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MITOCHONDRIOCENTRIC PATHWAY TO CARDIOMYOCYTE NECROSIS IN ALDOSTERONISM: CARDIOPROTECTIVE RESPONSES TO CARVEDILOL AND NEBIVOLOL

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Abstract

Foci of fibrosis, footprints of cardiomyocyte necrosis, are scattered throughout the failing myocardium and are a major component to its pathologic remodeling. Understanding pathogenic mechanisms contributing to hormone-mediated necrosis are therefore fundamental to developing cardioprotective strategies. In this context, a mitochondriocentric signal-transducer-effector (MSTE) pathway to necrosis is emerging. Our first objective, using cardiomyocytes and subsarcolemmal mitochondria (SSM) harvested from rats receiving 4 wks aldosterone/salt treatment (ALDOST), was to identify major components of this pathway. Secondly, to validate this pathway we used mitochondria-targeted pharmaceutical interventions as cardioprotective strategies using 4 wks cotreatment with either carvedilol (Carv) or nebivolol (Nebiv). Compared to controls, we found 4 wks ALDOST to be accompanied by: elevated cardiomyocyte free $[Ca^{2+}]_i$ and SSM free $[Ca^{2+}]_m$; increased H_2O_2 production and 8-isoprostane in SSM, cardiac tissue and plasma; and enhanced opening of mitochondrial permeability transition pore (mPTP) and myocardial scarring. Increments in antioxidant capacity augmented by increased cytosolic free $[Zn^{2+}]_i$ were overwhelmed. Cotreatment with either Carv or Nebiv attenuated $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ overloading, prevented oxidative stress and reduced mPTP opening while augmenting $[Zn^{2+}]_i$ and conferring cardioprotection. Thus, major components of the MSTE pathway to cardiomyocyte necrosis seen with ALDOST include intracellular Ca^{2+} overloading coupled to oxidative stress and mPTP opening. This subcellular pathway can be favorably regulated by Carv or Nebiv cotreatment to salvage cardiomyocytes and prevent fibrosis.

Keywords

aldosteronism; subsarcolemmal mitochondria; carvedilol; nebivolol; cardiomyocyte necrosis

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Introduction

Foci of fibrosis are present scattered throughout the myocardium of the right and left heart of the explanted failing human heart (1). Fibrosis, a morphologic footprint of cardiomyocyte necrosis (*vis-à-vis* apoptosis), is a major component of the pathologic remodeling found in the failing heart. Given its widespread distribution (*pari passu* cardiomyocyte loss), necrosis and fibrosis are likely ongoing events in myocardial remodeling. In support of this hypothesis are nonischemic-based elevations in serum troponins, a biochemical marker of cardiomyocyte necrosis found in patients when hospitalized because of their congestive heart failure (CHF), and which may again appear with recurrent admissions, in arguably a postmitotic organ unable to regenerate lost myocardium (2-11). Consequently, ongoing necrosis portends a poor prognosis.

Elevations in plasma angiotensin II, aldosterone and the catecholamines each contribute to the salt-avid state of CHF, where salt and water retention is the root cause of the symptoms and signs which are manifested with this clinical syndrome (12). Once signs and symptoms of CHF have resolved and patients are again compensated, the resolution in troponins implicates a pathogenic role for circulating effector hormones of the activated renin-angiotensin-aldosterone and adrenergic nervous systems in mediating cardiomyocyte necrosis. Understanding cellular and subcellular mechanisms contributing to hormone-induced cardiomyocyte necrosis is therefore of fundamental importance and represents the scientific underpinning to the development of cardioprotective strategies.

Mitochondria sustain the cellular energetics of this obligate aerobic muscular pump. These organelles also play a central role in the subcellular mechanisms leading to cardiomyocyte necrosis that occurs with acute and chronic stressor states. These include: ischemia/reperfusion or hypoxia/reoxygenation-induced forms of cardiac injury (13-15); acute volume overload that accompanies the opening of an aortocaval fistula (16); isoproterenol administration (17); Mg^{2+} deficiencies (18); and aldosterone/salt treatment (ALDOST) (19-23). In this context, a mitochondriocentric signal-transducer-effector (MSTE) pathway to cardiomyocyte necrosis is beginning to emerge. It involves the subsarcolemmal population of cardiac mitochondria (SSM) and associated iterations in Ca^{2+} , redox state and opening potential of the inner membrane permeability transition pore (mPTP) under a variety of pathogenic stimuli.

