CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

UNIDAD ZACATENCO
DEPARTAMENTO DE FARMACOLOGÍA

Los efectos anti-hipertróficos del diazóxido implican cambios en la expresión del miR-132 en cardiomiocitos de rata adulta.

Tesis que presenta

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Anti-hypertrophic effects of diazoxide involve changes in miR-132 expression in adult rat cardiomyocytes

Thesis

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Gayathri Narasimhan

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Abbreviations

TAC   -Transverse aortic constriction

ANP   - Atrial natriuretic peptide

BNP   -B- type natriuretic peptide

βMHC   -β-myosin heavy chain

ROS   -Reactive oxygen species

MPTP   - Mitochondrial permeability transition pore

SOD   -Superoxide dismutase

NOX-4   - NADPH oxidase 4

KR-31378   - (2*S*,3*S*,4*R*)-*N*″-cyano-*N*-(6-amino-3,4-dihydro-2-dimethoxymethyl-3-hydroxy-2-methyl-2*H*-1-benzopyran-4yl)-*N*′-benzylguanidine

RAAS   -Renin-angiotensin II-aldosterone system

TRX   -Thioredoxin

HDAC   -Histone deacetylases

CaMK   -Ca^{2+} -calmodulin dependent protein kinase

EPAC   -Exchange protein directly activated by cAMP

CREB   -cAMP response element binding protein

HAT   -Histone acetyltransferase

CBP   -CREB binding protein

MREs   -miR regulatory elements

RISC   -RNA induced silencing complex

ISO   -Isoproterenol

Dzx   -Diazoxide

H2O2   -Hydrogen peroxide

REST   -Repressor element-1 silencing factor

NRSF   -Neuron-restrictive silencer factor

SRF   -Serum response factor

AP-1   -Activator protein-1

NAC   -*N*-acetylcysteine

CICR   -Calcium induced calcium release

MEF2   -Myocyte enhancer factor 2

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Abstract

Background: Pathological hypertrophy of cardiomyocytes is regulated by microRNA-132 (miR-132). But the mechanisms involved in its increased expression caused by hypertrophic stimuli are largely unknown. Here we show in an isoproterenol (ISO) hypertrophic model in rats that miR-132 expression is regulated by reactive oxygen species (ROS) dependent and independent mechanisms and that the ISO induced increase in miR-132 expression is associated with overexpression and under expression of transcription factors pCREB and REST, respectively. We also show that diazoxide (Dzx), a mitochondrial K_{ATP} channel opener has anti-hypertrophic effects through regulation of miR-132 expression.

Methods: Hypertrophy was induced by injection of isoproterenol (ISO) in rats (5 mg/kg/day). Dzx (100 µM) actions were assessed in adult rat cardiomyocytes, paced externally at 1 Hz. The rate of ROS production was measured by a fluorescent probe. Mir-132 expression was estimated by the qRT-PCR technique as $2^{-\Delta\Delta CT}$. p-CREB, p-CaMKII and REST protein expression levels were analyzed by Western blot in total fractions of isolated cardiomyocytes.

Results: The heart/body weight ratio significantly increased in ISO treated rats, an effect that was prevented in part by Dzx and by the CAMKII inhibitor KN-93. ISO increased the rate of ROS production an effect that was antagonized by Dzx and KN-93. H_2O_2 (50-100 µM) increased the expression of miR-132 which was blocked by Dzx but not by KN-93. H_2O_2 also increased the expression of pCREB suggesting that up-regulation of miR-132 expression by ROS is mediated by this transcription factor. Consistent with this view, ISO increased the expression of pCREB which was prevented by Dzx and KN-93. Finally, ISO reduced the expression of the silencing transcription factor (REST) that targets miR-132, an effect prevented by Dzx but not by KN-93.

Conclusion: ISO-related hypertrophy involves up-regulation of miR-132 expression in ROS production dependent and independent manners in which p-CREB and REST play relevant roles.

1. Introduction

Cardiac hypertrophy is an adaptive response to a variety of extrinsic and intrinsic stimuli such as pressure overload or volume stress and to mutations of sarcomeric proteins or loss of contractile mass prior to infarction. Various forms of heart disease including ischemia, hypertension, myocardial infarction, aortic valve stenosis and heart failure are accompanied by hypertrophic growth. Cardiac hypertrophy is characterized by an increase in cell size, enhanced protein synthesis and heightened organization of the sarcomere. There are two types of hypertrophic phenotypes: i) concentric hypertrophy and ii) eccentric hypertrophy. Concentric hypertrophy which is caused by pressure overload is characterized by parallel addition of sarcomeres and lateral growth of individual cardiomyocytes. Eccentric hypertrophy is caused by volume overload and characterized by addition of sarcomeres in series and longitudinal cell growth (Frey 2004). Though initially this compensatory mechanism is beneficial, when prolonged it can lead to contractile dysfunction, heart failure and death (Shimizu and Minamino 2016).

Ventricular remodelling characterized by mild hypertrophy is observed in intermittent transverse aortic constriction (iTAC), chronic pressure overload by chronic TAC and independent forms of exercise such as voluntary wheel running and forced swimming activities. This intermediary phenotype shows the absence of fetal gene induction, a marked decrease in capillary density and preserved global left ventricular (LV) functions. Whereas, impaired contractility and relaxation parameters both at baseline and in response to β-agonist exposure in isolated ventricular myocytes from (iTAC) mice similar to those observed in chronic TAC myocytes are present.
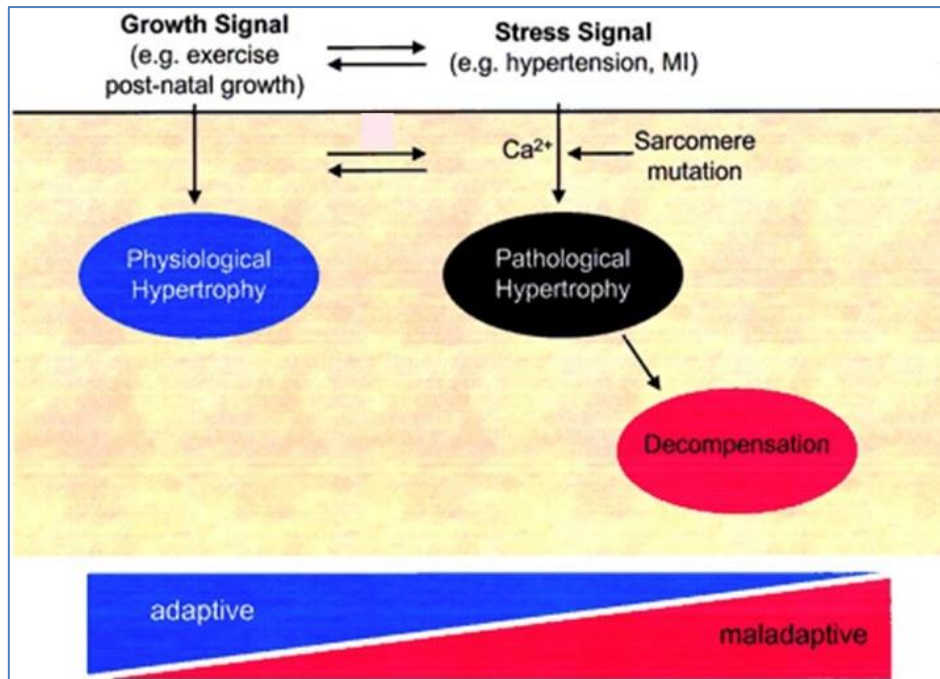
Figure 1.1. Physiological hypertrophy is an adaptive response to growth signals. Pathological hypertrophy develops in response to stress signals. It is not known whether stress signals are unique or whether overstimulation ("too much of a good thing") evokes a pathological response. Similarly, it is not known whether physiological versus pathological hypertrophy derives from activation of unique, "beneficial" signaling cascades or whether extreme activation of these same pathways evokes a pathological response (Frey 2004).

It is reported that the nature of the inciting signal must be pathogenic to induce ventricular remodelling. The duration of the pathogenic stimulus may only dictate the magnitude of ventricular hypertrophy and chronicity is not sufficient to cause hypertrophic heart disease (Tardiff 2006). In pathological cardiac hypertrophy diastolic dysfunction progressively leading to systolic dysfunction is caused by mechanical stiffness due to excessive and disproportionate accumulation of cardiac fibroblasts and extracellular matrix proteins (Brower et al. 2006). Up-regulation of fetal genes such as atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), skeletal α-actin and β-myosin heavy chain (MHC) are commonly associated with pathological cardiac hypertrophy (McMullen and Jennings 2007).

Several molecular changes are also involved in the induction of hypertrophy. Factors such as ion channels, basal calcium and calcium regulatory proteins, reactive oxygen species and micro RNAs are reported to be altered in cardiac hypertrophy.

Key elements and their regulation in beta-adrenergic stimulation- induced cardiac hypertrophy will be discussed in detail in section 1.1.

1.1 Factors involved in cardiac hypertrophy

1.1.1 Role of mitochondrial K_{ATP} channel activity in cardiac hypertrophy

An increase in workload is observed in cardiac hypertrophy depending on the stimulus such as repetitive or chronic pressure overload or volume overload. Since, energy demand and workload are closely linked there is always an impact on energy generation by mitochondria in loading conditions associated with cardiac hypertrophy.

Myocardial infarction, ischemia and heart failure are leading causes of morbidity and mortality in man. Several studies have been conducted to improve the functional recovery and reduce the extent of infarction after ischemic episodes. Preconditioning the heart by brief periods of ischemia or by administering a potassium channel opener is found to protect the heart from ischemia-reperfusion injury. Preconditioning requires opening of mitochondrial K_{ATP} channel which is cardio-protective through two distinct mechanisms. In low energy states, there is a high mitochondrial reactive oxygen species (ROS) production triggered by mitoK_{ATP} channel opening which will lead to the stimulation of several protein kinases such as PKC and MAPK involved in gene transcription and cell growth. In high energy states, matrix contraction due to high electron transport rates is prevented by mitoK_{ATP} channel opening. Mitochondrial K_{ATP} channel regulates mitochondrial volume by preventing the disruption of the structure-function of the intermembrane space. It also facilitates efficient energy transfers between mitochondria and myofibrillar ATPases (Garlid et al. 2003).
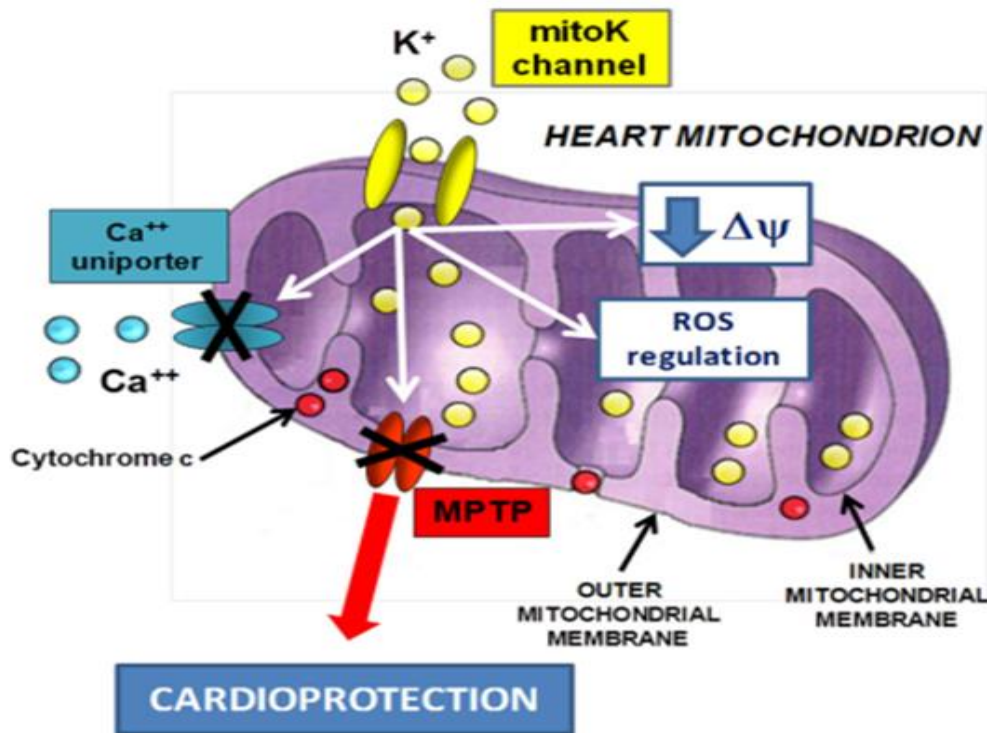
Figure 1.2. Schematic description of some of the different mechanisms, which links the activation of mitoK$_{ATP}$ channels to the control of mitochondrial calcium movements and modulation of the mitochondrial permeability transition pore (MPTP). The activation of mitoK$_{ATP}$ channels causes inward flow of potassium ions, weak membrane depolarization, and reduced driving force for calcium accumulation in the matrix, limiting the formation of MPTP and its opening. This mechanism can, at least in part, reduce the release of mitochondrial proapoptotic factors during reperfusion and thus preserve mitochondrial membrane integrity (Testai et al. 2015).

Although, the role of mitochondrial ATP sensitive K$^+$ channels have been studied in ischemia/reperfusion conditions and reported to prevent oxidative cardiac damage, studies on the effect of these channels in cardiac hypertrophy are limited. In hypertrophy models induced by angiotensin-II, alpha-adrenergic agonists, endothelin-1, cyclic stretch and tumor-necrosis factor alpha are all been shown to involve ROS production (Hirotani 2002; Pimentel et al. 2001). Treatment with antioxidants (Date et al. 2002; Dhalla et al. 1996) and overexpression of mitochondrial targeted catalase prevents protein oxidation, pressure-overload induced cardiac hypertrophy and diastolic dysfunction (Dai et al. 2009). Cardiomyocytes treated with H$_2$O$_2$ show a hypertrophic phenotype (Kwon 2003) and inhibition of superoxide dismutase (SOD) which increases superoxide anion radicals inside cells activates cardiac cells growth (Siwik et al. 1999). There are ample evidences that the source of ROS that leads to oxidative imbalance and cardiac damage is energy metabolism in

mitochondria (Madamanchi and Runge 2013). Oxidant species generation and mitochondrial damage are determinants for the transition from hypertrophy to heart failure (Osterholt et al. 2013). The mitochondrial electron transport chain is reported to be impaired in pressure-overload induced cardiac hypertrophy (Griffiths et al. 2010). Knock-out mice of mitochondrial-specific SOD is shown to present high levels of mitochondrial ROS production and reduced survival due to dilated cardiomyopathy (Koyama et al. 2013). Pressure overload-induced hypertrophy is developed due to mitochondrial superoxide anion radical generation in a cardiac specific NADPH oxidase 4 (NOX-4) deletion models of mice (Kuroda et al. 2010). Diazoxide, a mitochondrial K_{ATP} channel opener is shown to prevent cardiac hypertrophy induced by isoproterenol. It restores the levels of protein thiols, glutathione and superoxide dismutase activity that is all reduced in isoproterenol treated mice (Lemos Caldas et al. 2015).

