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Cellular and molecular pathways to myocardial necrosis and replacement fibrosis

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Abstract Fibrosis is a fundamental component of the adverse structural remodeling of myocardium present in the failing heart. Replacement fibrosis appears at sites of previous cardiomyocyte necrosis to preserve the structural integrity of the myocardium, but not without adverse functional consequences. The extensive nature of this microscopic scarring suggests cardiomyocyte necrosis is widespread and the loss of these contractile elements, combined with fibrous tissue deposition in the form of a stiff in-series and in-parallel elastic elements, contributes to the progressive failure of this normally efficient muscular pump. Cellular and molecular studies into the signal-transducer-effector pathway involved in cardiomyocyte necrosis have identified the crucial pathogenic role of intracellular Ca^{2+} overloading and subsequent induction of oxidative stress, predominantly confined within its mitochondria, to be followed by the opening of the mitochondrial permeability transition pore that leads to the destruction of these organelles and cells. It is now further recognized that Ca^{2+} overloading of cardiac myocytes and mitochondria serves as a prooxidant and which is counterbalanced by an intrinsically coupled Zn^{2+} entry serving as antioxidant. The prospect of raising antioxidant defenses by increasing intracellular Zn^{2+} with adjuvant nutriceuticals can, therefore, be preferentially exploited to uncouple this intrinsically coupled Ca^{2+}–Zn^{2+} dyshomeostasis. Hence, novel yet simple cardioprotective strategies may be at hand that deserve to be further explored.

Keywords Hypocalcemia · Hypomagnesemia · Hypozincemia · Secondary hyperparathyroidism · Intracellular calcium overloading · Mitochondria · Oxidative stress · Aldosteronism

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Abbreviations

ACTH	Adrenocorticotropin hormone
ALDOST	Aldosterone/salt treatment
ANS	Adrenergic nervous system
CHF	Congestive heart failure
EICA	Excessive intracellular Ca^{2+} accumulation
GSH-Px	Glutathione peroxidase
HPA	Hypothalamic–pituitary–adrenal
I/R	Ischemia/reperfusion
ICAM	Intercellular adhesion molecule
MCP	Monocyte chemoattractant protein
mPTP	Mitochondrial permeability transition pore
MT-1	Metallothionein-1
MTF-1	Metal-responsive transcription factor-1
NFκB	Nuclear transcription factor-κB
PBMC	Peripheral blood mononuclear cells
PDTC	Pyrrolidine dithiocarbamate
PTH	Parathyroid hormone
RAAS	Renin–angiotensin–aldosterone system

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SHPT	Secondary hyperparathyroidism
SOD	Superoxide dismutase
TNF	Tumor necrosis factor

Introduction

Fibrosis, including microscopic scarring, is a fundamental component of the adverse structural remodeling found in the myocardium of the failing human heart [1, 2]. Scarring, a morphologic footprint of earlier cardiomyocyte necrosis, serves to replace lost contractile cells and thereby plays a vital role in preserving myocardial structure and function. The extensive distribution of this replacement fibrosis suggests a widespread and ongoing necrosis of cardiomyocytes. Apoptosis also occurs in the failing heart, but to a lesser extent, often involving such noncardiomyocytes as macrophages and endothelial cells [3]. Furthermore, programmed cell death begets neither inflammatory cells nor fibroblast responses. As a consequence, fibrous tissue does not appear at the site of lost myocytes and, therefore, apoptosis has been referred to as a sterile form of cell death [4, 5]. The cumulative loss of contractile elements, together with the deposition of fibrous tissue, stiff in-series and in-parallel elastic elements composed primarily of type I fibrillar collagen having the tensile strength of steel, each contributes to the progressive failure of this previously efficient muscular pump during systolic and/or diastolic phases of the cardiac cycle [6].

Elevations in serum troponins, which do not stem from an acute coronary event or significant renal dysfunction, that compromises urinary troponin excretion have been reported in patients hospitalized because of the symptoms and signs of congestive heart failure (CHF) [7–15]. This would be consistent with an ongoing necrotic loss of cardiomyocytes. Coronary microembolization with microinfarcts could be held responsible for the rise in serum troponins [16]. We speculate neurohormonal activation could be another plausible mechanism. In either case, these serum-derived biomarkers of cardiomyocyte necrosis are associated with increased in-hospital and overall cardiac morbidity and mortality. Insights into cellular and molecular pathways involved in ongoing cardiomyocyte necrosis are quintessential in developing novel cardioprotective strategies that would salvage them and prevent myocardial fibrosis.

The failing heart and congestive heart failure syndrome

The heart fails as the contractility of this muscular pump declines and it becomes unable to propel sufficient amounts of blood and nutrients into the circulation for their delivery to metabolizing tissues at a rate commensurate with their prevailing metabolic demands. Likewise, the heart also fails when its ability to receive blood during diastolic filling is compromised by abnormalities in myocardial relaxation and/or tissue stiffness, and consequently atrial and ventricular filling pressures are inappropriately elevated and the displacement of blood is suboptimal due to the inadequately stretched ventricle. Systemic blood flow can, therefore, be impaired by ventricular dysfunction in either systole or diastole. In turn, renal perfusion is correspondingly reduced setting the primary prerequisite for the appearance of CHF.