The first objective of our study, using cardiomyocytes and SSM harvested from rats with a chronic stressor state that accompanies 4 wks of ALDOST, was to address major components of the MSTE pathway. This included monitoring responses in Ca^{2+} , as prooxidant, and Zn^{2+} , as antioxidant, that occur in these cells and organelles and which determines the respective equilibrium of their redox state, together with a propensity for mPTP opening. Our second objective was to validate the role of this pathway in leading to cardiomyocyte necrosis using mitochondria-targeted pharmaceutical interventions as cardioprotective strategies in preventing scarring (*pari passu* necrosis), which first appears at wk 4 ALDOST when antioxidant defenses have been overwhelmed (20). Toward this end, we compared rats receiving 4 wks ALDOST alone to those cotreated with either carvedilol or nebivolol, each of which are β_1 -adrenergic receptor antagonists, but whose pleiotropic actions target mitochondria with antioxidant properties (24, 25). Untreated age-/sex-matched rats served as controls.

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. It releases ALDO (0.75 $\mu\text{g/h}$) to raise circulating ALDO levels to those commonly found in human CHF, which suppresses both plasma renin activity and circulating levels of angiotensin II. Drinking water was fortified with 1% NaCl and with 0.4% KCl to prevent hypokalemia. A detailed accounting of this model, including various experimental controls have been reported previously (22, 26).

Separate groups of rats received ALDOST plus 4 wks cotreatment with either: a) carvedilol (5 mg/kg/day by daily gavage); or b) nebivolol (10 mg/kg/day) by gavage.

Cardiac pathology first appears at wk 4 ALDOST (27). We therefore restricted the present study to this time point. Rats were killed at 4 wks ALDOST alone or at wk 4 ALDOST plus either cotreatment. Unoperated, untreated age-/sex-matched rats served as controls.

Isolation of Cardiomyocytes and Mitochondria

Ventricular myocytes were isolated from excised hearts as previously reported (19). Mitochondria were isolated by differential centrifugation from whole heart homogenates with interfibrillar mitochondria discarded after $1500\times g$ and only the subsarcolemmal (SSM) population harvested at $10,000\times g$ as previously reported (19, 20). The purity of the SSM preparation and integrity of their membranes were routinely assessed as we previously reported (19, 20).

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

Cytosolic free $[\text{Ca}^{2+}]_i$ was measured ratiometrically using the Ca^{2+} -specific fluorophore Fura-2 (Invitrogen, Eugene OR) as reported previously (22). Cytosolic free Zn^{2+} concentration ($[\text{Zn}^{2+}]_i$; nmol/L) of viable cardiomyocytes was measured by flow cytometry (BD FACSCalibur; Becton, Dickinson, Franklin Lakes, NJ) using Zn^{2+} -specific dye FluoZin (Invitrogen) as previously reported (28).

Mitochondrial Free $[\text{Ca}^{2+}]_m$

Mitochondrial free $[\text{Ca}^{2+}]_m$ was determined by the ratiometric method using fura-2 as we previously reported (21, 29)

Mitochondrial H_2O_2 Production

We monitored H_2O_2 released by these organelles in response to succinate stimulation as we previously reported (21).

Mitochondria, Cardiac Tissue and Plasma 8-Isoprostane

Mitochondria, cardiac tissue and plasma 8-isoprostane levels were measured using a competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) as previously reported (20).

Mitochondrial mPTP Opening

mPTP opening was determined by CaCl_2 -induced swelling of isolated cardiac mitochondria as previously reported (20).

Cardiac Pathology

The extent of myocardial fibrosis was assessed by collagen-specific picrosirius red staining in 6 μm coronal sections of the ventricles using a computer image system as previously reported (30).