Myocardial protective effects of mitochondrial K_{ATP} channel opening in ischemia preconditioning is reported (Ghosh 2000). Mitochondrial K_{ATP} channel opening leads to reduced intracellular Ca^{2+} accumulation which will further lead to reduced calcium-dependent activation of hypertrophic factors. Diazoxide (100 µM) is reported to produce anti-hypertrophic effects in phenylephrine-induced hypertrophy in cultured neonatal cardiomyocytes (Xia et al. 2004a). Mitochondrial K_{ATP} channel antagonists, 5-HD (100 µM) and glibenclamide (50 µM) reverse the inhibitory effect of diazoxide on cell area.

Another mitochondrial K_{ATP} channel opener, (2*S*,3*S*,4*R*)-*N''*-cyano-*N*-(6-amino-3,4-dihydro-2-dimethoxymethyl-3-hydroxy-2-methyl-2*H*-1-benzopyran-4yl)-*N'*-benzylguanidine, known as KR-31378 directly blocks hypertrophy in H9c2 cells treated with hypertrophic agonists and improves cardiac dysfunction in rats with chronic heart failure (Hwang et al. 2006). It has been shown to reduce myocardial infarction induced by ischemia/reperfusion in rats and dogs (Lee et al. 2001; Yoo et al. 2001). Also, KR-31378 is shown to have anti-ischemic effects in focal ischemic brain-damage in rats (Hong et al. 2002; Kim et al. 2004). Fig. 1.3 shows that 10 µM KR-31378 reverses the cell area induced by angiotensin II (100 nM) to the untreated control level. Mitochondrial K_{ATP} channel blockers 5-HD and glibenclamide (Jabůrek et al. 1998) either partially or completely reverse the inhibitory effects of KR-31378 on cell area induced by angiotensin II.

In addition, mitochondrial ATP sensitive K^+ channels are thought to provide adaptations of cardiac myocytes to ischemia, hypoxia, oxidative stress and hypertrophy (Flagg et al. 2010).

An effect similar to pressure overloading induced by acute aortic constriction is observed in a genetic knockout model of K_{ATP} channels (Yamada et al. 2006). Mitochondrial K_{ATP} channel opening is modulated by p70S6 kinase activity in infarction and mitochondrial K_{ATP} channel agonists attenuate ventricular remodelling and cardiac hypertrophy by reducing myocyte sizes, cardiac fibrosis and p70S6 kinase protein and mRNA levels (Lee et al. 2008). Hence, considering all these cardioprotective effects of mitochondrial K_{ATP} channel, we assessed the anti-hypertrophic effect of a mitoK_{ATP} channel opener, diazoxide in an isoproterenol-induced cardiac hypertrophy model.

Further details on pharmacological interventions in mitochondria during heart disease are described in appendix A.

1.1.2   Reactive oxygen species in cardiac hypertrophy

Reactive oxygen species (ROS) are implicated in cardiovascular diseases. Oxidative stress is involved in several conditions including atherosclerosis, ischemia-reperfusion injury, diabetic vascular disease, cardiomyopathy, myocardial infarction, arrhythmia, hypertrophy and heart failure (Brown and Griendling 2015). Angiotensin-II activates H_2O_2-sensitive signalling pathways in vascular smooth cells indicating its role in vascular hypertrophy. Increase in intracellular H_2O_2 leads to phosphorylation of p38MAPK (Ushio-Fukai et al. 1998) which plays a major role in cell growth mediating cardiac hypertrophy (Zechner et al. 1997). SOD-mimetics inhibits α_1-adrenergic receptor stimulated hypertrophy in both neonatal and adult rat ventricular myocytes indicating the role of ROS in hypertrophy (Amin et al. 2001). In heart failure, there is a decrease in antioxidant reserve and an increase in oxidative stress. This mismatch between oxygen radical production and available antioxidants suggests a potential role of antioxidants such as vitamin E therapy in modulating the pathogenesis of heart failure (Dhalla et al. 1996). In addition, ROS stimulates growth and apoptosis in neonatal rat ventricular myocytes by activating ERK1/2 and JNKs and inducing cardiomyocyte apoptosis at high levels of mechanical stretch (Pimentel et al. 2001).

Cellular sources of ROS are mitochondria, endoplasmic reticulum, NADPH oxidases, endothelial and neuronal uncoupled NO synthases, monoamine oxidases, cytochrome P450 oxidase and xanthine/xanthine oxidase. Initiation of stress-induced cardiac hypertrophy and its disease progression towards heart failure depends not only on ROS but also on ROS source. Targeting specific ROS sources or specific downstream molecular pathway is essential for a successful therapeutic approach (Sag et al. 2014).
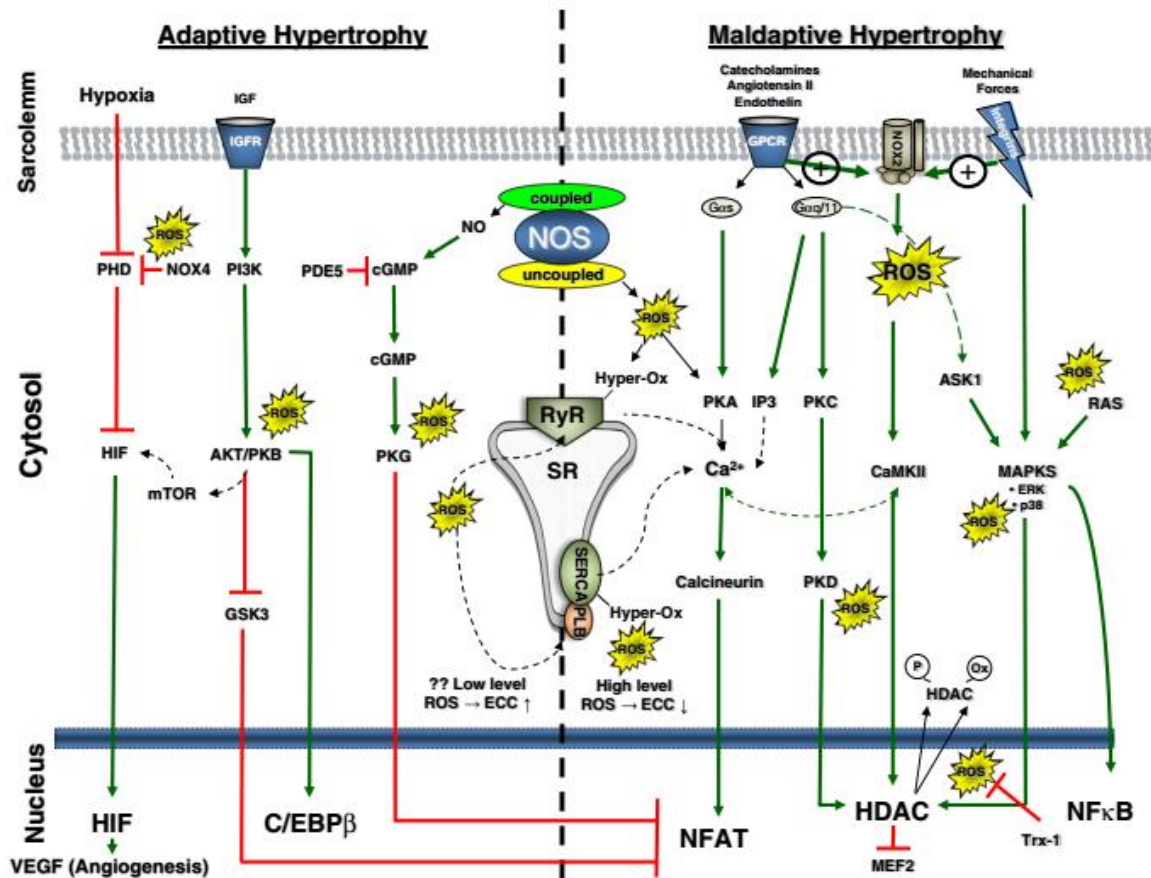
Figure.1.3. Key redox-sensitive signaling pathways involved in cardiomyocyte hypertrophy. Redox-regulated signaling pathways involved in adaptive hypertrophy are shown on the left, while pathways involved in maladaptive hypertrophy are illustrated on the right. Schematically shown are sarcolemmal receptors, cytosolic signaling cascades and their main nuclear transcription factor targets. Red lines indicate inhibition whereas green lines indicate activation of downstream targets (Sag et al. 2014).

The major sources of ROS in cardiomyocytes are NOX and the complexes I, II and III of the mitochondrial respiratory chain. Prolonged exposure of cells to ROS and Ca^{2+} destroys cells` equilibrium leading to arrhythmias and cardiac hypertrophy (Terentyev et al. 2008; Zhao et al. 2011; Maulik and Kumar 2012). Increase in mitochondrial ROS leads to mPTP opening and cytochrome C release, irreversible cell damage and ultimately cell death (Wang et al. 2008). ROS-induced-ROS release mechanism is a process involved in the development of cardiac hypertrophy and the transition to heart failure. In this process ROS generated by mitochondria amplifies NOX-produced ROS which is reported in different models of heart failure with (renin-angiotensin II-aldosterone system) RAAS activation (De Giusti et al. 2013).
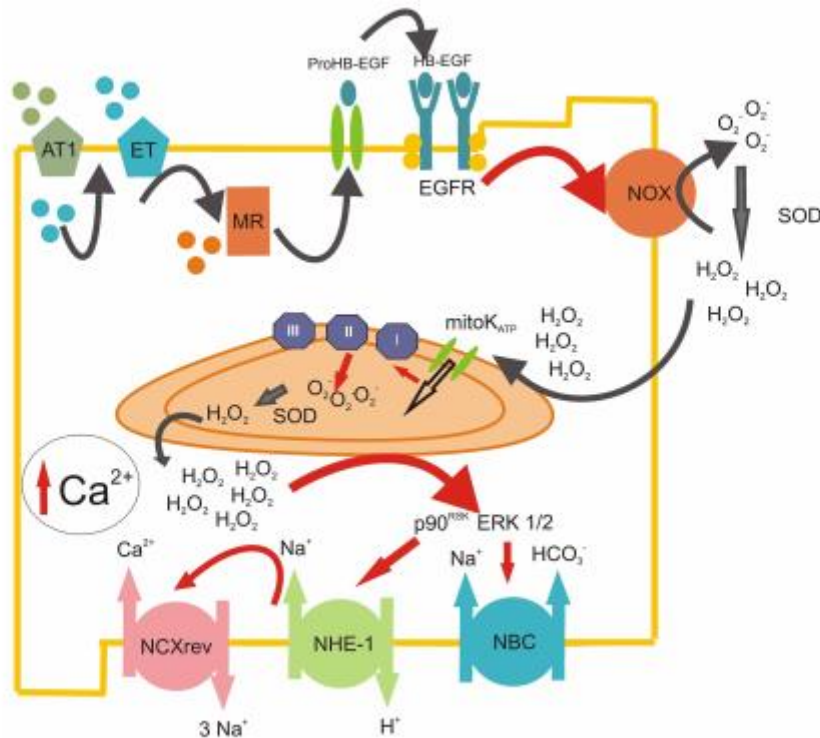
Figure.1.4. ROS-induced ROS-release mechanism triggered by RAAS. Scheme representing the sequential steps involved in the production of mitochondrial ROS after the initial Ang II stimulation. Ang II acting on AT1 receptors induces the release of intracellular ET-1, which, in turn, acts in an autocrine manner on ETA receptors. This autocrine action leads to activation of the mineralocorticoid receptor (MR), which induces transactivation of EGFR, possibly via the release of membrane heparin-bound EGF (HB-EGF). The stimulation of EGFR leads to activation of the NADPH oxidase (NOX), which produces superoxide anion (O_2-) and quickly dismutate by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). This permanent and stable oxidant molecule produces the opening of mitochondrial K_{ATP} channels (mitoK$_{ATP}$) with the subsequent enhanced production of mitochondrial O_2- by the electron transport chain (mainly by complex II). These mitochondrial ROS are released to the cytosol (ROS-induced ROS-release mechanism), where they stimulate redox sensitive MAP kinases ERK 1/2 and p90RSK, which, in turn, activate NHE-1 and NBC, pH regulation transporters that induce the increase in intracellular Na^+. Finally, cytosolic Na^+ increase favors the operation of the reverse mode of NCX, promoting the influx of Ca^{2+} into the cell. The enhancement of intracellular Ca^{2+} in the cardiomyocyte could lead to a positive ionotropic effect in the short term and/or the development of cardiac hypertrophy in a time-prolonged scenario (De Giusti et al. 2013).

In vascular smooth muscle cells angiotensin II activates c-Src via H_2O_2 generated from protein kinase C-dependent activation of NADPH oxidase. For Rac and NADPH oxidase activation, epidermal growth factor receptor transactivation by c-Src is necessary (Seshiah et

al. 2002). Also, it is reported that c-Src is upstream of NADPH oxidase in rat aortic vascular smooth muscle cells under angiotensin II stimulated H_2O_2 production (Touyz et al. 2003).

Several signalling molecules are redox modified in cardiac hypertrophy as shown in Fig 1.6 (appendix B) that include protein kinases such as calcium-calmodulin kinase II, protein kinase A and protein kinase G, sarcoplasmic reticulum Ca ATPase (SERCA2a), GTPases (e.g. RAS), antioxidant proteins such as thioredoxin (TRX) and histone deacetylases (HDAC). Regulation of HDACs may be influenced by ROS. In response to α-adrenergic stimulation, HDAC4 which inhibits the expression of MEF-2 dependent genes undergoes oxidation at Cys 274/276 resulting in its nuclear export and dis-inhibition of gene transcription (Sag et al. 2014; Ago et al. 2008). Protein kinase B (Akt) pathway that drives adaptive hypertrophy is redox regulated linking HIF (hypoxia inducible factor) via the activation of mTOR (Pouysségur and Mechta-Grigoriou 2006). cGMP/PKG pathway shows anti-hypertrophic effects which is redox regulated in transverse aortic constricted mice model (Zhang et al. 2012). Constitutive activation of CaMKII on methionine residues (Met-281/282) is associated with the development of cell death and ventricular rupture after myocardial infarction (Erickson et al. 2008; He et al. 2011). CaMKII oxidation may play a major role in cardiac hypertrophy development as CaMKII-dependent misregulation of RyR2 phosphorylation is implicated in altered calcium cycling in a mouse model of hypertrophy (Toischer et al. 2010). Since ROS plays a key role in the development of hypertrophy by activating several kinases leading to cell growth and apoptosis, we assessed the involvement of ROS and ROS-dependent signalling in isoproterenol-induced hypertrophy model.