In order to sustain a steady-state filtration fraction at equilibrium over a wide range of conditions where the intake of water is either plentiful or limited, renal blood flow normally constitutes 20% of the cardiac output. However, when renal perfusion is reduced, juxtaglomerular cells elaborate renin leading to an ensuing activation of the renin–angiotensin–aldosterone system (RAAS). A concomitant activation of the hypothalamic–pituitary–adrenal (HPA) axis stimulates the adrenergic nervous system (ANS) and release of catecholamines. The HPA axis also contributes to adrenocorticotropin (ACTH)-mediated release of glucocorticoids and mineralocorticoids by the adrenal glands. Activation of the RAAS and ANS normally involves short-term, physiologic homeostatic responses to reduced dietary Na^+ intake or intravascular volume contraction. In the patient with heart failure, however, sustained homeostatic neurohormonal responses beget dyshomeostasis, which over time is expressed as an inappropriate (relative to dietary Na^+ and intravascular volume) salt-avid state. The accompanying retention of salt and water, and subsequent expansion of intra- and later extravascular volumes, leads to the symptoms and signs of the clinical syndrome CHF. CHF occurs when these homeostatic responses are inappropriate and persistent. Treatment of the decompensated, often-hospitalized, patient with CHF will restore euvolemia and ameliorate their signs and symptoms returning them to a state of compensation. Episodes of neurohormonal activation that beget CHF disrupt the stable state of compensation and as such represent *an acute stressor state*, which proves integral to the pathologic remodeling of myocardium. Less well recognized are adverse consequences of effector hormones of the RAAS and ANS that extend beyond salt and water retention to involve a dyshomeostasis of several cations, including K^+, Ca^{2+}, Mg^{2+}, and Zn^{2+}, which have been shown to adversely influence intracellular homeostasis and survival of cardiomyocytes [17–21].

Acute stressor state

Bodily injury, such as accompanies burns, head trauma, subarachnoid hemorrhage, or acute myocardial infarction,

represents a hyperadrenergic acute stressor state with marked elevations in circulating catecholamines. Acting through $\beta 2$ receptors, epinephrine and norepinephrine promote the translocation of K^+ from blood into the intracellular compartment of various cells, including cardiac myocytes and peripheral blood mononuclear cells (PBMC), leading to hypokalemia [22–24]. Likewise, plasma-ionized hypocalcemia and hypomagnesemia, and hypozincemia are common findings at the time of or soon after admission in patients hospitalized in the medical/surgical intensive care unit or the coronary care unit [25, 26]. The magnitude to which their respective plasma concentrations fall correlates well with the severity of injury, and therefore they may have predictive roles as relevant clinical biomarkers of poor prognosis. Necrosis of cardiomyocytes, with an elevation in serum troponins (albeit modest vis-à-vis myocardial infarction), occurs in response to the marked rise in circulating catecholamines and which can be ascertained postmortem from the morphologic evidence of contraction band necrosis and microscopic scarring of the right and left heart.

Acute stressor state: an animal model

The cellular and molecular pathways leading to this adverse myocardial remodeling during the systemic hyperadrenergic state have been investigated in animal models. In rodents, the administration of a single dose of a catecholamine leads to acute intracellular Ca^{2+} overloading of cardiac myocytes and mitochondria, as well as the concomitant generation of reactive oxygen and nitrogen species that overwhelm antioxidant defenses [27]. Cardiomyocyte necrosis, for example, occurs within hours of isoproterenol infusion and can be prevented by cotreatment with either a β_1 adrenergic receptor antagonist or Ca^{2+} channel blocker [20, 21, 28]. The induction of oxidative stress that accompanies consequent excessive intracellular Ca^{2+} accumulation (EICA) begins in mitochondria with the necrotic cell death pathway initiated by the opening of the mitochondrial permeability transition pore (mPTP) regulated by cyclophilin D [21, 27, 29, 30]. Cyclophilin D, a prolyl isomerase located within the matrix of mitochondria, is required for mediating EICA- and oxidative stress–induced cardiomyocyte necrosis (vis-à-vis apoptosis). Its pathophysiologic significance has been underscored by cyclophilin D null mice which are protected from cardiomyocyte necrosis due to ischemia/reperfusion (I/R) injury, including Ca^{2+} overloading and oxidative damage. Contrariwise, cyclophilin D-overexpressing mice have spontaneous cardiomyocyte necrosis with mitochondrial swelling [31, 32]. Inhibitors of mPTP opening of cyclophilin D have each been proven cardioprotective in the isoproterenol-induced experimental model, as well as in I/R-induced injury, another example of acute EICA [33–35].

Chronic stressor state

Persistent neurohormonal activation involving the RAAS and ANS occurs with protracted CHF, especially in newly diagnosed and previously untreated patients or those who have discontinued taking their medications, or in whom the dosage of pharmacologic agents interfering with these effector hormones is suboptimal. A dyshomeostasis of divalent cations is frequently found in patients hospitalized with decompensated biventricular failure due to a dilated (idiopathic) cardiomyopathy [36–45]. These include plasma-ionized hypocalcemia and hypomagnesemia that account for secondary hyperparathyroidism (SHPT) and elevated plasma parathyroid hormone (PTH) levels, together with hypovitaminosis D that further compromises Ca^{2+} balance, hypozincemia, and hyposelenemia. Systemic evidence of oxidative stress, expressed as elevated plasma malondialdehyde and 8-isoprostane, has also been reported in patients with CHF [46–48].