Statistical Analysis

Group data are presented as mean $\pm$ SEM, and analyzed by one-way ANOVA in SPSS software (ver. 18.0; SPSS, Inc., Chicago, Illinois, USA) with $p < 0.05$ as significant. Multiple-group comparisons between two groups were made by Scheffé's F -test.

RESULTS

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

As presented in the upper panel of Figure 1 and compared to controls, cardiomyocyte free $[\text{Ca}^{2+}]_i$ was significantly increased at 4 wks ALDOST. This excessive accumulation of intracellular Ca^{2+} which accompanied chronic aldosteronism was attenuated by cotreatment with either carvedilol or nebivolol with values comparable to those found in untreated control rats.

Mitochondrial Free $[\text{Ca}^{2+}]_m$

In subsarcolemmal (SSM) mitochondria harvested from rat hearts at 4 wks ALDOST and compared to controls, mitochondria free $[\text{Ca}^{2+}]_m$ was significantly increased (see lower panel of Figure 1). Cotreatment with either carvedilol or nebivolol attenuated mitochondrial $[\text{Ca}^{2+}]_m$ overloading.

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

Compared to controls, 4 wks ALDOST was associated with significantly increased cardiomyocyte free $[\text{Zn}^{2+}]_i$ (see Figure 2), which could be further increased by cotreatment with either carvedilol or nebivolol. Furthermore, nebivolol raised $[\text{Zn}^{2+}]_i$ to levels greater than carvedilol.

Mitochondrial H_2O_2 Production

H_2O_2 production by SSM harvested from rat hearts at 4 wks ALDOST was significantly increased compared to those organelles from hearts of untreated controls (see Figure 3). Cotreatment with either carvedilol or nebivolol attenuated this evidence of ALDOST-induced oxidative stress in mitochondria.

Mitochondria, Cardiac Tissue and Plasma 8-Isoprostane Levels

At 4 wks ALDOST, 8-isoprostane levels in SSM were increased above those found in organelles harvested from control hearts (see Figure 4, upper panel). This evidence of oxidative stress and lipid peroxidation was abrogated by cotreatment with carvedilol or nebivolol.

In cardiac tissue, 8-isoprostane levels were likewise increased at 4 wks ALDOST above control values (see Figure 4, lower panel), which was also abrogated by cotreatment with either carvedilol or nebivolol.

Plasma 8-isoprostane levels were significantly ($p < 0.05$) raised above controls (357.0 ± 36.4 pg/mL) at 4 wks ALDOST (802.6 ± 46.3 pg/mL) in keeping with the systemic nature to the altered redox state and which was attenuated ($p < 0.05$) by carvedilol or nebivolol cotreatment (314.2 ± 27.8 and 339.3 ± 22.5 pg/mL, respectively).

mPTP Opening

The decrease in spectrophotometer absorbance due to mPTP opening induced by a 200 μ M CaCl_2 challenge in mitochondria harvested at 4 wks ALDOST was greater than that seen in controls ($p < 0.05$). This response implicates an increased propensity of cardiomyocytes to mPTP opening and a greater susceptibility to necrosis during chronic ALDOST. This response was significantly attenuated by cotreatment with either carvedilol or nebivolol.

Cardiac Pathology

At 4 wks ALDOST and compared to controls, microscopic scars were seen scattered throughout the left and right ventricles. A more than two-fold rise in collagen volume fraction for ventricular myocardium was found at 4 wks ALDOST, and was attenuated by cotreatment with either carvedilol or nebivolol (see Figure 5) in keeping with the cardioprotective properties of these agents in abrogating cardiomyocyte necrosis and consequent myocardial scarring.