Involvement of oxidative stress and ROS-induced signalling kinases in cardiac hypertrophy developed by several hypertrophic agonists are described in detail in appendix B.

1.1.3 Calcium and its regulatory proteins

Calcium and calcium dependent signalling is vital for proper cardiac function and abnormalities in its homeostasis leads to myocardial pathologies such as hypertrophy, ventricular arrhythmias and heart failure (Marks et al.,2013; George 2013).

Calcineurin, a calcium-calmodulin-activated serine/threonine phosphatase plays an important role in transducing calcium dependent signals. It is reported to be a modulator of cardiac hypertrophy using different approaches such as calcineurin inhibitors, calcineurin inhibiting proteins and transgenic mice overexpressing active calcineurin (Schaeffer et al. 2009).

Myocardial hypertrophy *in vivo* is induced by calcineurin activation or its target transcription factor NFAT3. Increased calcineurin activation and GATA-4 activity, and its expression in the hypertrophic myocardium lead to translocation of dephosphorylated NFAT3 to the nucleus of hypertrophied myocardium. Calcineurin-NFAT signalling is more evident in decompensated hypertrophy compared to the compensated hypertrophy (Diedrichs et al. 2007). Calcineurin-NFAT signaling is co-dependent on MEK1-ERK1/2 kinases in such a way that hypertrophic response induced by activated MEK1 is blunted by inhibiting calcineurin or NFAT activation in cultured neonatal cardiomyocytes. In addition, inhibiting MEK1/ERK1/2 signaling reduces hypertrophic growth response driven by activated calcineurin (Sanna et al. 2005).

CaMKs regulate several transcription factors such as AP-1, CREB (cAMP response element binding protein), MEF2 and SRF. Activation of MEF2 by CaMK is not a direct phosphorylation effect but via class II HDAC interaction (Gordon et al. 2009). Cytoplasmic CaMKIIδ_C not only phosphorylates RyR2 and PLB but also activates MEF2 and other transcription factors. CaMKIIδ_C phosphorylates calcineurin and reduces its activity resulting in decreased nuclear translocation of NFAT (MacDonnell et al. 2009). Ca^{2+} -calmodulin dependent protein kinases-1 and IV (CaMKI and CaMKIV) are involved in development of hypertrophy *in vitro* and CaMKIV overexpressing mice develop cardiac hypertrophy with increased left ventricular end-diastolic diameter and decreased fractional shortening (Passier et al. 2000). Both calcineurin-NFAT and CaMK-MEF2 pathways are involved in development of hypertrophic heart *in vivo*. It is also demonstrated that MEF2 is a downstream target of CaMK signalling and that calcineurin is less efficient in activating MEF2-dependent transcription (Passier et al. 2000). MEF2c is involved in Ca^{2+} triggered endoplasmic reticulum stress (ERS) induced cardiac hypertrophy. Thapsigargin (Tg), a specific irreversible inhibitor of the SERCA2a pump functions as ERS inductor and induces a significant increase in intracellular Ca^{2+}, calcineurin activation, MEF2c in the cardiomyocyte. Cyclosporin A (calcineurin inhibitor) inhibits Tg induced cardiac hypertrophy by suppressing MEF2c nuclear translocation. This indicates that calcineurin-MEF2c pathway is involved in ERS induced cardiac hypertrophy in
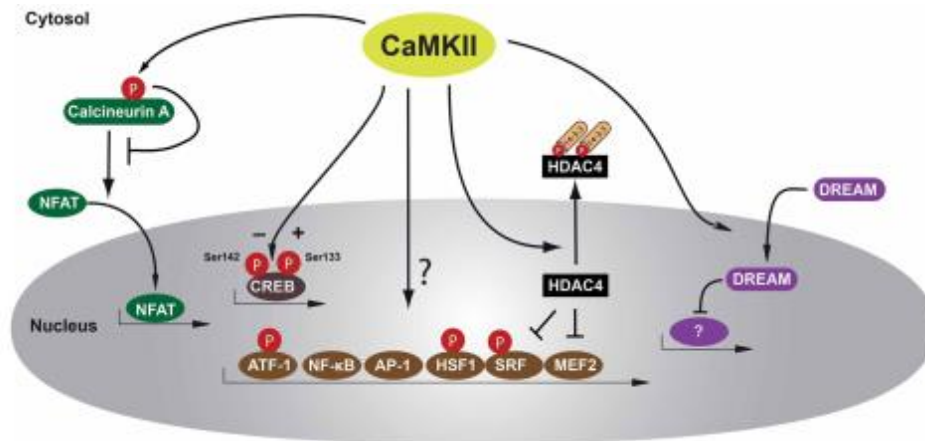
Figure.1.5. Scheme of transcription factors and transcriptional repressors regulated by CaMKII in cardiomyocytes. CaMKII phosphorylates HDAC4 at Ser-467 and Ser-632, allowing binding of the chaperone protein 14-3-3, leading to nucleo-cytoplasmic shuttling of a phospho-HDAC4/14-3-3 complex out of the nucleus and resulting in repression of transcription factors such as MEF2 that regulates genes responsible for adverse cardiac remodelling. Other transcription factors such as NF-κB or HSP-1 may play maladaptive or adaptive roles and these factors can be directly or indirectly regulated by CaMKII. Another transcription factor regulated after β-adrenergic stimulation is CREB that can be phosphorylated by CaMKII at two serine residues, Ser-133 and Ser-142, resulting in opposing effects in regard to activation of CREB. However, the functional effects of CREB/CaMKII interaction during cardiac remodelling remain unclear. Another recently recognized mechanism is translocation of the transcriptional repressor DREAM from the cytosol to the nucleus. Calcineurin/NFAT interaction may also be inhibited by direct phosphorylation by CaMKII at Ser-411, leading to decreased NFAT translocation to the nucleus and subsequent reduced transcriptional activity. AP-1 activation protein-1, ATF-1 activating transcription factor-1, CaMKII Calcium/Calmodulin-dependent kinase II, CREB cAMP-response element binding protein, DREAM downstream regulatory element agonist modulator, HDAC4 histone deacetylase 4, HSF-1 heat shock factor 1, MEF2 myocyte elongation factor, NFAT nuclear factor of activated T-cells, NF-κB nuclear factor κB, and SRF serum response factor (Kreusser and Backs 2014).

cardiomyocytes (Zhang et al. 2010). The exchange protein directly activated by cAMP (EPAC) activation increases pro-hypertrophic transcription factor MEF2 in a CaMKII dependent manner in primary cardiac myocytes. EPAC is a cAMP sensor and activates hypertrophic signalling pathways including CaMKII. H-Ras, PLC and HDAC4 nuclear export (Métrich et al. 2010).

Cardiac hypertrophy development requires the histone acetyltransferase (HAT) activity of CREB binding protein (CBP) and p300 in phenylephrine (PE)-treated cardiac cells. p42/p44

MAPK phosphorylates C terminal region of CBP and enhances HAT activity encoded by the molecule (Gusterson et al. 2003). CREB is phosphorylated at Ser-133 by angiotensin II which is mediated by a p38 MAPK, MEK-ERK and PKA-dependent pathways. Angiotensin II activates ERK through transactivation of EGFR which is important for angiotensin II induced CREB phosphorylation. Inhibition of CaMKII by KN-93 inhibits angiotensin II induced CREB phosphorylation indicating that an increase in intracellular calcium and activation of calcium/CaM pathway is crucial for angiotensin II induced cardiac hypertrophy. Angiotensin II induced CREB phosphorylation is inhibited by H89, a PKA inhibitor. Also, AG1478 (EGFR inhibitor) inhibits angiotensin II induced CREB phosphorylation which may be ascribed to suppression of MAPK pathways (Funakoshi et al. 2002).

More details on calcium and calcium regulatory proteins crucial for excitation-contraction coupling and their role in hypertrophic signalling are described in appendix C.

Name	Abbrev.	Type	Effect	Phosphorylation site	Kinase assay	References
cAMP-response element binding protein	CREB	Transcription factor	Unknown	Ser-133, Ser-142	Yes	Sun et al., 1994
Activating transcription factor 1	ATF-1	Transcription factor	Unknown	Ser-63	Yes	Shimomura et al., 1996
Myocyte elongation factor 2	MEF2	Transcription factor	Hypertrophy/remodeling	Unknown	/	Passier et al., 2000
Serum response factor	SRF	Transcription factor	Unknown	Ser-103, Thr-160	Yes	Fluck et al., 2000
Nuclear factor κB	NF-κB	Transcription factor	Hypertrophy/remodeling	Indirect via IκB kinase	/	Kashiwase et al., 2005; Ling et al., 2013
Histone deacetylase 4	HDAC4	Transcriptional repressor	Hypertrophy/remodeling	Ser-467, Ser-632	Yes	Backs et al., 2006
Histone deacetylase 5	HDAC5	Transcriptional repressor	Hypertrophy/remodeling	Unknown	/	Wu et al., 2006; Backs et al., 2008
GATA4	/	Transcription factor	Antiapoptotic	Unknown	/	Little et al., 2009
Activation protein 1	AP-1	Transcription factor	Calcium homeostasis	Unknown	/	Mani et al., 2010
Heat shock factor 1	HSF-1	Transcription factor	Antiapoptotic	Ser-230	Yes	Holmberg et al., 2001; Peng et al., 2010
Downstream regulatory element agonist modulator	DREAM	Transcriptional repressor	Calcium homeostasis	Unknown	/	Ronkainen et al., 2011
Histone H3	H3	Histone	Hypertrophy/remodeling	Ser-10	Yes	Awad et al., 2013

CaMKII interacts with various transcription factors, transcriptional repressors, and histone 3 and thereby influences cardiac gene expression. This interaction can be a direct phosphorylation of Ser/Thr residues by CaMKII, indirect via other proteins (other kinases or cardiac repressors) or by unknown mechanisms. Known phosphorylation site and proof of direct phosphorylation are indicated. CaMKII Calcium/Calmodulin-dependent kinase II.

Table 1. CaMKII-dependent regulators of cardiac transcription (Kreusser and Backs 2014).

Although, several studies documenting the role of CaMKII and its calcium-dependent signalling have been published, the relationship between mitoK_{ATP} channel and CaMKII is not established. There are reports that mitochondrial-targeted inhibition of CaMKII could prevent myocardial death and heart failure (Joiner et al. 2012). We assessed the effect of

diazoxide on CaMKII in a cardiac hypertrophy model treated with isoproterenol. In addition, the expression of downstream transcription factor, CREB was also studied in both isoproterenol and diazoxide conditions.

1.1.4 Micro RNAs in cardiac hypertrophy

MicroRNAs (miRs) are small non-coding RNAs that repress gene expression post transcriptionally by binding to discrete miR regulatory elements (MREs) typically located in the 3'-untranslated region (UTR) of target mRNAs (Kiriakidou et al. 2004; Cipolla 2014). miRs can repress expression of target genes by promoting mRNA degradation, inhibiting translation, or both (He and Hannon 2004; Farh et al. 2005; Jackson and Standart 2007).
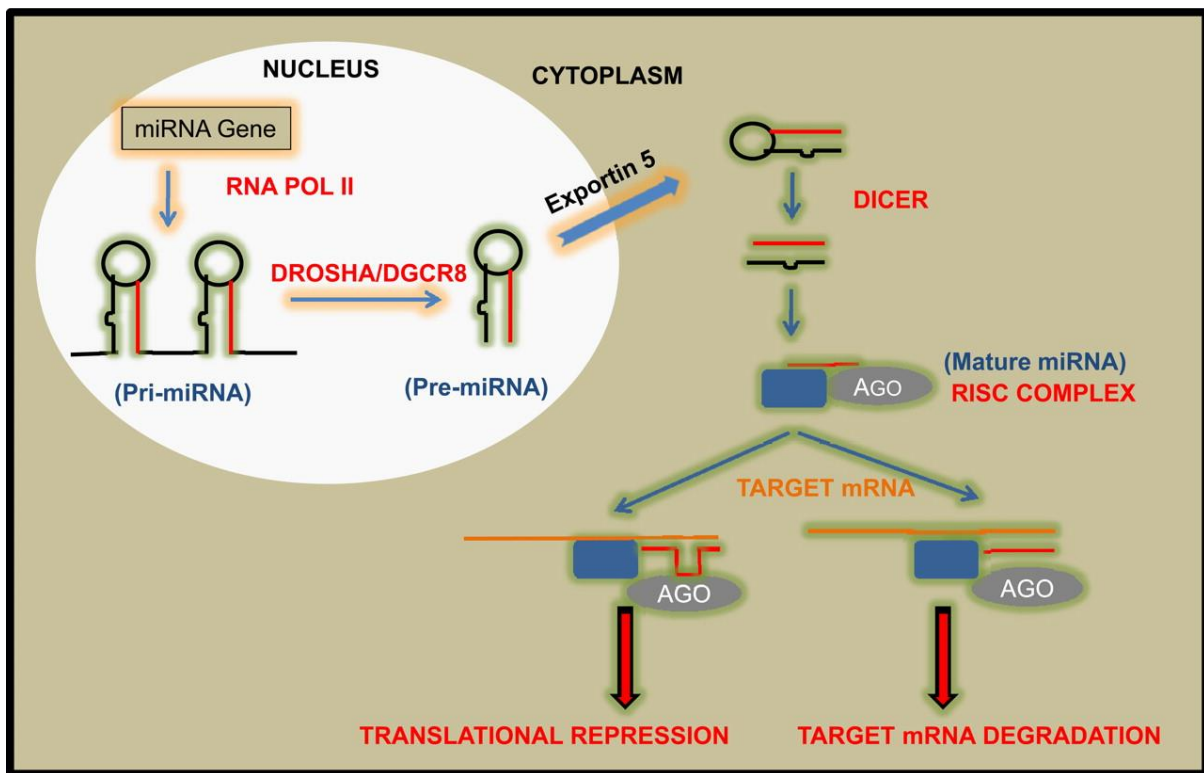
Figure.1.6. Biogenesis and function of microRNAs (miRNA). Most of miRNA genes are transcribed via RNA polymerase II, leading to the generation of pri-miRNAs that are long transcripts with multiple hairpin loop structures. Pre-miRNAs are processed by the Microprocessor protein complex containing Drosha, resulting in the generation of smaller precursor molecules called pre-miRNAs. pre-miRNAs are then exported from the nucleus via exportin-5. In the cytoplasm, the pre-miRNAs are further processed via Dicer to generate short, double-stranded miRNAs, which are then converted to mature, single-stranded miRNAs via RISC, a protein complex containing argonaute (AGO). Finally, mature miRNAs direct RISC to target gene mRNA, resulting in mRNA degradation (miRNA finds

perfect complementary sequences in mRNA) or translational repression (imperfect complementary sequences in mRNA) (da Costa Martins et al. 2008).