Chronic stressor state: an experimental model

Eight-week-old male Sprague–Dawley rats are uninephrectomized, followed by the implantation of an osmotic minipump containing aldosterone. Infusion of aldosterone (0.75 μg/h) raises their plasma levels to those found in CHF which are inappropriate relative to dietary Na^+ intake. Drinking water is fortified with 1% NaCl and 0.4% KCl to prevent hypokalemia. At week 1 of aldosterone/salt treatment (ALDOST), animals are clinically healthy and the myocardium appears normal by light microscopy. This *preclinical stage* gives way to a *clinical stage* with anorexia and a failure to gain weight at week 2 and beyond. Cardiac pathology first appears at week 4. This *pathologic stage* features microscopic scarring scattered throughout both the right and left atria and ventricles [49]. A perivascular/interstitial fibrosis involving the coronary, renal, and mesenteric circulations is also found. This topic has been reviewed extensively elsewhere [50, 51].

A series of studies have addressed the relevance of hemodynamic factors that could potentially contribute to cardiac fibrosis and have concluded those are not directly involved (reviewed in [52]). This viewpoint was primarily based on: (a) the presence of fibrosis in nonpressure-overloaded right atria and ventricle; (b) the absence of fibrosis when the LV pressure overload is created by infrarenal aortic banding without subsequent RAAS activation or when treatment is based on aldosterone together

with a low-Na^+ diet, or when 1% NaCl alone is given; (c) the prevention of fibrosis with either a small (nondepressor) or large (depressor) dose of spironolactone, which respectively fails to or does prevent hypertension; (d) an intracerebroventricular infusion of a mineralocorticoid receptor antagonist that prevents hypertension, but not fibrosis [53]; and (e) when a cardiospecific upregulation of aldosterone synthase accounts for increased tissue levels of aldosterone unaccompanied by cardiac fibrosis [54]. Thus, the evidence gathered to date indicates the adverse remodeling of myocardium during ALDOST is both (a) independent of hypertension and (b) unrelated to plasma or tissue-derived aldosterone per se, but some other circulating factor that accompanies aldosteronism (vide infra).

Cellular and molecular pathways leading to cardiomyocyte necrosis

Pathways accounting for cardiomyocyte necrosis and subsequent scarring of myocardium found at 4 weeks ALDOST have been examined and the identity and pathogenic role of circulating factors elucidated.

Oxidative stress

Evidence of oxidative stress in the myocardium is found during chronic mineralocorticoidism [55–59]. This includes (a) the presence of 3-nitrotyrosine, a byproduct of the reaction involving superoxide and nitric oxide; (b) an activation of the $gp91^{phox}$ subunit of NADPH oxidase found in inflammatory cells invading the injured myocardium that contributes to superoxide generation; (c) upregulated redox-sensitive nuclear transcription factor (NF)-κB and a proinflammatory gene cascade it regulates that includes intercellular adhesion molecule (ICAM)-1, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF)-alpha; and (d) increased tissue levels of 8-isoprostane and malondialdehyde, biomarkers of lipid peroxidation [58, 59]. There is also considerable evidence of oxidative stress in blood and urine consistent with the systemic nature of an altered redox state during chronic aldosteronism.

Intracellular Ca^{2+} overloading

Our hypothesis for the induction of oxidative stress during ALDOST would draw upon Albrecht Fleckenstein's original concept that intracellular Ca^{2+} overloading of the heart is an integral and adverse pathophysiologic feature leading to myocardial necrosis [60]. In rats receiving 1 and 4 weeks ALDOST, we monitored intracellular Ca^{2+} levels in several tissues that included the heart and PBMC. We found increased Ca^{2+} levels in the myocardium and PBMC during preclinical, clinical, and pathologic stages, accompanied by biomarker evidence of oxidative stress that included increased levels of malondialdehyde and 8-isoprostane in the heart and increased H_2O_2 production by PBMC [59, 61–63].

Calcium and magnesium dyshomeostasis

Why intracellular Ca^{2+} overloading occurred during ALDOST was next addressed. Elevations in dietary Na^+, albeit modest but inappropriate during ALDOST, are accompanied by increased tubular Na^+ and, in turn, elevated urinary concentrations of Ca^{2+} and Mg^{2+}. Aldosterone promotes epithelial cell Na^+ channel-mediated Na^+ reabsorption without influencing Ca^{2+} and Mg^{2+} excretion, which in turn accounts for the marked urinary losses of Ca^{2+} and Mg^{2+} [61]. A similar scenario unfolds in the Na^+ channels of the colon's epithelial cells that represent another site of high-density aldosterone receptor binding. The fecal excretion of Ca^{2+} and Mg^{2+}, in fact, is many-fold greater than their urinary losses [61].