DISCUSSION

Our study led to several major findings. Chronic aldosteronism in rats is accompanied by a Ca^{2+} overloading of cardiomyocytes and SSM, as evidenced by increased cytosolic free $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_m$. The calcitropic peptide, parathyroid hormone (PTH), contributes to the increase in cardiac tissue $[\text{Ca}^{2+}]$ that accompanies chronic aldosteronism (31). Secondary hyperparathyroidism (SHPT) with a marked demineralization of bone is invoked by the Ca^{2+} -sensing receptor of the parathyroid glands in response to plasma ionized hypocalcemia, a consequence of the marked increments in fecal and urinary excretion of Ca^{2+} that accompany ALDOST (32). Spironolactone, an aldosterone receptor antagonist, prevents SHPT and is thereby cardioprotective (27, 31). Parathyroidectomy, cotreatment with a calcimimetic, or a Ca^{2+} -fortified diet, coupled with vitamin D, each prevent SHPT and intracellular Ca^{2+} overloading (33-35). Massry and colleagues (36) have demonstrated the calcitropic properties of PTH in cultured cardiomyocytes that can be prevented by Ca^{2+} channel blockade. Intramitochondrial Ca^{2+} overloading involving SSM is the *signal* to the MSTE pathway leading to cardiomyocyte necrosis.

The rise in $[\text{Ca}^{2+}]_m$ is accompanied by the induction of oxidative stress in these organelles, as evidenced by the increased H_2O_2 production and their increased 8-isoprostane content. The Ca^{2+} overloading and oxidative stress in SSM is accompanied by an increased propensity to the opening of their mPTP during CaCl_2 provocation. The subsequent degeneration of these organelles leads to cardiomyocyte necrosis and consequent tissue repair with myocardial scarring, expressed as a contemporaneous rise in collagen volume fraction of the right and left ventricles. Previous studies indicated that fibrosis also involves both right and left atria (37). The systemic nature of the aggravated redox state which accompanies ALDOST and is attributed to elevated circulating levels of PTH is evidenced by pathologic effects involving diverse tissues (29, 33-35, 38-40). Presence of Ca^{2+} overloading, coupled with the appearance of oxidative stress and mPTP opening in SSM, represent the major components of a mitochondriocentric MSTE pathway and mechanistic insight to cardiomyocyte necrosis seen in chronic aldosteronism.

This prooxidant pathway to death of cardiomyocytes infers that endogenous antioxidant defenses have been overwhelmed. In response to intracellular Ca^{2+} overloading and oxidative stress, cardiomyocytes invoke a variety of endogenous responses innately designed for their survival. One such strategy includes a rise in biologically active cytosolic free $[\text{Zn}^{2+}]_i$ serving as antioxidant. Cardiomyocyte $[\text{Zn}^{2+}]_i$, increased at wk 4 ALDOST, involves several mechanisms. Increased Zn^{2+} entry via L-type Ca^{2+} channels (LTCC) is

intrinsically coupled to intracellular Ca^{2+} overloading (22, 41). The primary facilitator of increased Zn^{2+} entry, however, is the upregulation of Zn^{2+} transporters augmented by oxidative stress (22, 42). Another source is its release from an inactive form, compartmentalized and bound to metallothionein (MT) (43, 44). Reactive oxygen species trigger the release of bound Zn^{2+} via a nitric oxide (NO)-derived pathway in which $[\text{Zn}^{2+}]_i$ protects against NO cytotoxicity (45-47). Hence, the cardiomyocyte redox state regulates intracellular $[\text{Zn}^{2+}]_i$ homeostasis (41). Finally, NO-mediated formation of cyclic guanosine monophosphate (cGMP) downregulates Ca^{2+} entry via LTCC (48-50). This intrinsic competition between coupled Ca^{2+} and Zn^{2+} entry allows for enhanced $[\text{Zn}^{2+}]_i$ and relatively reduced Ca^{2+} overloading.

The rise in $[\text{Zn}^{2+}]_i$ serves as an antioxidant. This includes its upregulation of antioxidant defense genes, which is facilitated through the activation of its sensor, metal-activated transcription factor (MTF)-1. Upon its translocation to the nucleus, MTF-1 activates the transcription of target genes that include MT, glutathione synthase and Cu/Zn-superoxide dismutase (22). Thus in cardiomyocytes, an increase in antioxidant capacity, including activity of Zn^{2+} -dependent antioxidant proteins and enzymes, is invoked to restore this delicate equilibrium between prooxidant and antioxidant. During wk 1 ALDOST, we found these defenses were capable of preventing cardiomyocyte necrosis (20, 27). However at wk 4, they are overwhelmed by the persistence of intracellular Ca^{2+} overloading. The marked rise in oxidative stress now accounts for the appearance of cardiomyocyte necrosis and consequent myocardial scarring (20).