Dicer, an endonuclease processes pre-microRNAs after being exported to the cytoplasm into 22-nucleotide double-stranded microRNAs. The mature strand of microRNA binds to Argonaute protein in RNA induced silencing complex (RISC) which utilizes this strand to target the 3` untranslated region (UTR) of the target gene by Watson-crick base pairing as shown in Fig.1.9. Cardiac-specific Dicer deletion leads to structural and functional abnormalities in animal models implicating the significance of microRNAs in the cardiovascular system (da Costa Martins et al. 2008).
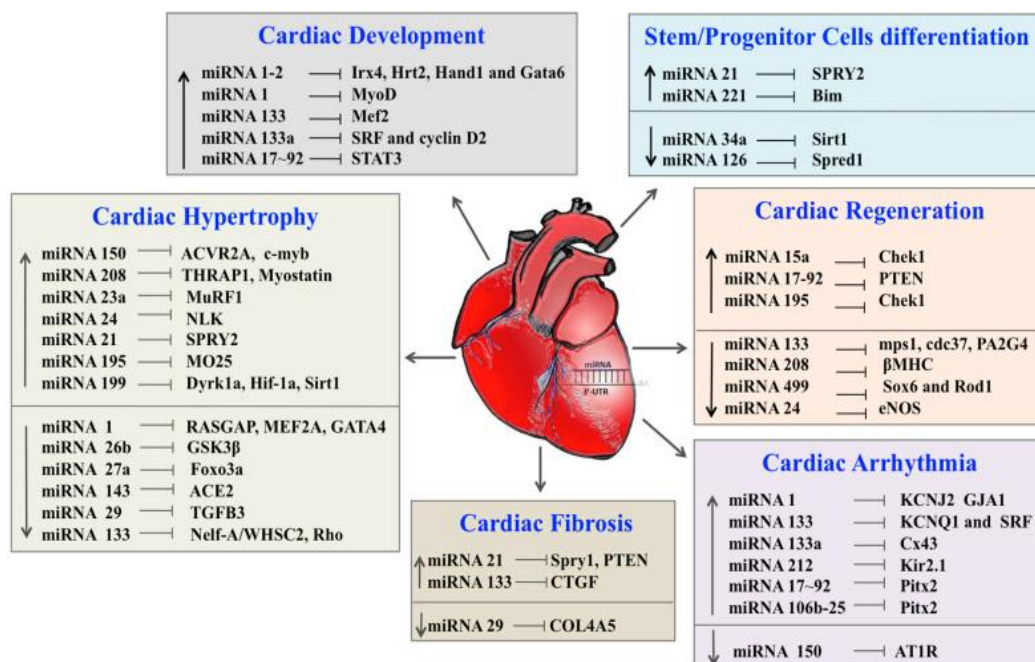
Figure.1.7. Role of miRNAs in cardiac development, disease and regeneration.↑ Increasing, ↓Decreasing, Inhibiting (Joladarashi et al. 2014).

Several hypertrophic stimuli up-regulate miR-132 expression to drive the hypertrophic growth of cardiomyocytes (Ucar et al. 2012). Consistent with this study sustained beta-adrenergic activation led to the increase in miR-132 expression in an animal model (Carrillo et al. 2015). miR-132 is a target of the transcription factor CREB and it is a rapidly responding microRNA with long-lasting effects (Vo et al. 2005) as shown in Fig.1.12. miR-132 up-regulation in isoproterenol (ISO) treated rats is developed within hours after administration and reaches a plateau at 12 h. Such a fast response of miR-132 is consistent with its role in the development of hypertrophy (Carrillo et al. 2011).

Both miR-132 and miR-212 regulate cardiac hypertrophy and cardiomyocyte autophagy and pharmacological inhibition of miR-132 by antagomir injection significantly reduces cardiac hypertrophy and heart failure in mice. FoxO3, an anti-hypertrophic and pro-autophagic factor is negatively regulated by both miR-132 and miR-212 in cardiomyocytes (Sengupta, Molkentin, and Yutzey 2009; Yan et al. 2015). miR-132/212 overexpression downregulates FoxO3 leading to hypertrophy by hyperactivation of calcineurin/NFAT signalling in cardiomyocytes. In addition, knockdown of miR-132 by antagomir-132 or the genetic loss-of-function of miR-132/212 suppresses pressure-overload induced cardiac hypertrophy by downregulating calcineurin/NFAT signalling (Ucar et al. 2012).
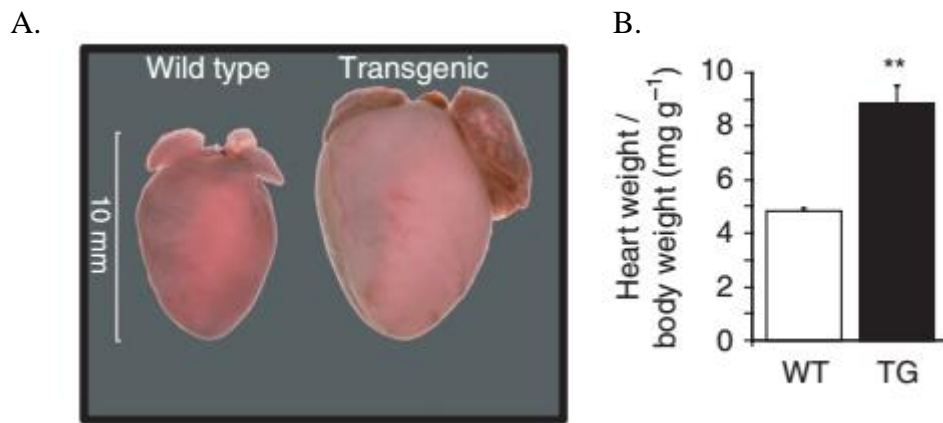
Figure.1.8. A. Morphology of explanted hearts from transgenic and WT mice at 10 weeks after birth. B. Heart-to-body-weight ratios (Ucar et al. 2012).
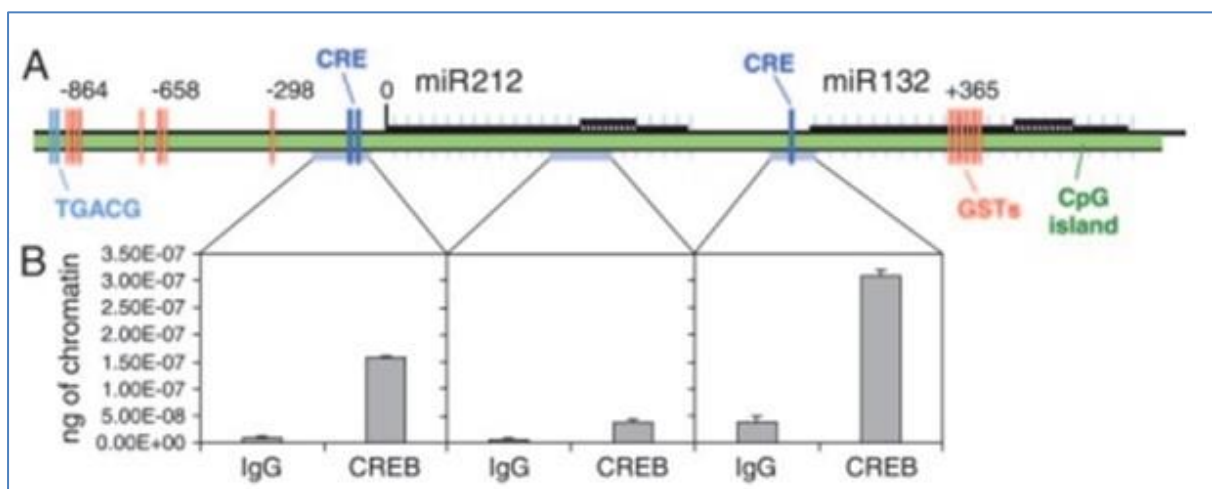
Figure.1.9. The neuronal miRNA miR132 is regulated by CREB. (A) A diagram indicating the relative positions of genomic signature tags (GSTs, red), CRE motifs (blue), and predicted premiRNA

15

sequences for the miR212 and miR132 cluster. B) Neocortical neurons are subjected to chromatin immunoprecipitation by using a CREB Ab or IgG control. Real-time PCR is conducted by using primers that interrogate the regions indicated in A (Vo et al. 2005).

Aberrant expression of miRNAs in VSMCs treated with H_2O_2

Down-regulated miRNAs		Up-regulated miRNAs	
miR-290	miR-107	rno-miR-351	rno-miR-20a
miR-193	miR-103	rno-miR-30d	rno-let-7e
miR-181c	miR-328	rno-let-7b	rno-miR-26b
miR-29b	miR-34a	rno-miR-30b	rno-miR-10b
miR-30e	miR-181b	rno-let-7f	rno-miR-15b
miR-145	miR-19b	rno-miR-18	rno-miR-92
miR-181a	miR-324-5p	rno-let-7i	rno-miR-352
miR-199a	miR-101b	rno-miR-342	rno-miR-21
miR-22	miR-214	rno-let-7d	rno-miR-20b
miR-130a	miR-23b	rno-miR-361	rno-miR-10a
miR-30a-5p	miR-23a	rno-miR-424	rno-miR-98
miR-99b	miR-143	**rno-miR-132**	rno-miR-7
miR-101a	miR-151	rno-miR-30c	rno-miR-195
miR-301	miR-31	rno-miR-25	rno-miR-365
miR-99a			

Table 2. The effect of H_2O_2 on miR-21 expression in cultured rat VSMCs. Micro array analysis of micro RNAs that are up-regulated and dysregulated by H_2O_2 in vascular smooth muscle cells (VSMCs). Cultured rat VSMCs were treated with vehicle or H_2O_2 (10–200 µM) for 6 h (Lin et al. 2009).

ROS such as H_2O_2 controls cellular functions such as proliferation, cell migration, cell differentiation, apoptosis and cell death (Irani 2000). Cultured rat vascular smooth muscle cells (VSMC) treated with H_2O_2 (10-200 µM) for 6 h revealed that several microRNAs are either up-regulated or down-regulated and are responsible for H_2O_2-mediated cellular effects. miR-21 is up-regulated in these cells and inhibited H_2O_2-mediated VSMC apoptosis and death. miR-21 is involved in H_2O_2-mediated gene regulation and injury response through its target gene PDCD4, a pro-apoptotic protein and AP-1 pathway. Hence, ROS-dependent

dysregulated microRNAs may have implications in cardiovascular diseases including atherosclerosis, hypertension and diabetic vascular complications (Lin et al. 2009).