Secondary hyperparathyroidism (SHPT)

Metabolic studies accounted for the marked increase in urinary and fecal excretion of Ca^{2+} and Mg^{2+} during ALDOST, which leads to plasma-ionized hypocalcemia and hypomagnesemia. The calcium-sensing receptor of the parathyroid glands, in turn, responds to hypocalcemia with increased secretion of PTH. Accordingly, plasma PTH levels rise [61] and SHPT is evidenced by marked and progressive reductions in bone mineral density and bone strength [64]. We therefore hypothesized the intracellular Ca^{2+} overloading and induction of oxidative stress that accompanies ALDOST leading to cardiomyocyte necrosis, and scarring is mediated by the calcitropic hormone, PTH and not aldosterone (see Fig. 1). It represents an example of the Ca^{2+} paradox associated with SHPT as characterized by Fujita and Palmieri [65]. Furthermore, PTH-mediated intracellular Ca^{2+} overloading is coupled to an induction of oxidative stress in diverse tissues that includes cardiomyocytes and their mitochondria, as well as PBMC. The generation of reactive oxygen (ROS) and nitrogen (RNS) species appear to overwhelm their rate of detoxification by the cumulative capacity of antioxidant defenses. In mitochondria, Ca^{2+} overloading and oxidative stress lead to a nonphysiologic opening of the mPTP, with the ensuing osmotic-based structural and functional degeneration of these organelles that triggers the downhill final common cell death pathway leading to cardiomyocyte necrosis and subsequent replacement fibrosis [66].

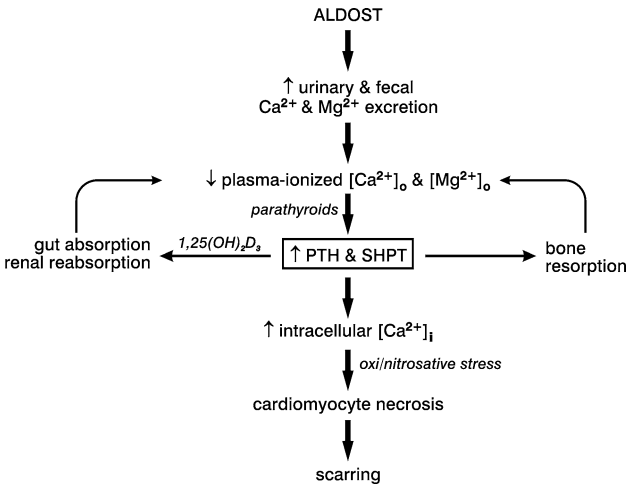

Fig. 1 The appearance of secondary hyperparathyroidism (*SHPT*) during aldosterone/salt treatment (*ALDOST*) is associated with increased excretory losses of Ca^{2+} and Mg^{2+}, and the consequent appearance of plasma-ionized hypocalcemia and hypomagnesemia. Elevations in parathyroid hormone (*PTH*) seek to restore extracellular homeostasis of these divalent cations through their resorption from bone, absorption from gut, and reabsorption by kidney promoted by the steroid hormone $1,25(OH)_2D_3$. Paradoxically, PTH elaboration is responsible for intracellular Ca^{2+} overloading and the induction of oxi-/nitrosative stress, which leads to cardiomyocyte necrosis and consequent replacement fibrosis, or scarring. Adapted from Alsafwah et al. [115]

A series of site-directed, sequential pharmacologic interventions targeted along the cellular–molecular cascades to block downstream events leading to cardiomyocyte necrosis and myocardial scarring were conducted. They collectively validated our hypothesis regarding the pathologic sequelae of events leading to this structural remodeling of myocardium in rats with chronic aldosteronism. These interventions included (a) cotreatment with spironolactone, an aldosterone receptor antagonist that attenuated the enhanced urinary and fecal losses of these cations to prevent hypocalcemia and hypomagnesemia and thereby ensuing SHPT [61]; (b) cotreatment with a Ca^{2+}- and Mg^{2+}-supplemented diet, together with vitamin D_3 to enhance Ca^{2+} absorption, which prevented hypocalcemia and SHPT [67]; (c) parathyroidectomy, performed prior to starting ALDOST, to prevent SHPT [68]; (d) cotreatment with cinacalcet, a calcimimetic that resets the threshold of the parathyroid glands' Ca^{2+}-sensing receptor to prevent SHPT despite modest hypocalcemia [69]; (e)cotreatment with amlodipine, a Ca^{2+} channel blocker, which prevents intracellular Ca^{2+} overloading [62]; and finally (f) cotreatment with N-acetylcysteine, an antioxidant that abrogated oxidative stress [58].

Thus, taken together, the multitude of evidence gathered to date congruently supports the mechanism of PTH-mediated intracellular Ca^{2+} overloading that leads to the induction of oxidative stress during aldosteronism where ROS and RNS, primarily derived from mitochondria, overwhelm cellular antioxidant defenses. This scenario anticipates whether the overall consequence of an excessive generation of prooxidants or cumulative endogenous antioxidant defenses in combating ROS and RNS had been compromised. In this context, we next addressed plausible association of Zn^{2+} dyshomeostasis during ALDOST given its importance to these endogenous defenses, including Cu/Zn-superoxide dismutase (SOD).

Zinc dyshomeostasis

Chronic inappropriate excess of aldosterone is accompanied by increased urinary and fecal excretory Zn^{2+} losses, hypozincemia, and a fall in plasma Cu/Zn-SOD activity [70]. The hyperzincuria seen with ALDOST is related to urinary acidification, which contributes to the consequent metabolic alkalosis of aldosteronism [59]. Also contributory to hypozincemia is a coordinated selective translocation of Zn^{2+} to the sites of tissue injury, facilitated by corresponding upregulation of a Zn^{2+}-binding protein, metallothionein (MT)-1 in targeted tissues [59, 70].