Our second major finding relates to pharmaceutical-based interference with the MSTE pathway using carvedilol, a combined β_1/β_2 and α_1 adrenergic receptor antagonist. This agent has additional properties which extend beyond adrenergic receptor blockade, including its role as an inhibitor of mitochondrial Ca^{2+} uptake and antioxidant (51). Cotreatment with carvedilol led to several salutary responses. These included its preventing the rise in both $[\text{Ca}^{2+}]_m$ and $[\text{Ca}^{2+}]_i$ and attendant increase in mitochondrial H_2O_2 production and 8-isoprostane content, and promoting a rise in cardiomyocyte $[\text{Zn}^{2+}]_i$. Its cardioprotective properties were evidenced by an attenuation in the appearance of myocardial scarring and attendant decrease in collagen volume fraction. These findings suggest that antioxidant properties of carvedilol are severalfold. We cannot discount its role in blocking hyperadrenergic activity during chronic mineralocorticoidism (52, 53).

Our third major finding was the cardioprotective properties of nebivolol. Like carvedilol, this β_1 adrenergic receptor antagonist also prevented the rise in cardiomyocyte $[\text{Ca}^{2+}]_i$ and mitochondrial $[\text{Ca}^{2+}]_m$ and the appearance of oxidative stress in SSM. Furthermore, a marked rise in $[\text{Zn}^{2+}]_i$ was seen with nebivolol cotreatment and which may be associated with the competitive inhibition of LTCC-mediated Ca^{2+} entry. Furthermore, the β_3 agonistic properties of nebivolol could increase NO production from eNOS in cardiomyocytes, which promotes the release of inactive Zn^{2+} bound to MT while reducing Ca^{2+} entry by cGMP-mediated impact on LTCC (54, 55). Recent studies suggest that upregulation of Zn^{2+} transporters would also serve as an inhibitor of LTCC (42). Figure 6 depicts our current concept on an emerging schema of pro- and antioxidant events involving the MSTE pathway that leads to cardiomyocyte necrosis during aldosteronism.

Our study had several limitations. We did not contrast responses seen with carvedilol and nebivolol to more specific β_1 receptor blocker, such as atenolol. Such a comparison would distinguish the pleiotropic actions of this newer generation of β blockers from their antiadrenergic activity. Second, and as previously reported with ZnSO_4 (19, 21), we could have combined cotreatment with these agents with a Zn^{2+} supplement to further raise cardiomyocyte antioxidant defenses. Thirdly, we did not address the role of β_3 receptor in

NO generation and its derivation in regulating $[Zn^{2+}]_i$. Our current findings, nevertheless, suggest a salutary response putatively invoked by this pathway to assuage concerns for its negative inotropic response (56, 57).

In summary, our study using β receptor blockers has provided crucial mechanistic insights and identified the major components of the mitochondriocentric MSTe pathway leading to cardiomyocyte necrosis in rats receiving ALDOST. Cotreatment with either carvedilol or nebivolol, mitochondria-targeted interventions, attenuated the dyshomeostasis of intracellular Ca^{2+} while raising $[Zn^{2+}]_i$ and $[Zn^{2+}]_m$ as antioxidants. Together, these novel iterations in cellular and subcellular events salvaged degenerating myocardium and are therefore cardioprotective. Other relevant mitochondria-targeted cardioprotective strategies we have most recently explored include quercetin, a flavonoid and mitochondria-specific antioxidant, and cyclosporine A, an mPTP opening inhibitor (23).

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Figure 1.

Cardiomyocyte cytosolic free $[Ca^{2+}]_i$ (upper panel) and mitochondrial free $[Ca^{2+}]_m$ (lower panel) for control hearts, and those harvested at 4 wks aldosterone/salt treatment (ALDOST) alone or together with carvedilol (Carv) or nebivolol (Nebiv) cotreatment. Chronic aldosteronism is accompanied by increased $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$, which is abrogated by Carv or Nebiv cotreatment. * $p < 0.05$ ALDOST vs. controls and † $p < 0.05$ ALDOST vs. Carv or Nebiv cotreatment.