In non-neuronal cells, miR-132/212 locus is transcriptionally repressed by the repressor element-1 silencing factor/neuron-restrictive silencer factor (REST/NRSF). One REST and several CREB binding sites are conserved among mammals in the vicinity of miR-132/212 locus, suggesting an evolutionary conserved involvement of CREB and REST in the control of miR-132/212 expression as shown in Fig.1.13 (Wanet et al. 2012). REST mediates silencing of its target genes through the recruitment of histone deacetylases by corepressors associated with its repressor domains. Repressor element 1 (RE1) sites are present in the promoter regions of certain microRNAs including miR-124a, miR-9 and miR-132 in mouse and humans and are functionally validated targets of REST in mammalian cell lines (Conaco et al. 2006; J. Wu and Xie 2006). REST is a key transcriptional regulator in heart development and vascular smooth muscle growth (Kuwahara et al. 2003; Cheong et al. 2005). Dominant negative form of REST expression disrupts REST function in the heart resulting in cardiomyopathy, arrhythmias and sudden death. REST represses fetal cardiac gene program in the adult heart and these effects are thought to be the result of re-expression of fetal cardiac genes under dominant negative REST expression (Kuwahara et al. 2003).
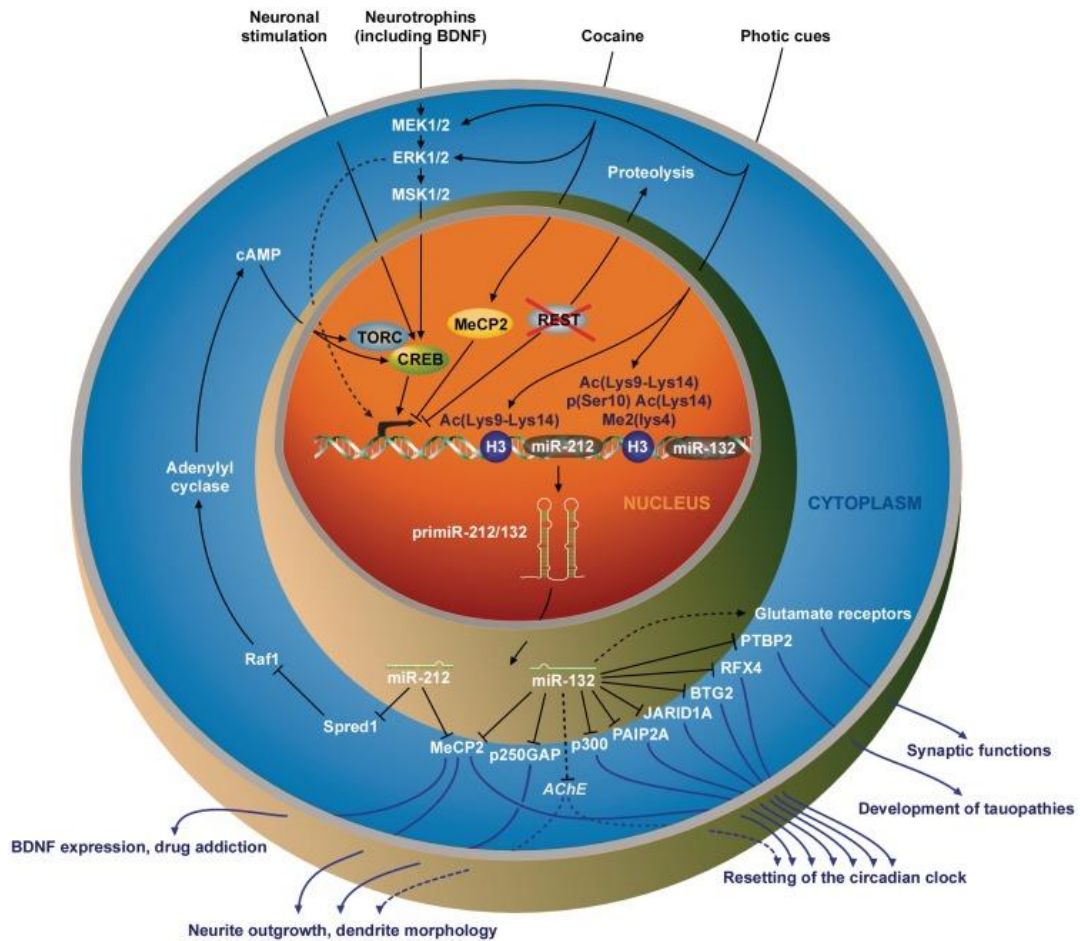
Figure.1.10. Inducers and targets of the miR-212/132 locus in the neuronal compartment. In neurons, the transcriptional repressor REST is targeted to proteolysis, enabling the transcription of its target genes. Various stimuli (such as the exposition to neurotrophins or photic cues, or an extended access to cocaine) lead to the transcription of the miR-212/132 locus through CREB activation, although an unidentified ERK1/2-dependent, MSK1/2- and CREB-independent mechanism may also contribute to miR-212/132 expression in BDNF-stimulated neurons (dashed arrow). Histone 3 post-translational modifications are also involved in pri-miR-212/132 expression following light exposure. By repressing the expression of several mRNA targets (AChE is a probable but not yet demonstrated target of miR-132 in neurons), miR-212/132 are involved in neurite outgrowth and dendrite morphology as well as in the resetting of the circadian clock and would participate to synaptic functions by up-regulating the expression of the glutamate receptors NR2A, NR2B and GluR1. miR-132 expression deregulation is also associated with the development of tauopathies through the targeting of PTBP2. Besides, miR-212 may be involved in regulating the vulnerability to cocaine addiction by targeting Spred1 mRNA (Wanet et al. 2012).
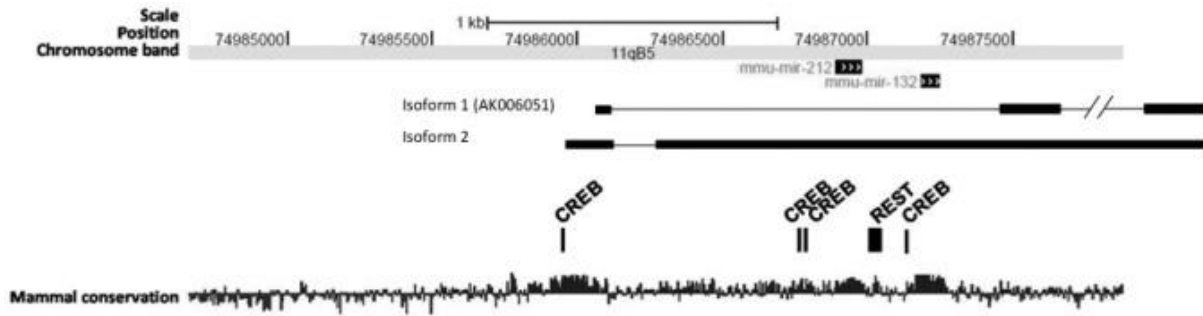
Figure.1.11. Genomic features of the miR-212/132 locus in the mouse genome. The diagram indicates the genomic localization of miR-212 and miR-132 pre-miRNA sequences, the two isoform transcripts by which they are encoded in mice (thick lines indicate exons; thin lines indicate introns), as well as the mammal conservation (the sites predicted to be conserved are assigned positive scores, while sites predicted to be fast-evolving are assigned negative ones). These annotations are from the UCSC Genome Center and Ucar et al. (2010). Besides, the position of CREB and REST binding sites that involved in miR-212/132 transcription (Vo et al. 2005; Remenyi et al. 2010; Conaco et al. 2006) are indicated. Interestingly, all of the indicated binding sites for transcription factors are conserved among the mouse, rat and human genomes (Wanet et al. 2012).

Genes encoding the brain and atrial natriuretic peptides (*Nppb* and *Nppa* encoding BNP and ANP, respectively), potassium/sodium hyperpolarisation-activated cyclic nucleotide-gated channels 2 and 4 (*Hcn2* and *Hcn4*), α-skeletal actin (*Acta1*) and voltage-gated calcium channel subunit alpha Cav3.2 (*Cacna1h*) are repressed by REST in myocytes (Kuratomi et al. 2007; Ogawa et al. 2002). In addition, both ANP and BNP expression increase in cardiac hypertrophy in adult ventricular myocytes leading to increased levels of circulating peptides (Mukoyama et al. 1991; Saito et al. 1989). In transgenic mice removal of REST function within the heart results in increased ANP and BNP expression and cardiac hypertrophy and repression of these genes by REST is essential for normal heart function (Kuwahara et al. 2003). REST represses *Nppb* transcription in H9c2 cells using its repression domains which is achieved by a combination of targeted histone deacetylation and histone methylation (Bingham et al. 2007).

Three classes of HDAC are expressed by vertebrates: class I HDACs, class II HDACs and Sir2 family proteins. Calcium/calmodulin kinase (CaMK) phosphorylates two conserved phosphorylation sites induced by hypertrophic stimulus that causes class II HDACs to be exported from the nucleus resulting in derepression of their gene targets (McKinsey et al, 2001; McKinsey et al. 2000). It has been shown that class II HDACs act as signal-responsive repressors of cardiac hypertrophy (Lu et al. 2000; McKinsey et al, 2000; McKinsey et al.

2001; Zhang et al. 2002). Class II HDACs, HDAC 4 and HDAC5 form a complex with NRSF in ventricular myocytes and the interaction mediates the CaMK-dependent signalling that promotes ANP and BNP gene transcription in cardiac hypertrophy. Endothelin-1 treated neonatal rat ventricular myocytes and the hearts from mouse model of pressure-overload induced cardiac hypertrophy show a disrupted NRSF- class II HDAC complex. CaMK is also involved in regulating various transcription factors such as CREB, serum response factor (SRF), activator protein-1 (AP-1) and MEF2 which are involved in the regulation of ANP gene expression. It is reported that CaMK may regulate ANP and BNP gene transcription in both NRSF-dependent and independent manners (Zhang et al., 2004, Cornelius et al., 1997, von Harsdorf et al. 1997 and Sprenkle, Murray, and Glembotski 1995). In addition, in the hearts of TAC operated mice NRSF protein and mRNA levels are down-regulated (Kuwahara et al. 2001). NRSF and HDAC5 interaction in sham operated mice is significantly decreased in TAC mice as determined by coimmunoprecipitation assays with lysates from ventricles of mice subjected to TAC and sham operation. Hence, NRSF is reported as a target of CaMK-class II HDAC signalling pathways in the reactivation of fetal cardiac gene program (Nakagawa et al. 2006).

Several pro-hypertrophic micro-RNAs that are dysregulated in transverse aortic constriction and hypertrophic agonists-induced hypertrophic models are illustrated in appendix D.

The role of miR-132 and the transcription factors, CREB and REST on cardiac hypertrophy and regulation of fetal cardiac genes have been reported. However, the detailed mechanism of regulation of miR-132 by CREB and REST in cardiac hypertrophy is not established. We characterized the mechanisms involved in the regulation of miR-132 in an isoproterenol cardiac hypertrophy model. We also studied the effect of diazoxide on miR-132 expression to assess its anti-hypertrophic effect.

2. Justification

Heart responds to a myriad of physiological and pathophysiologic stimuli to maintain sufficient cardiac output. Sustained or progressive demands on the heart results in pathological cardiac hypertrophy due to a disruption in the myocardial reserve that reversibly alters cardiac output in response to a sudden increase in demand. This initial adaptive response will eventually lead to cardiac failure if the inciting pathogenic stimulus is not relieved. Also, there are substantial evidences that cardiac hypertrophy is associated with an increased risk of cardiovascular morbidity and mortality. Hence, understanding the pathogenesis of hypertrophic heart disease is essential to develop therapeutic strategies to abate the transition from adaptive to maladaptive process. In addition, several pharmacological agents have proven effective against cardiac hypertrophy but the underlying mechanism is unclear. A clear understanding of the basic molecular pathways that lead to development of cardiac hypertrophy is therefore essential.

3. Hypothesis

The micro RNA, miR-132 is involved in the anti-hypertrophic actions of diazoxide and its expression is regulated by ROS-dependent and independent pathways in adult rat cardiomyocytes.

4. Aim

To test whether diazoxide mediates anti-hypertrophic effects through the regulation of miR-132 expression.

5. Specific aims

   a. To study the effect of diazoxide in isoproterenol induced cardiac hypertrophy in rats
   b. To assess the role of pCREB and pCaMKII in the anti-hypertrophic actions of diazoxide
   c. To study the possible action of ROS on miR-132 expression
   d. To evaluate the role of REST in isoproterenol induced hypertrophy
   e. To assess whether regulation of miR-132 expression involves CREB and REST

6. Materials and Methods:

6.1 The composition of solutions used is given in the tables described below:

Tyrode solution

Reagents	Concentration
NaCl	137 mM
KCl	5.4 mM
$MgCl_2$	1 mM
HEPES	10 mM
Glucose	10 mM

pH set to 7.47 with 1N NaOH at 25 °C; Osmolality: 305 mOsm

10X PBS

Reagents	Concentration/Litre
KCl	2 g
NaCl	80 g
KH_2PO_4	2 g
Na_2HPO_4	11.5 g

pH set to 7.4 with 1N NaOH

10X protein running buffer

Reagent	Concentration/Litre
Tris Base	30.2 g
Glycine	144 g
SDS	10 g

pH set to 8.3 with concentrated HCl

RIPA buffer for tissues

Reagents	Concentration
Tris HCl pH 7.5	20 mM
NaCl	350 mM
β-Mercaptoethanol	0.05%

Tween-20	0.1%
PMSF	100 µM
Na$_3$VO$_4$	100 µM
NaF	10 mM
Protease cocktail	1X
Aprotinin	1.53 nM
Leupeptin	2.1 nM

Lysis buffer for isolated cardiomyocytes

Reagents	Concentration
Tris Hcl pH 7.5	50 mM
NaCl	300 mM
Glycerol	10%
Triton-X	10%
Protease cocktail	1X

SOD 2 buffer

Reagents	Concentration
HEPES pH-7.2	20 mM
EGTA	1 mM
Mannitol	210 mM
Sucrose	70 mM
PMSF	1mM

4X sample loading buffer

Reagents	Concentration
Tris HCl (pH 6.8)	0.2 M
SDS	0.8 g/10ml
Glycerol	40%
β-Mercaptoethanol 14.7 M	0.4 ml/10 ml

EDTA 0.5M	0.05 M
Bromophenol blue	8 mg/10 ml

Protein transfer buffer

Reagents	Concentration/100 ml
Tris base	0.532 g
Glycine	0.293 g
SDS 10%	0.375 ml
Methanol	20%

Blocking solution

4.5% non-fat milk powder in 1X PBS solution

Coomassie blue staining solution

Reagents	Concentration/200 ml
Glacial acetic acid	10%
Methanol:H_2O	1:1
Coomassie brilliant blue R-250	0.5 g/200 ml

Ponceau red staining solution

Reagents	Concentration
Acetic acid	1%
Ponceau red	0.5 g/100 ml

Destaining solution

Reagents	Concentration
Methanol	30%
Acetic acid	10%

6.2 Pharmacological agents

Reagents	Concentration
Isoproterenol (ISO)	0.5 µM; 5 mg/kg
Diazoxide (Dzx)	100 µM; 20 mg/kg
5-HD	100 µM
KN-93	1 µM; 0.501 µg/kg
Mito-tempo	25 µM
H_2O_2	100 µM

6.3 Adult male Wistar rats

Experiments with male Wistar rats (300-350 g) conformed to protocols approved by the Division of Laboratory Animal Units, Cinvestav-IPN, in compliance with federal law, federal statute and Consejo Nacional de Ciencia y Tecnología (CONACYT) regulations.

Rats were injected subcutaneously with isoproterenol (ISO), 5 mg/kg every 24 h for 2 d unless otherwise indicated. Diazoxide (Dzx) was administered intraperitoneally, 10 or 20 mg/kg every 24 h for 3 d. Rats were anaesthetized with 50 mg·kg^{-1} of pentobarbital sodium injected intraperitoneally. A 500 U·kg^{-1} heparin sodium solution (Sigma) was also administered intraperitoneally.

6.4 Adult rat cardiomyocytes isolation

Adult rat ventricular myocytes were isolated by enzymatic digestion using Langendorff apparatus as described previously (Sánchez et al. 2001). Briefly, hearts were perfused for 5 min at 37ºc with Tyrode solution while oxygenating the solution continuously. Hearts were re-circulated for ~60 min with Tyrode solution containing collagenase (Worthington, Lakewood, NJ, USA, type II) and protease type XIV (Sigma, 0.5 mg/100 ml). Digested ventricle was cut into fragments and shaken in bath for 2-3 times at 45 rpm for 5 min in the same solution. Cells were filtered through a cell stainer (100 µm nylon BD Falcon) and centrifuged at 72 x g for 2 min. The pellet was dissolved in Tyrode solution containing bovine serum albumin and 0.1 mM Ca^{2+} and gradually changing to solutions containing 0.5 mM and finally 1 mM Ca^{2+} for several washes.

Myocytes were stimulated externally using platinum electrodes at a frequency of 1 Hz, with 65 V and 4 ms duration square pulses for 5-10 min without the addition of drugs and then ISO (0.5 µM) or ISO and KN-93 (1 µM; 5 min before ISO) were added. Stimulation continued for 20 min. Dzx (100 µM) was added to cardiomyocytes 1 h prior to stimulation, then Dzx was washed out and cells were stimulated for 5-10 min after which ISO was added and stimulated during 20 min. 5-HD was added 30 min prior to Dzx if indicated in experimental condition. Drugs were removed by washing three times with Tyrode solution containing BSA (1 mg/ml) and 1 mM Ca^{2+}. Thereafter cells were centrifuged at 72 x g for 2 min and total protein extracts were obtained for Western blot analysis following the procedure described below.

To test the effect of ROS on miR-132 expression cardiomyocytes were treated with H_2O_2 (100 µM) for ~5 min, washed three times with Tyrode solution and then following a 6h waiting period, Qiazol reagent was added to enable RNA isolation with a miRNeasy Mini kit (Qiagen). cDNA was synthesized using Taqman micro RNA reverse transcription kit (4366596).