We also used a ^{65}Zn tracer to systematically monitor Zn^{2+} kinetics during 1 and 4 weeks of ALDOST. A simultaneous fall in plasma ^{65}Zn and a selective accumulation of ^{65}Zn was found at sites of injury that included its translocation to recently incised skin at week 1 used for osmotic minipump implantation, as well as the injured heart and kidneys at week 4. This intracellular trafficking of ^{65}Zn to injured tissues was facilitated by the upregulation of MT-1 [71]. However, at week 4, there was a decline in ^{65}Zn in healed skin and bone, which serve as Zn^{2+} reservoirs and participate in resolving acute hypozincemia. Thus, the preferential translocation of circulating Zn^{2+} to injured tissues contributes to hypozincemia found with ALDOST, where increased tissue Zn^{2+} is essential in wound healing at these sites [72]. Since dyshomeostasis of Zn^{2+} proved to be another integral feature of aldosteronism, it became crucial to investigate whether the rise in myocardial tissue Zn^{2+} involved both its cardiac myocytes and mitochondria.

Zinc and antioxidant defenses

Cardiac myocytes and mitochondria were harvested from rats with 4 weeks of ALDOST, involving the pathologic stage, as well as from experimental controls. We found increased cytosolic free $[Zn^{2+}]_i$ in cardiac myocytes and total Zn^{2+} concentration in mitochondria [59]. The rise in cardiomyocyte Zn^{2+} was facilitated by the increased expression of membranous Zn^{2+} transporters. Increased $[Zn^{2+}]_i$ serves to augment the antioxidant defenses of cardiomyocytes, including their upregulation of MT-1 and

activation of metal-responsive transcription factor (MTF)-1, which encodes genes related to various antioxidant defenses, such as Cu/Zn-SOD, MT-1, and glutathione synthase. Thus, intracellular Zn^{2+} loading in chronic aldosteronism is contemporaneous with intracellular Ca^{2+} overloading and relevant biomarkers of oxidative stress [73]. Pathophysiologically and in terms of innate redox states, Zn^{2+} serves as antioxidant and Ca^{2+} as prooxidant in our experimental model. This concept offers the prospect of exploiting Zn^{2+} supplementation as a novel therapeutic strategy to uncouple the intrinsically coupled Ca^{2+} and Zn^{2+} dyshomeostasis in favor of increasing $[Zn^{2+}]_i$, thus enhancing the overall endogenous antioxidant defense capacity and attenuating adverse myocardial remodeling.

The efficacy of a Zn^{2+} supplement in augmenting intracellular $[Zn^{2+}]_i$, and thereby antioxidant defenses, in rats receiving ALDOST was examined using $ZnSO_4$. Cotreatment of ALDOST rats with $ZnSO_4$ prevented hypozincemia and a fall in plasma Cu/Zn-SOD activity, while significantly increasing cardiomyocyte cytosolic $[Zn^{2+}]_i$. It attenuated biomarkers of oxidative stress, such as cardiac 8-isoprostane, and microscopic scarring [59]. Thus, increased tissue Zn^{2+} in the heart serves as an antioxidant, and intracellular Ca^{2+} overloading as prooxidant, with cardiomyocyte necrosis that highlights the intrinsic codependency of these two biologically essential and dynamic divalent cations (vide infra). Others have also reported a Zn^{2+} supplement to be cardioprotective in mice with streptozocin-induced diabetic cardiomyopathy, in rat hearts with ischemia/reperfusion injury or following isoproterenol administration [28, 33, 59, 74].

Coupled Ca^{2+} and Zn^{2+} dyshomeostasis

The dyshomeostasis of extra- and intracellular Ca^{2+} and Zn^{2+} that accompanies ALDOST contributes to a deleterious but reversible dysequilibrium between pro- and antioxidants. We hypothesized that intrinsic coupling of intracellular Ca^{2+} and Zn^{2+} dyshomeostasis regulates the redox state of cardiac myocytes and mitochondria. To test our hypothesis, we monitored each of these two cations using relevant fluorescent tags and fluorescence microscopy in cardiac myocytes and mitochondria harvested from rats receiving 4 weeks ALDOST alone, or in combination with spironolactone or amlodipine cotreatment. Compared to untreated, age-/sex-matched controls, we found (see Fig. 2) increased cardiomyocyte cytosolic free $[Ca^{2+}]_i$ and $[Zn^{2+}]_i$, together with increased mitochondrial $[Ca^{2+}]_m$ and $[Zn^{2+}]_m$, each of which could be prevented by spironolactone and attenuated by amlodipine cotreatment [73]. These iterations in divalent cation composition were accompanied by increased levels of 3-nitrotyrosine and 4-hydroxy-2-nonenal in cardiomyocytes, together with