Figure 2.

Cardiomyocyte cytosolic free $[Zn^{2+}]_i$ for controls, 4 wks ALDOST alone or cotreated with Carv or Nebiv. An increment in $[Zn^{2+}]_i$ accompanies 4 wks ALDOST, which can be further augmented by cotreatment with either Carv or Nebiv. * $p < 0.05$ ALDOST vs. controls and † $p < 0.05$ ALDOST vs. Carv or Nebiv cotreatment.

Figure 3.

Subsarcolemmal mitochondrial H₂O₂ production for controls, 4 wks ALDOST alone or with Carv or Nebiv cotreatment. The rise in H₂O₂ production, a biomarker of oxidative stress, seen at 4 wks ALDOST was attenuated by Carv or Nebiv cotreatment. * $p < 0.05$ ALDOST vs. controls and † $p < 0.05$ ALDOST vs. Carv or Nebiv cotreatment.

Figure 4. Subsarcolemmal mitochondria 8-isoprostane (upper panel) and cardiac tissue 8-isoprostane (lower panel) for controls, 4 wks ALDOST alone or with Carv or Nebiv cotreatment. An increase in this biomarker of lipid peroxidation in mitochondrial and cardiac tissue was seen at 4 wks ALDOST, and was abrogated to near normal levels by cotreatment with either Carv or Nebiv. * $p < 0.05$ ALDOST vs. controls and † $p < 0.05$ ALDOST vs. Carv or Nebiv cotreatment.

Figure 5.

Collagen volume fraction, a morphometric estimate of the portion of myocardium occupied by picrosirius red-labeled fibrous tissue, in controls, 4 wks ALDOST alone or with Carv or Nebiv cotreatment. Myocardial scarring, a biomarker of cardiomyocyte necrosis, contributed to a rise in collagen volume fraction at 4 wks ALDOST. The extent of necrosis and, by inference, the rise in collagen volume fraction was attenuated by cotreatment with either Carv or Nebiv. * $p < 0.05$ ALDOST vs. controls and † $p < 0.05$ ALDOST vs. cotreatment with either Carv or Nebiv.

Figure 6.

An evolving schema of the pathophysiologic paradigm contributing to the dyshomeostasis of cardiomyocyte cytosolic free $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ as prooxidant, and subsarcolemmal mitochondria and $[Zn^{2+}]_i$ and $[Zn^{2+}]_m$, as antioxidant, during chronic aldosteronism is presented, which is accompanied by secondary hyperparathyroidism evoked by ionized hypocalcemia (decreased $[Ca^{2+}]_o$). Elevations in plasma parathyroid hormone (PTH) cause intracellular Ca^{2+} overloading via L-type Ca^{2+} channels (LTCC), and the induction of oxidative stress with reactive species (ROS) generation contributing to the opening of the mitochondrial permeability transition pore (mPTP) and ensuing cardiomyocyte necrosis followed by tissue repair and a replacement fibrosis, or myocardial scarring. Antioxidant defenses are mobilized to counteract the prooxidant state. These include: ROS-mediated generation of nitric oxide (NO) from endothelial eNOS, present in both endothelial cells and cardiomyocytes, where NO and its generation of cyclic guanosine monophosphate (cGMP) via guanylyl cyclase (GC) serves to inhibit LTCC. NO also promotes the release of inactive Zn^{2+} bound to metallothionein (Zn/MT) to raise both $[Zn^{2+}]_i$ and $[Zn^{2+}]_m$. The rise in $[Zn^{2+}]_i$ activates its sensor, metal-responsive transcription factor (MTF)-1 which, in turn, upregulates transcription of antioxidant defense genes. Zn^{2+} transporters, induced by ROS, also contribute to increased Zn^{2+} entry and a resultant rise in $[Zn^{2+}]_i$ and $[Zn^{2+}]_m$. In response to catecholamine receptor-ligand binding, β_3 adrenergic receptors (β_3R) evoke the NO-mediated pathway to Zn^{2+} release and inhibition of Ca^{2+} entry via LTCC.