6.5 Primary culture of neonatal rat cardiomyocytes

Ventricular cardiomyocytes from 1–2-day-old neonatal rats were isolated by enzymatic digestion as described elsewhere (Xia et al. 2004). Cardiomyocytes were cultured for 48 h in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), NaHCO3 1.5 g/l, penicillin (50 IU), and streptomycin (50 µg/ml) under atmospheric conditions of 95% air and 5% CO_2 at 37 °C in a humidified incubator. Cells were serum starved by changing to a medium containing 0.4% fetal bovine serum, and then treated for 48 h with ISO (1 µM) to induce hypertrophy. When experimentally indicated, Dzx (100 µM) was administered 30 min prior to ISO stimulation and the specific mitoK$_{ATP}$ channels blocker, 5-hydroxydecanoic acid (5-HD, 500 µM) (Jabůrek et al. 1998) was added 30 min prior to Dzx. Cells were cultured for 48 h, washed thrice with 1× phosphate buffered saline (PBS), fixed in 4% formaldehyde in PBS, and then stained with haematoxylin. We imaged the stained cells at various magnifications using Motic Images plus 2.0 software (Motic China Group Co.,LTD) and measured cell areas using Adobe Photoshop.

6.6 Western blotting

Dissociated myocytes were prepared for immunoblotting as described elsewhere with minor modifications (González et al. 2010). Protein content was measured with a Bradford Protein Assay kit (Bradford 1976). We subjected total fraction samples (45–60 μg) to 11% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (140 V, 120 min), transferred the resultant proteins bands onto nitrocellulose membranes, blocked the membranes with 4.5% non-fat dried milk in PBS, and probed the membranes with anti-CREB monoclonal antibody (1:750, Abcam, Cambridge, MA, USA), anti-CREB S133 monoclonal antibody (1:1000, Abcam, Cambridge, MA, USA), and anti-GAPDH monoclonal antibody (1:10000, Sigma Aldrich, St. Louis, MO, USA) in PBS for 12-14 h at room temperature. After rinsing the blots with PBS-tween20, they were incubated with anti-rabbit (1:90,000) or anti-mouse (1:90,000) horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) in PBS. Antibody labeling was detected with Immobilon Western reagent (Millipore Co, Billerica, MA) according to the manufacturer's instructions.

6.7 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The relative expression levels of rno-miR-132 were quantified using TaqMan miR assays (4427975, ID 000457, Applied Biosystems, Foster City, CA, USA) and an iCycler iQ (Bio-Rad, Hercules, CA) using the TaqMan Gene Expression Master Mix (4369016). miR expression was assessed relative to the small nucleolar RNA U87 (442795, ID 001712), as recommended by the manufacturer. Changes in expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

6.8 Fluorescence measurements

6.8.1. ROS measurements

Levels of ROS were measured as described previously (García, Hernández, and Sánchez 2009) using the cell-permeant fluorescent probe 5-(and-6)-chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA, Molecular Probes/Invitrogen, USA). Fluorescence (505 nm excitation, 545 nm emission) was measured in arbitrary units (a.u.) for 30 ms in user defined segments of cardiomyocytes. Images were acquired at 15 s intervals. Myocytes were stimulated externally at a frequency of 1 Hz throughout the experiment except at the end of experiments when H_2O_2 (0.2 mM) was applied to estimate maximal ROS production rate. The effect of ISO on ROS production was

estimated by measuring fluorescence signals firstly for 10 min under control conditions and secondly for 15–20 min 0.5 µM ISO stimulation (ISO group); Dzx group myocytes were treated as in ISO group except that myocytes were incubated with Dzx (100 µM) for 1 h under quiescent conditions prior to being externally paced at 1 Hz. ROS measurements under each experimental condition were fitted to a straight line, the slope of which was taken as a ROS production rate estimate. The ratio between the slope obtained for each experimental condition and that obtained under control conditions was used as the relative ROS production rate for that condition (Bovo, Lipsius, and Zima 2012).

6.9 Assays

6.9.1 BCA protein assay

Neonatal rat ventricular myocytes were collected in 1% SDS solution after 48 h of treatment. 10 µl of each sample was used in triplicates to measure the absorbance using the protocol indicated in the Pierce^{TM} BCA protein assay kit (Thermo Scientific, Pierce Biotechnology, IL, USA). To determine the protein concentration of each sample a BSA standard curve was plotted as indicated in the protocol. The total protein in µg measured was normalized to the number of cells plated in each condition and the differences in relative total protein content were plotted.

6.9.2 Superoxide dismutase 2 enzyme assay

Ventricular tissue was homogenized in 5–10 ml of cold SOD buffer (20 mM HEPES 7.2, 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) per gram of tissue. The homogenate was centrifuged at 1,500 $\times g$ for 5 min at 4 °C to produce supernatant containing total SOD lysate, the supernatant was centrifuged at 10,000 $\times g$ for 15 min at 4 °C to separate cytosolic and mitochondrial enzyme into supernatant and pellet portions, respectively. The pellet was homogenized in cold SOD buffer in preparation for mitochondrial SOD activity assessment. SOD1 and SOD3 were inhibited with 1–3 mM KCN to isolate SOD2 activity. SOD2 assays were performed with a SOD assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the instructions of the manufacturer; SOD2 activity was measured as the amount of enzyme necessary to exhibit 50% dismutation of O_2-.

6.10 Confocal Immunofluorescence

Adult rat cardiomyocytes were attached to cover slips using 5% Laminin for confocal microscopy imaging. Cells were stimulated and treated in Tyrode solution containing 1mM Ca^{2+} and 5% fetal bovine serum and immediately washed and fixed with 4% cold paraformaldehyde for 20 min. Afterwhich cells were washed with 1X PBS thrice and incubated with cold permeabilizing (1% triton X-100) and blocking solution (1% BSA) for 30 min. The coverslips were incubated overnight at 4 °C with primary antibody (anti-CREB S133, monoclonal antibody 1:100, Abcam, Cambridge, MA, USA prepared in the same permeabilizing and blocking solution. Serial 10 min washes were performed with cold permeabilizing and blocking solution after bringing the coverslips to room temperature. Cells were incubated with secondary antibody (Alexa555, 1:200, Life Technologies) for 1 h followed by serial 10 min washes. Nuclear staining was performed using cold Hoechst dye (1:1000) for 20 min prepared in 1X PBS. After washing with 1X PBS, cover slips were mounted on glass slides with Vectashield® and labelled cells were observed through a Zeiss LSM700 imager. Z2 confocal laser microscope at RT. Images were acquired with a 100x objective (ECPlanarNeofluar 40x/1.30 oil dichroic M27) and high-resolution camera Axiocam Hrm. Acquisition and analysis of images were performed using Leica Application Suite X software (Leica Microsystems CMS GmbH).

6.11 Statistical analysis

Data are expressed as means ± SE. Statistical analyses were performed in GraphPad Prism 4.0 (GraphPad Software) and Sigma Stat 2.0. For two-group comparisons, Student's t test was performed. For multiple comparisons, data with a normal distribution were analyzed by one-way analyses of variance (ANOVAs) followed by Turkey's honest significant difference test. A $p < .05$ was considered statistically significant.

7. Results

7.1 Hypertrophy Index

7.1.1. Dzx reduced cell area induced by ISO in neonatal rat cardiomyocytes

Cell area measurement of cultured neonatal rat cardiomyocytes revealed that ISO induced an increase in cell area after 48 hours of treatment. Dzx treatment one hour before ISO treatment reduced the increase in cell area produced by ISO. In order to verify whether the effect of Dzx on cell area is mediated by mitochondrial K_{ATP} channel opening, 5-HD a specific blocker of mitochondrial K_{ATP} channel was used 30 minutes before the addition of Dzx (Fig. 3.1.1.A). In Fig 3.1.1.B., it is shown that 5-HD reverses the effect of Dzx and hence there was an increase in cell area in the presence of 5-HD+Dzx+Iso. 5-HD did not show any effect by itself on cell area.

A.

| Vehicle | Iso | Dzx + Iso | 5-HD (500 μM) | 5-HD (500 μM)+Dzx+Iso |
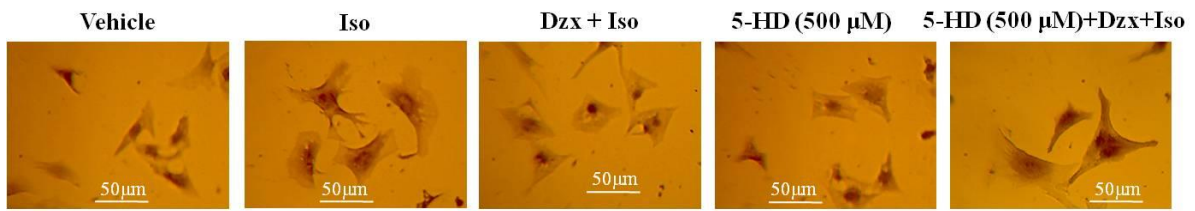
B.
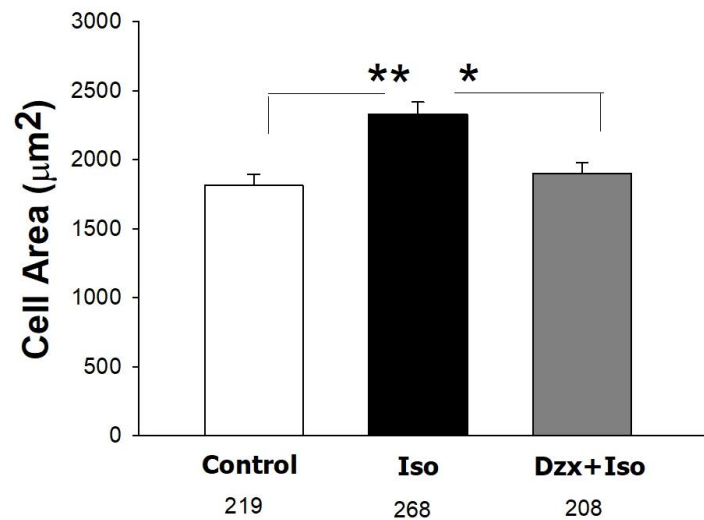
C.
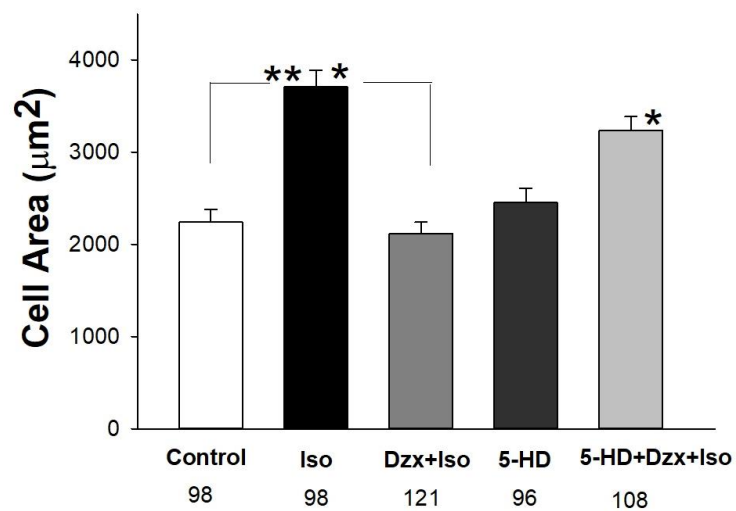
Figure7.1.1. Effect of Dzx on cell area induced by ISO. A. Haematoxylin –stained images of cultured neonatal rat ventricular myocytes treated for 48 h under control (0.01% DMSO), ISO, Dzx+ISO, 5-HD and 5-HD+Dzx+ISO conditions. B. Bar graphs showing the cell area (μm^2) under different treatment conditions. C. Bar graphs showing the effect of 5-HD on the

cell area in control and Dzx+ISO treated conditions. Values represent mean ± SE, number indicated below each experimental condition represents the number of cells measured, $*p < 0.05$, $**p < 0.01$.

7.1.2 Total protein content increased by ISO in neonatal rat cardiomyocytes and this effect was reversed by Dzx

In addition to phenotypic changes such as cell area and ventricle-to-body weight ratio total protein content has been reported to be altered by hypertrophy (Simpson 1985; Wang et al, 2002). Likewise, we found that cells treated with ISO for 48 hours showed a significant increase in total protein content compared to control cells. In contrast, treatment with Dzx for one hour prior to ISO reduced the total protein content induced by ISO significantly as shown in Fig 3.1.3.
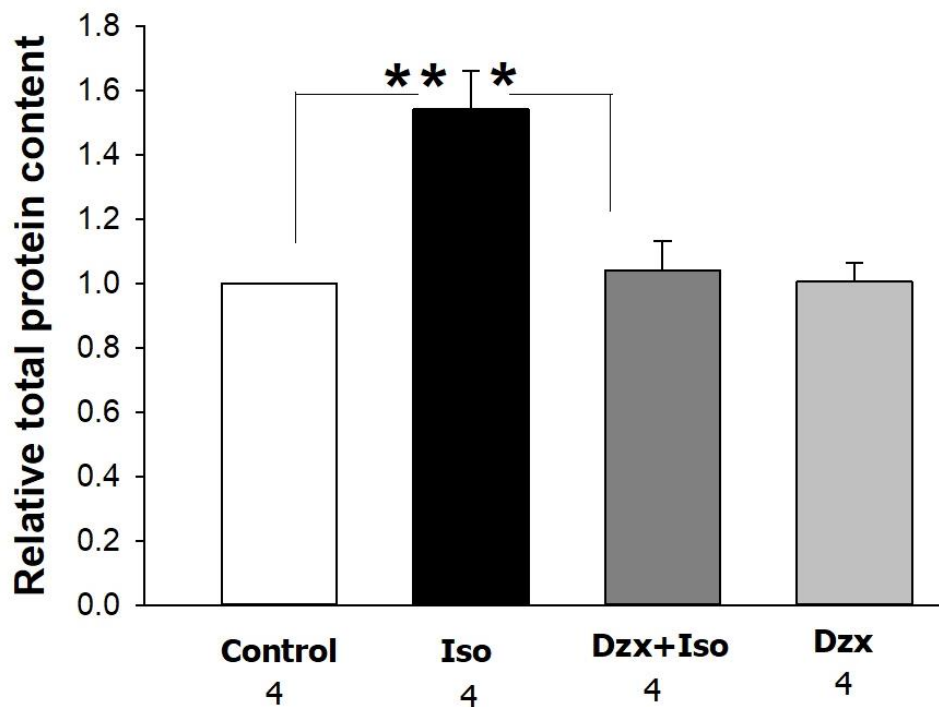
Figure 7.1.2. Total protein content induced by ISO was reduced by Dzx. Bar graphs show the relative total protein content under different treatment conditions measured by BCA assay.Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, $*p < 0.05$, $**p < 0.01$.