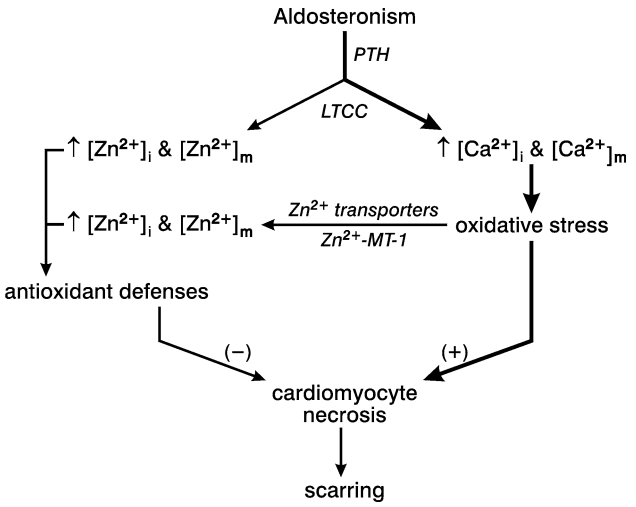

Fig. 2 Our current understanding of the pathways involving intrinsically coupled dyshomeostasis of Ca^{2+} and Zn^{2+} found in ALDOST. Increased excretory losses of these divalent cations lead to hypocalcemia and hypozincemia. Consequent secondary hyperparathyroidism with persistent elevations in circulating 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 intracellular $[Ca^{2+}]_i$ overloading and excessive Ca^{2+} sequestration within mitochondria. An induction of oxidative stress and generation of reactive oxygen species (*ROS*) ensue involving mitochondria. The rise in $[Zn^{2+}]_i$ and $[Zn^{2+}]_m$ involves increased Zn^{2+} entry via LTCC to a minor extent, while the majority of $[Zn^{2+}]_i$ is regulated by membrane-bound Zn transporters, including importers and exporters, Zip1 and ZnT-1, respectively, and its binding to metallothionein (MT)-1 to minimize cytotoxicity. Reprinted from Kamalov et al. [66]

increased H_2O_2 production, malondialdehyde and oxidized glutathione in mitochondria that were coincident with increased activities of Cu/Zn-SOD and glutathione peroxidase (GSH-Px) [59, 66, 73]. Furthermore, alterations in intracellular $[Zn^{2+}]_i$ were accompanied by the contemporaneous upregulation of MT-1, a Zn^{2+} importer and exporter (Zip1 and ZnT-1, respectively) and MTF-1.

Thus, in cardiac myocytes and mitochondria, an intrinsically coupled dyshomeostasis of intracellular Ca^{2+} and Zn^{2+} serves to regulate the redox state via induction of oxidative stress and generation of antioxidant defenses, respectively. These findings underscore the clinical relevance of both pharma- and nutriceutical strategies that can uncouple the coupled dyshomeostasis of these biologically essential cations and modulate them in favor of sustained antioxidant defenses. The coupled Ca^{2+} and Zn^{2+} dyshomeostasis seen in aldosteronism resembles the Ca^{2+} overloading and oxidative stress that exists in the hearts of hamsters with hereditary muscular dystrophy which is also accompanied by increased tissue Zn^{2+} [75–79]. This divalent cation dyshomeostasis seen in muscular dystrophy could be prevented by parathyroidectomy or a Ca^{2+} channel blocker. 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 is present [80].

The temporal response to coupled Ca^{2+} and Zn^{2+} dyshomeostasis

Intracellular $[Ca^{2+}]_i$ overloading, coupled with the induction of oxidative stress, is present at 4 weeks ALDOST. This prooxidant reaction in cardiac myocytes and mitochondria accounts for necrotic cell death and subsequent myocardial scarring. The rise in $[Ca^{2+}]_i$, a prooxidant, is intrinsically linked to increased $[Zn^{2+}]_i$ serving as antioxidant. We addressed the temporal responses in coupled Ca^{2+} and Zn^{2+} dyshomeostasis, reflecting the prooxidant/antioxidant equilibrium, by examining preclinical and pathologic stages of ALDOST and by observing whether endogenous antioxidant defenses were ultimately overwhelmed accounting for the delay in cardiac remodeling. Responses in cardiomyocyte free $[Ca^{2+}]_i$ and $[Zn^{2+}]_i$ and mitochondrial total $[Ca^{2+}]_m$ and $[Zn^{2+}]_m$, together with biomarkers of oxidative stress and antioxidant defenses, during 1 and 4 weeks ALDOST were monitored and compared. At week 1 and compared to controls, we found (i) elevations in $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ to be coupled with $[Zn^{2+}]_i$ and $[Zn^{2+}]_m$; (ii) increased mitochondrial H_2O_2 production, cardiomyocyte xanthine oxidase activity, and cardiac and mitochondrial 8-isoprostane levels, counterbalanced by increased activity of antioxidant proteins, enzymes, and the nonenzymatic antioxidants that can be considered as cumulative antioxidant capacity. Some of these enzymes and proteins (e.g., metallothionein-1, Cu/Zn-superoxide, glutathione synthase) are regulated by MTF-1; and (iii) although these augmented antioxidant defenses were sustained at week 4, overall they fell short in combating the persistent intracellular Ca^{2+} overloading together with a marked rise in cardiac tissue 8-isoprostane and mPTP opening.

