be a dominant source of prooxidants (13).

One limitation to our study relates to our use of fluorescent indicators of $[Ca^{2+}]_i$ and $[Zn^{2+}]_i$, where possible leakage of indicator from the cells and intracellular organellar sequestration can occur. To avoid this shortcoming we minimized incubation time spent in loading cardiomyocytes and promptly conducted our subsequent measurements. We further acknowledge that a minor cross-reactivity of the fluorescent labeling can occur with $[Co^{2+}]_i$ and $[Mn^{2+}]_i$, however, the relative concentrations of these cations is abysmal compared to $[Zn^{2+}]_i$ and $[Ca^{2+}]_i$.

In closing, a schematic diagram depicting our current understanding of the coupled dyshomeostasis of extra- and intracellular Ca^{2+} and Zn^{2+} that occurs during aldosteronism is presented in Figure 6. This potential scenario encompasses a hitherto less well-appreciated reciprocal regulation of cardiac myocyte and mitochondrial redox state. We do not suggest this perspective overlooks the potential importance of a simultaneous imbalance in other divalent (Mg^{2+} and Se^{2+}), and monovalent (Na^+ and K^+) cations that could also occur with aldosteronism. These limitations of our paradigm notwithstanding, a unidirectional movement of Ca^{2+} and Zn^{2+} regulating the heart's redox state is brought to light and deserving of further in-depth investigation. The coupled Ca^{2+} and Zn^{2+} dyshomeostasis seen in aldosteronism is reminiscent of the Ca^{2+} overloading and oxidative stress that exists in the hearts of rodents with hereditary muscular dystrophy which is also accompanied by increased tissue Zn^{2+} (50-55). This divalent cation dyshomeostasis seen in muscular dystrophy can be prevented by parathyroidectomy, a Ca^{2+} channel blocker, or by a nitric oxide synthase antagonist. Furthermore, our findings with ALDOST resemble the protective role of increased $[Zn^{2+}]_i$ induced by a Zn^{2+} supplement or Zn^{2+} ionophore when intracellular $[Ca^{2+}]_i$ overloading of the heart accompanies diabetic cardiomyopathy or ischemia/reperfusion injury (56,57). We therefore would suggest the importance of therapeutic interventions that would uncouple Ca^{2+} and Zn^{2+} to change their intracellular ratio in favor of antioxidant defenses.

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Figure 1.

Intracellular levels of cytosolic free $[Ca^{2+}]_i$ and $[Zn^{2+}]_i$ found in cardiomyocytes harvested from: unoperated, untreated, age-/gender-matched Sprague-Dawley rats (controls); 4 wks aldosterone/salt treatment alone (ALDOST); 4 wks ALDOST plus cotreatment with either spironolactone (Spiro) or amlodipine (Amlod). ALDOST was associated with a significant rise in $[Ca^{2+}]_i$ and $[Zn^{2+}]_i$. The increase in $[Ca^{2+}]_i$ could be prevented by Spiro and Amlod cotreatment, whereas the rise in $[Zn^{2+}]_i$ was prevented by Spiro and attenuated by Amlod.

* $p < 0.05$ vs. control; † $p < 0.05$ vs. ALDOST.

Figure 2. Mitochondrial concentrations of $[Ca^{2+}]_m$ and $[Zn^{2+}]_m$ for the 4 groups of rats presented in Figure 1. * $p < 0.05$ vs. control; † $p < 0.05$ vs. ALDOST. A significant rise in $[Ca^{2+}]_m$ and $[Zn^{2+}]_m$ was seen at 4 wks ALDOST, which could be prevented by Spiro cotreatment and attenuated by Amlod.

Figure 3.

The expression of Zn transporters in cardiomyocytes seen for controls, 4 wks ALDOST (A) alone, and 4 wks ALDOST plus cotreatment with either Spiro or Amlod. The expression of the Zn^{2+} importer, Zip1, and exporter ZnT-1 was increased at 4 wks of aldosteronism, which was attenuated by cotreatment with Spiro, but not Amlod (* $p < 0.01$ vs. control).

Figure 4.

Western blot is shown together with quantitative expression of the metal-response element transcription factor (MTF)-1, which was increased at 4 wks ALDOST, prevented by Spiro cotreatment and attenuated by Amlod (* $p < 0.05$ vs. control).

Figure 5.

Mitochondrial concentration of a biomarker of lipid peroxidation, malondialdehyde (MDA), and an antioxidant, glutathione peroxidase (GSHPx), for the 4 groups shown in Figure 1. The appearance of oxidative stress at 4 wks ALDOST was coupled to a rise in antioxidant defense. Cotreatment with Spiro or Amlod prevented these responses and likely relates to their impact on increased $[Ca^{2+}]_m$ and $[Zn^{2+}]_m$, respectively.

Figure 6.

Our current understanding of the intrinsically coupled dyshomeostasis of Ca^{2+} and Zn^{2+} found in ALDOST. Increased excretory losses of these divalent cations lead to hypocalcemia and hypozincemia. Consequent secondary hyperparathyroidism with persistent elevations in circulating parathyroid hormone (PTH) are accompanied by uncontrolled Ca^{2+} entry via L-type Ca^{2+} channels (LTCC) to saturate intracellular binding and storage sites, and ultimately to $[\text{Ca}^{2+}]_i$ overloading and excessive Ca^{2+} sequestration within mitochondria. An induction of oxidative stress and generation of reactive oxygen species (ROS) ensues involving mitochondria. The rise in $[\text{Zn}^{2+}]_i$ and $[\text{Zn}^{2+}]_m$ involves increased Zn^{2+} entry via LTCC to a minor extent, while the majority of $[\text{Zn}^{2+}]_i$ is regulated by membrane-bound Zn transporters, including Zip1 and ZnT-1 and its binding to metallothionein (MT), which minimizes cytotoxicity.

ROS serve as signals that initiate an activation of molecular transducers: a) stress-response kinases, MAPKs, and their activation of redox-sensitive nuclear transcription factor (NF)- κB and a proinflammatory gene cascade it induces including adhesion molecules (ICAM-1), chemokines (MCP-1) and cytokines ($\text{TNF-}\alpha$ and $\text{TGF-}\beta$); and b) activation of inducible or endothelial nitric oxide synthases (iNOS and eNOS), where nitric oxide (NO) generation releases Zn^{2+} from the Zn/MT complex. Increased $[\text{Zn}^{2+}]_i$ serves to activate its sensor, metal-response element transcription factor (MTF)-1, and the antioxidant genes it induces, including MT-1, Cu/Zn-SOD, and glutathione (GSH) synthase. Increased $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_m$ are prooxidants, which in toxic amounts threaten the fate of cardiomyocytes, while increments in $[\text{Zn}^{2+}]_i$ and $[\text{Zn}^{2+}]_m$ inhibit apoptosis and necrosis.