7.1.3. Dzx reduced the ventricle-to-body weight ratio and miR-132 expression induced by ISO

To induce hypertrophy β-agonists were applied and ventricle-to-body weight ratios and cell areas were measured after 48 hours treatment. Ventricle-to-body weight ratio was measured from control rats (0.9% NaCl), rats injected with ISO or Dzx+ISO. A significant increase in the ratio was found in ISO-treated rats while this ratio was decreased in Dzx+ISO-treated rats with different Dzx concentrations. This ratio is indicative of the hypertrophy index. As seen in Fig 3.1.2, Iso induced hypertrophy was reduced significantly by Dzx.
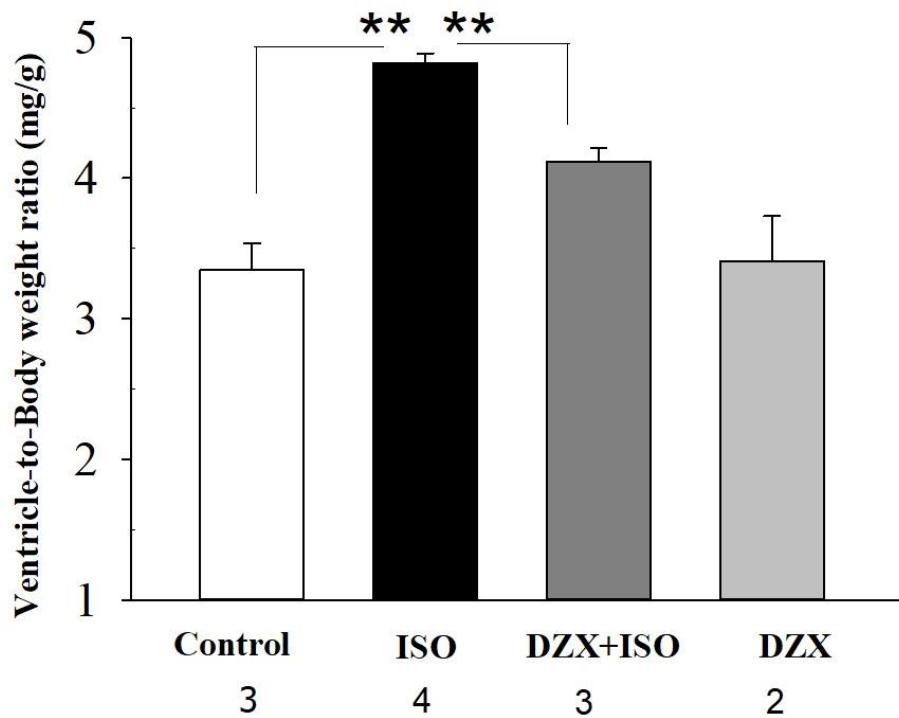
Figure7.1.3. Effect of Dzx on ventricle-to-body weight ratio and miR-132 expression. Bar graphs show the ventricle-to-body weight ratio (mg/g) obtained under different treatment conditions. Rats were treated with 20 mg/kg Dzx; 5 mg/kg ISO. Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, $**p < 0.01$.

7.2 Dzx reversed miR-132 expression induced by ISO in adult rat cardiomyocytes

miR-132 regulates cardiac hypertrophy (Ucar et al. 2012). Therefore, we tested the hypothesis that the effects of ISO and Dzx on cardiac hypertrophy that we characterized are mediated by changes in the expression of miR-132. In support of this idea our group previously reported that ISO up-regulates the expression of miR-132 (Carrillo et al. 2015). And my experiments revealed that Dzx decreased the expression of miR-132 induced by ISO as shown in Fig 3.2. This indicates that anti-hypertrophic effects of Dzx involve miR-132 regulation. Acute treatment with ISO led to up-regulation of miR-132 indicating the involvement of fast transcriptional changes (see below) and early responsive proteins in the process.

A.
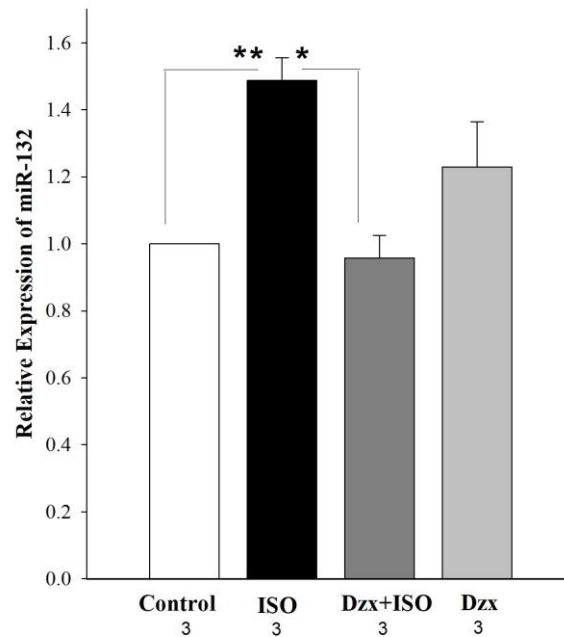
B.
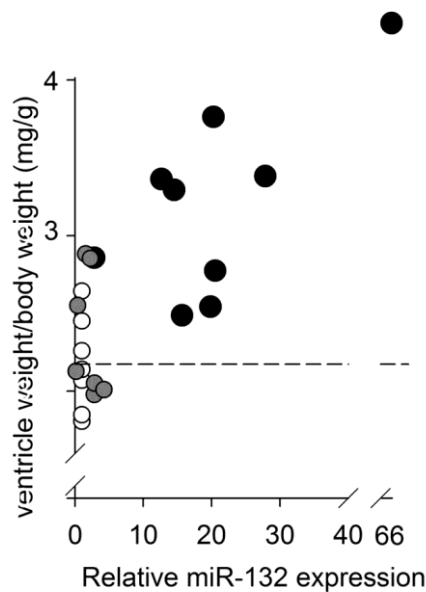
Figure7.2. A. Dzx reversed the expression of miR-132 induced by ISO. Bar graphs show relative expression of miR-132 in adult rat cardiomyocytes treated with ISO or Dzx+ISO compared to control cells. B. The relationship between miR-132 and ventricle-to-body weight ratios. Open circles, control; black and grey circles, ISO and ISO + Dzx treated rats, respectively.Values represent mean $\pm$ SE, number indicated below each experimental condition represents the number of groups, $*p < 0.05$, $**p < 0.01$.

3.3: ROS regulated miR-132 expression

H_2O_2 regulates the expression of various micro RNAs, including miR-132 in vascular smooth muscle cells (Lin et al. 2009) in time frame of 6-8 h. Therefore, we studied the effect of H_2O_2 on miR-132 in cardiomyocytes to assess whether ROS also regulates miR-132 expression in this preparation. Indeed, we found that H_2O_2 (100µM) increased the expression of miR-132 6 h after its application, as shown in Fig 3.3. H_2O_2 was applied only for 3-5 min and then cells were washed and left at room temperature for 6 h. We conclude that acute generation of ROS in cells treated with ISO is sufficient to up-regulate miR-132 expression.
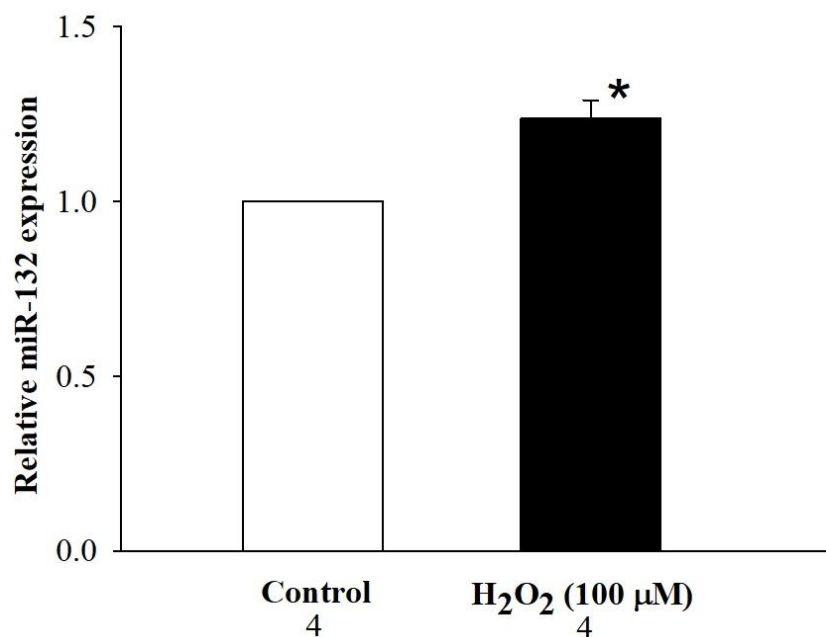
Figure7.3. ROS increases the expression of miR-132. A. Bar graphs show the relative expression of miR-132 in control and H_2O_2 (100µM) treated cells. Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, *p < 0.05.

7.4. Rate of ROS production induced by ISO decreased with Dzx treatment

We next checked the effects of ISO and Dzx on ROS productions. We found that the relative rate of ROS production increased in cells treated with ISO for 25-30 min. In contrast, in cells pretreated with Dzx for 1 h the relative rate of ROS production did not increase by ISO, being like that of control cells. We reached the conclusion that Dzx pre-treatment prevents the increase in relative ROS production rate induced by ISO.

A.

B.

C.

D.
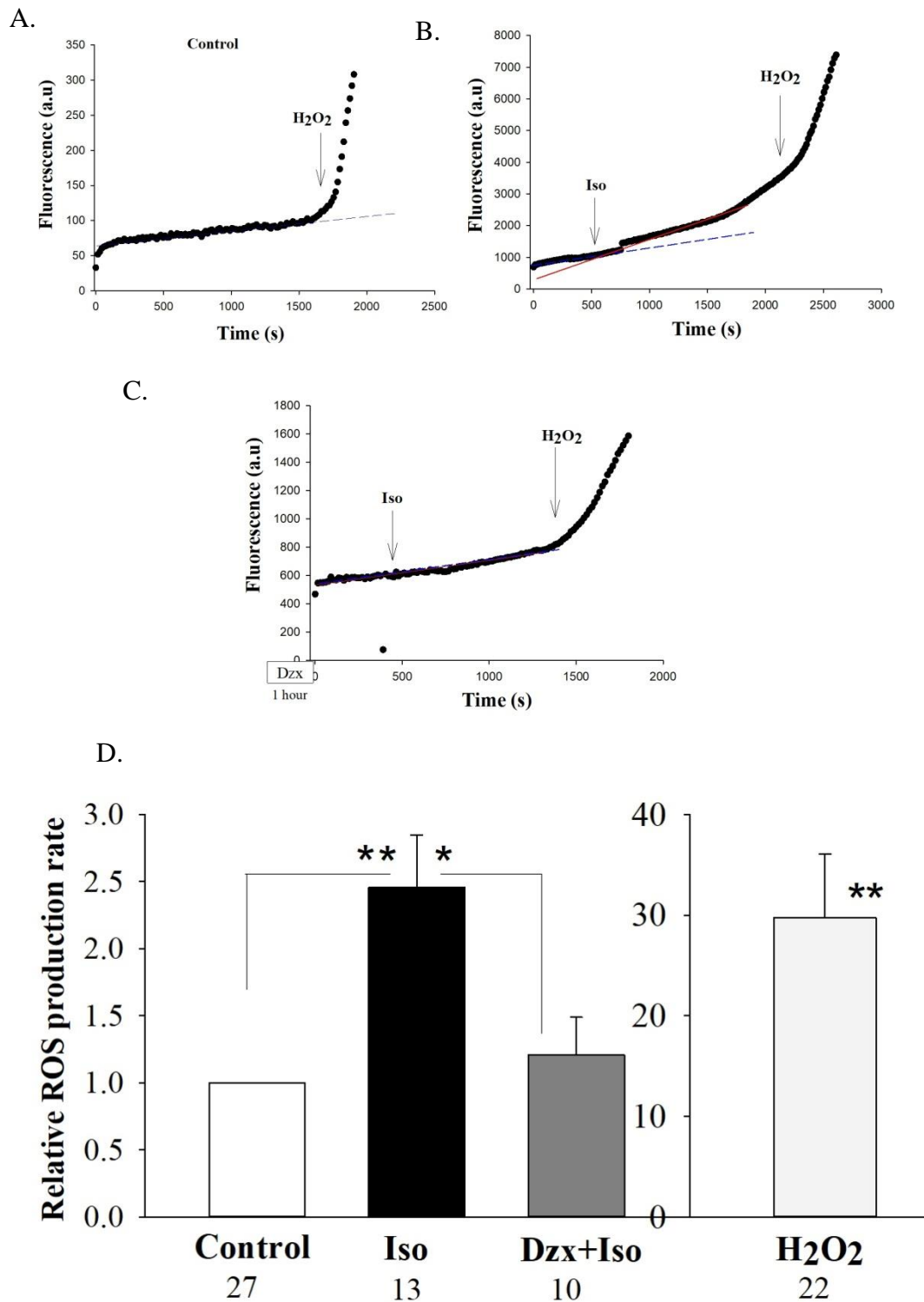
Figure 7.4. Relative rate of ROS production is increased by ISO and antagonized by Dzx. ROS production over time from representative experiments. In B-C, ISO and H_2O_2 were applied at the time indicated (arrows). In C, Dzx was applied 1hour before ROS measurements. Blue dotted lines are best fit to data points prior to ISO. Red solid lines are the

corresponding fits to data in the presence of ISO. D. The graphs show the relative rate of ROS production in control cells, cells treated with ISO, and cells treated with Dzx+ISO. For comparison, the effects of H_2O_2 on relative fluorescence are shown. Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, *p<.05, **p<.01.