Thus, the intrinsically coupled Ca^{2+} and Zn^{2+} dyshomeostasis occurs early during ALDOST in cardiac myocytes and mitochondria that regulate redox equilibrium until week 4, when ongoing intracellular Ca^{2+} overloading and the accelerated rate of prooxidant generation overwhelm their rate of detoxification by antioxidant defenses. These observations support our contention that intracellular $[Ca^{2+}]_i$ overloading accounts for the induction of oxidative stress that leads to necrotic cell death and consequent replacement fibrosis or myocardial scarring.

Uncoupling the coupled dyshomeostasis of Ca^{2+} and Zn^{2+}

The prooxidant response to Ca^{2+} overloading in cardiac myocytes and mitochondria has been shown to be intrinsically coupled to simultaneous increased Zn^{2+} entry serving as an antioxidant [73]. Later, we investigated whether Ca^{2+} and Zn^{2+} dyshomeostasis and prooxidant/antioxidant dysequilibrium seen at 4 weeks, the pathologic stage of ALDOST, could be uncoupled in favor of antioxidants, using cotreatment with a $ZnSO_4$ supplement, pyrrolidine dithiocarbamate (PDTC), a Zn^{2+} ionophore, or $ZnSO_4$ in combination with amlodipine. Responses in cardiomyocyte free $[Ca^{2+}]_i$ and $[Zn^{2+}]_i$, together with biomarkers of oxidative stress in cardiac myocytes and mitochondria, were monitored and statistically contrasted. At week 4 ALDOST and compared to controls, we found (i) an elevation in $[Ca^{2+}]_i$ was coupled with $[Zn^{2+}]_i$ and (ii) increased mitochondrial H_2O_2 production, and increased mitochondrial and cardiac 8-isoprostane levels. Cotreatment with the $ZnSO_4$ supplement alone, PDTC alone, or $ZnSO_4$+ amlodipine augmented the rise in cardiomyocyte $[Zn^{2+}]_i$ beyond that seen with ALDOST alone, while attenuating the rise in $[Ca^{2+}]_i$ which together served to reduce oxidative stress. Furthermore, $ZnSO_4$, PDTC, and $ZnSO_4$ + amlodipine were cardioprotective attenuating myocardial fibrosis [58, 59, 80].

Thus, the intrinsically coupled dyshomeostasis of intracellular Ca^{2+} and Zn^{2+} found in cardiac myocytes and mitochondria during 4 weeks ALDOST could be uncoupled in favor of antioxidant defenses by selectively increasing free $[Zn^{2+}]_i$ and/or reducing $[Ca^{2+}]_i$ using cotreatment with $ZnSO_4$ or PDTC alone or $ZnSO_4$+ amlodipine in combination. Each intervention proved to be cardioprotective at varying degrees. These cumulative salutary observations raise the therapeutic prospect that nutriceuticals capable of influencing extra- and intracellular Ca^{2+} and Zn^{2+} balance could prevent cardiac myocyte necrosis and myocardial scarring.

Translational research: divalent cation dyshomeostasis in human CHF

The secondary aldosteronism of CHF is accompanied by ionized hypocalcemia with SHPT [36–38, 45, 81]. As noted earlier, dyshomeostasis of divalent cations frequently occurs in patients hospitalized with decompensated biventricular failure due to a dilated (idiopathic) cardiomyopathy. Elevated serum PTH with SHPT is an established feature of primary and secondary aldosteronism [82]. Increased PTH levels found in patients with primary aldosteronism can be reduced by either an aldosterone receptor antagonist, spironolactone, or by adrenal surgery which also corrects ionized hypocalcemia [82]. SHPT is especially prevalent in African-Americans (AA) with protracted decompensated biventricular failure, where chronic elevations in plasma aldosterone account for symptoms and

signs of CHF [38]. This is also related to the prevalence of hypovitaminosis D in AA with CHF [38]. The increased melanin content of darker skin in AA serves as a natural sunscreen. Accordingly, the prevalence of hypovitaminosis D, often of marked severity, compromises Ca^{2+} homeostasis predisposing AA patients to hypocalcemia and consequent SHPT [38, 83, 84]. Hypomagnesemia is another common clinical feature of aldosteronism, which too is corrected by spironolactone or adrenal surgery [85–87]. In addition to the ionized hypocalcemia and hypomagnesemia that accompany increased urinary and fecal losses of these divalent cations, other studies have now identified a concomitant dyshomeostasis of Zn^{2+} with hypozincemia [88, 89]. Urinary Zn^{2+} excretion is increased in response to angiotensin-converting enzyme inhibitor or angiotensin receptor antagonist [90, 91].

We documented for the first time serum Zn^{2+} and Se^{2+} levels to be reduced in our AA patients followed here in Memphis [36, 37]. This included those with decompensated failure and compensated failure, as well as with heart disease but not heart failure. Reasons for the deficiency of these divalent cations, including their reduced dietary intake, are under investigation. Tennessee soil is not deficient in these minerals. Se is an essential trace mineral and cofactor of antioxidant selenoenzymes, such as GSH-Px and thioredoxin reductase, that promote optimal antioxidant/oxidant balance [92]. Furthermore, a Se-deficient diet is associated with a diminished activation potential of NFκB and downregulation of Se-GSH-Px activity, which detoxifies peroxides and hydroperoxides in such diverse tissues as kidney, cardiovasculature, and red cells [93–98], which is readily reversible when dietary Se is adequately fortified [96, 97, 99]. Monitoring serum Se, Se-dependent enzyme activities, and Se-GSH-Px mRNA expression are clinically useful in addressing optimal Se supplementation [100, 101].

There is an appearance of a dilated cardiomyopathy in greater abundance in general populations where dietary Se^{2+} deficiencies are found, such as in the Se-poor soil of the Keysan Province of China, or when parenteral nutrition is deficient in Zn and/or Se, [102–104]. It has been suggested that the myopathic process in these scenarios is more closely related to altered immunocompetence and predisposition to viral pathogens than a direct effect of the specific trace mineral deficiencies on the myopathic heart. However, this is arguable. A redistribution of Se from the vascular compartment into tissues that may occur in chronic illness has also been suggested as contributory to the genesis of a dilated (idiopathic) cardiomyopathy [105–109].

Therefore, the metabolic–hormonal profile of CHF with aldosteronism depicts a concerted and contemporaneous dyshomeostasis of multiple nutrients that include Ca^{2+}, Mg^{2+}, Zn^{2+}, and Se^{2+}. Vitamin D deficiency is another crucial and common confounding variable. Other factors which may relate to compromised Ca^{2+} stores and contribute to the appearance of SHPT in AA with CHF include reduced dietary Ca^{2+} intake because of lactose intolerance and an active avoidance of dairy products rich in Ca^{2+} [110] and a preference for a high Na^{+} diet that enhances urinary Ca^{2+} excretion. A high-salt diet and consequential calciuria is well known for predisposing a patient to ionized hypocalcemia and SHPT with a resorption of bone which is invoked to restore extracellular Ca^{2+} homeostasis. Over time, osteopenia and osteoporosis accompany the calciuria of long-term dietary Na^{+} excess further predisposing them to atraumatic bone fractures [111, 112]. The risk of such fractures is now shown to be increased in patients with heart failure and appears to be preventable by spironolactone together with today's standard of care [113, 114].

Summary

Previous myocardial infarction, hypertensive heart disease, or a dilated (idiopathic) cardiomyopathy may each contribute to the heart's failure as a muscular pump that is perpetuated by a sporadic and progressive necrosis of cardiomyocytes, replaced by fibrous tissue, and promoted by inappropriate neurohormonal activation and effector hormones of the RAAS and ANS. The hyperadrenergic, acute stressor state in CHF is accompanied by a translocation of K^{+}, Ca^{2+}, Mg^{2+}, and Zn^{2+} from the vascular to intracellular compartment. In the case of cardiac myocytes and mitochondria, intracellular Ca^{2+} overload and the induction of oxidative stress leads to mPTP opening, organellar degeneration, cardiomyocyte necrosis with myocardial fibrosis and subsequent scarring. A similar signal-transducer-effector pathway is at play with the chronic stressor state of aldosteronism where inappropriate elevations in plasma aldosterone, together with the heightened excretory losses of Ca^{2+} and Mg^{2+}, lead to resultant hypocalcemia and hypomagnesemia and each contributes to the appearance of SHPT. Elevations in plasma PTH seek to restore extracellular homeostasis of these divalent cations through their resorption from bone. At the same time, PTH-mediated intracellular Ca^{2+} overloading becomes highly prevalent in affected cardiac myocytes and mitochondria. This Ca^{2+} paradox accounts for an induction of oxidative stress, leading to cardiomyocyte necrosis and subsequent reparative fibrosis. Fibrosis contributes to the adverse structural remodeling of the right and left heart with its attending pathologic influences on myocardial stiffness and contractility while it also serves as substrate for re-entrant arrhythmias. An intrinsically

coupled dyshomeostasis of Zn^{2+} and Se^{2+} is also seen, which compromises antioxidant defenses.

Hence, several pathways are involved in the necrosis of cardiomyocytes and appearance of cardiac fibrosis. Included among these are catecholamine- and PTH-mediated intracellular Ca^{2+} overloading and induction of oxidative stress where the latter has been characterized as a Ca^{2+} paradox [65]. Another pathway relates to impaired antioxidant defenses that accompany Zn^{2+} dyshomeostasis with hypozincemia and Se^{2+} deficiency with hyposelenemia. The dyshomeostasis of intracellular Ca^{2+} and Zn^{2+} in cardiac myocytes and mitochondria is indeed intrinsically coupled where Ca^{2+} serves as prooxidant and Zn^{2+} as antioxidant [66, 73].

Conclusions

In unraveling these intricate cellular and molecular pathways leading to myocardial necrosis and fibrosis, it raises the prospect for nutriceutical uncoupling of Ca^{2+} and Zn^{2+} dyshomeostasis in favor of antioxidant defenses by raising $[Zn^{2+}]_i$ through Zn^{2+} supplementation alone, or in combination with a Ca^{2+} supplement or Ca^{2+} channel blocker. In addressing the importance of a simultaneous dyshomeostasis involving multiple macro- and micronutrients, particularly in African-Americans with CHF, it is plausible to prevent the adverse structural remodeling of myocardium, which inevitably contributes to increased cardiovascular risk. The time is propitious to develop novel strategies that combine current pharmacologic interventions with inexpensive nutriceutical adjuvants to achieve the greatest therapeutic potential in CHF, which is fast becoming the most serious burden to health care cost containment in today's industrialized world.

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