7.5. SOD2 activity was reduced by ISO and restored by Dzx

Superoxide dismutase 2 catalyzes dismutation of superoxide anion radical into either hydrogen peroxide or molecular oxygen (Zelko et al. 2002). A reduction in superoxide dismutase 2 activity indicates oxidative stress (Koyama et al. 2013), therefore, we tested the effects of ISO on SOD2 activity and found that it significantly reduced its activity. As expected, Dzx prevented the reduction in SOD2 by ISO. Dzx by itself had inhibitory effects on SOD 2 activity probably related to its ambivalent effect on ROS production. During preconditioning, Dzx inhibits complex II and promotes transient generation of signalling ROS at complex III. While, during ischemia and reperfusion Dzx attenuates the production of ROS at complex I. Hence, depending on the metabolic state and the membrane potential of mitochondria Dzx modulated ROS production (Dröse et al. 2009).
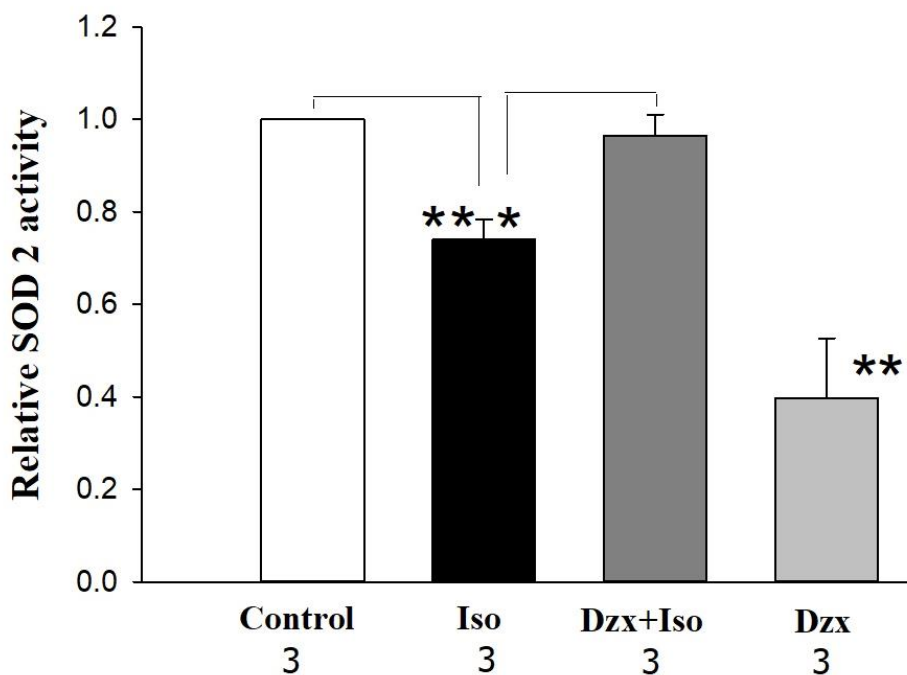
Figure 7.5. SOD2 activity is reduced by ISO. Bar graphs show the activity of SOD 2 in rats treated with ISO and Dzx+ISO for 12 h compared to control rats (injected s.c with 0.9%

NaCl). Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, $*p < 0.05$, $**p < 0.01$.

7.6. ROS promoted phosphorylation of CREB, an effect antagonized by Dzx

Phosphorylation of CREB up-regulates transcription of genes involved in hypertrophy (Funakoshi et al. 2002; Somvanshi et al. 2013) and it also increases transcription of miR-132 in neurons (Vo et al. 2005). However, the mechanism involved is unknown. We tested the hypothesis that ROS are involved in this process. In fact, we observed that H_2O_2 induced the phosphorylation of CREB and that Dzx pre-treatment to cells prevented this effect. Hence, we propose that ISO leads to phosphorylation of CREB via ROS. CREB phosphorylation by ROS is ESK-MEK dependent (Ozgen et al. 2009) and this pathway may be involved in ISO treated adult rat cardiomyocytes.
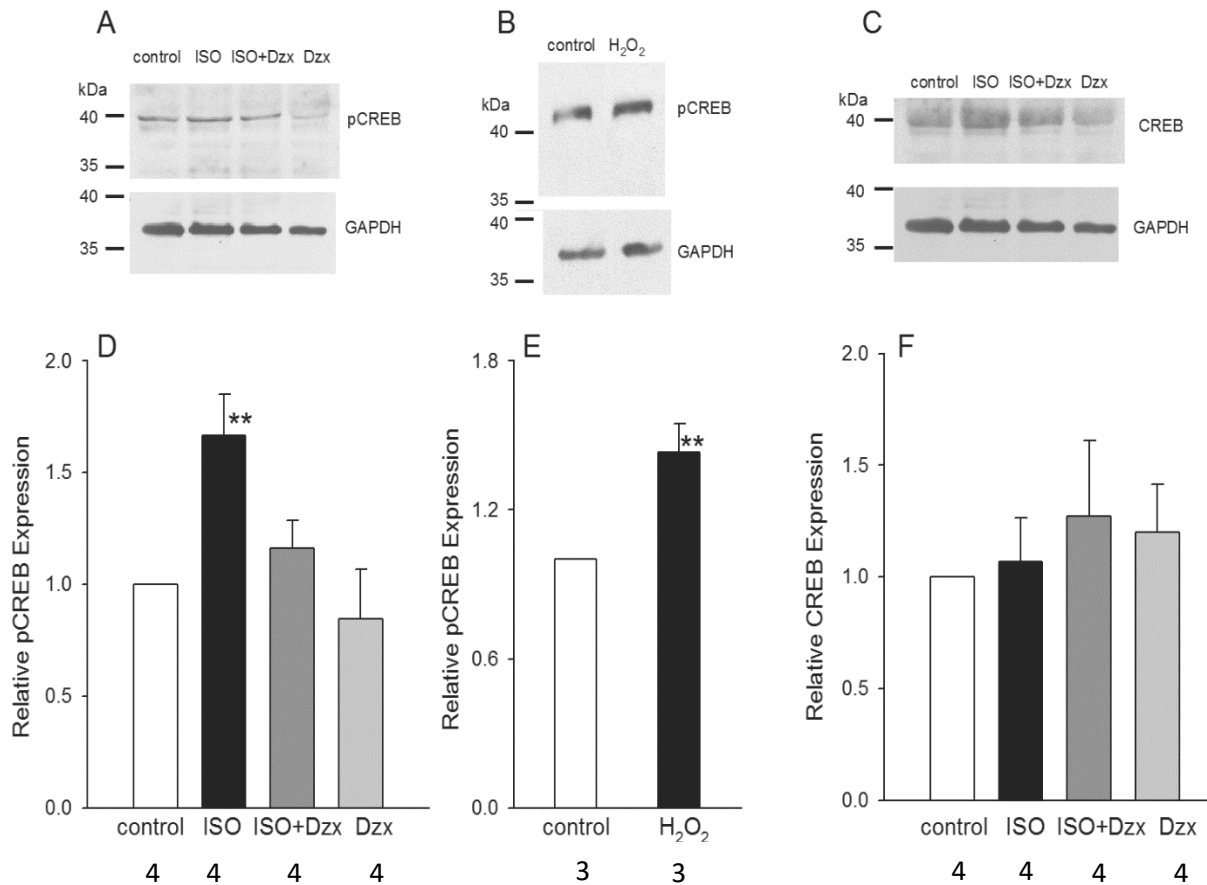
Figure 7.6. Phosphorylation of CREB is induced by ROS. A. Western blots of pCREB and corresponding GAPDH bands from control, ISO, Dzx+ISO and Dzx treated cells. B. Western blots of pCREB and GAPDH protein bands from H_2O_2 treated cells compared to control condition. C. Western blots of total CREB and corresponding GAPDH bands for the

indicated experimental conditions. D. Bar graphs show relative expression of pCREB in control, ISO treated cells, ISO treated cells pre-treated with Dzx and Dzx treated cells. E. Bar graphs show relative expression of pCREB in H_2O_2 treated cells compared to control cells. F. Bar graphs show the expression of total CREB under control, ISO, Dzx+ISO and Dzx conditions. Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, $**p < 0.01$.

7.7. Phosphorylation of CaMKII induced by ISO was prevented by Dzx

We confirmed that ISO phosphorylates CaMKII in a rat cardiac hypertrophy model as reported by (Liu et al. 2013). The effect of Dzx on the effect of ISO on CaMKII phosphorylation was investigated next. We found that 1 h pre-treatment with Dzx prevented this early event.
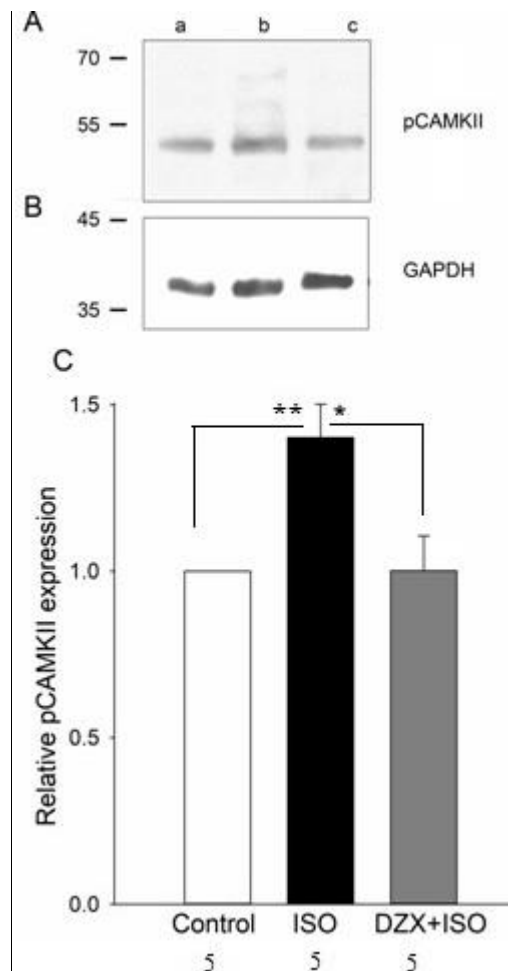
Figure 7.7. CaMKII phosphorylation is induced by ISO and blocked by Dzx. Western blots of A. pCAMKII and B. GAPDH protein bands under control, ISO and Dzx+ISO conditions. C. Bar graphs show the relative expression of pCAMKII under the same experimental

conditions. Values represent mean $\pm$ SE, number indicated below each experimental condition represents the number of groups, $*p < 0.05$, $**p < 0.01$.

7.8 The CaMKII inhibitor, KN-93 reduced hypertrophy induced by ISO

KN-93 is reported to prevent arrhythmic activity induced by beta adrenergic agonists in rabbit pulmonary veins (Lo et al. 2007). We tested the action of this inhibitor on our hypertrophy model and found that it showed anti-hypertrophic effects. Although KN-93 could not completely reverse the ventricle-to-body weight ratio, there was a significant reduction as seen in Fig 3.8.A. The anti-hypertrophic effect of KN-93 was also observed in neonatal rat ventricular cardiomyocytes. Fig. 3.8.B shows the images of cells under different treatment conditions. The cell area significantly increased in ISO-treated cells and it was significantly reduced by KN-93 as shown in Fig.3.8.C.

A.
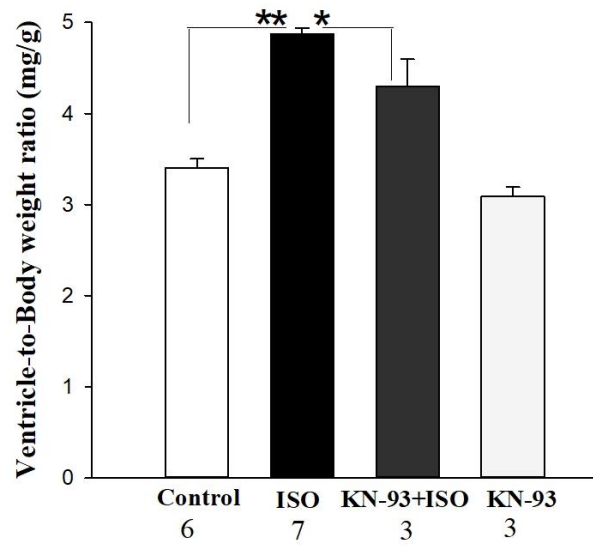
B.
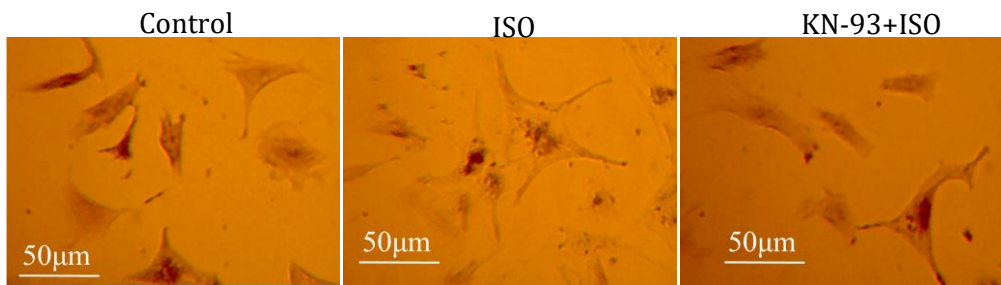
C.
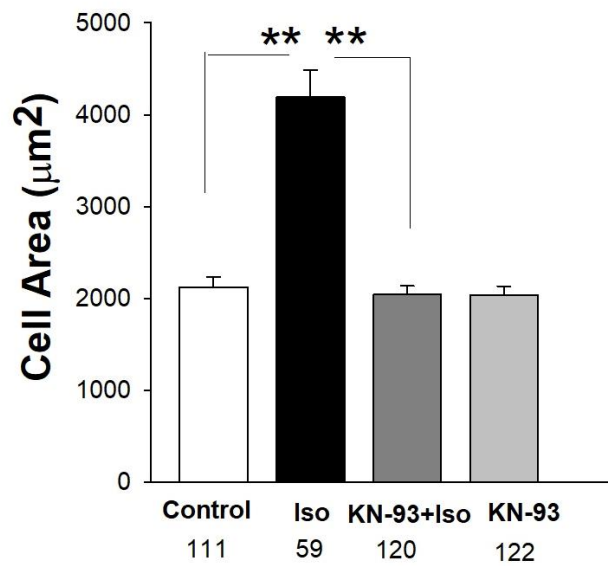
Figure 7.8. Effect of KN-93 on hypertrophy. A. Bar graphs show the ventricle-to body weight ratio of rats treated with ISO, KN-93+ISO and KN-93 compared to control rats

(injected s.c with 0.9% NaCl). B. Images of cultured neonatal rat ventricular myocytes under control, ISO and KN-93+ISO conditions. C. Bar graphs show the cell area measured under the indicated experimental conditions. Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, $*p < 0.05$, $**p < 0.01$.

7.9. KN-93 blocked the increase in the rate of ROS production by ISO

It is reported that increase in intracellular calcium activates CaMKII and stimulates ROS production (Nishio et al. 2012). We observed a similar effect in ISO stimulated cells. CaMKII phosphorylation by ISO increased the rate of ROS production. KN-93 completely blocked this increase as seen in Fig 3.9. KN-93 and Dzx (Fig.3.4) showed similar effects on ROS production rate.

A.

B.

C.
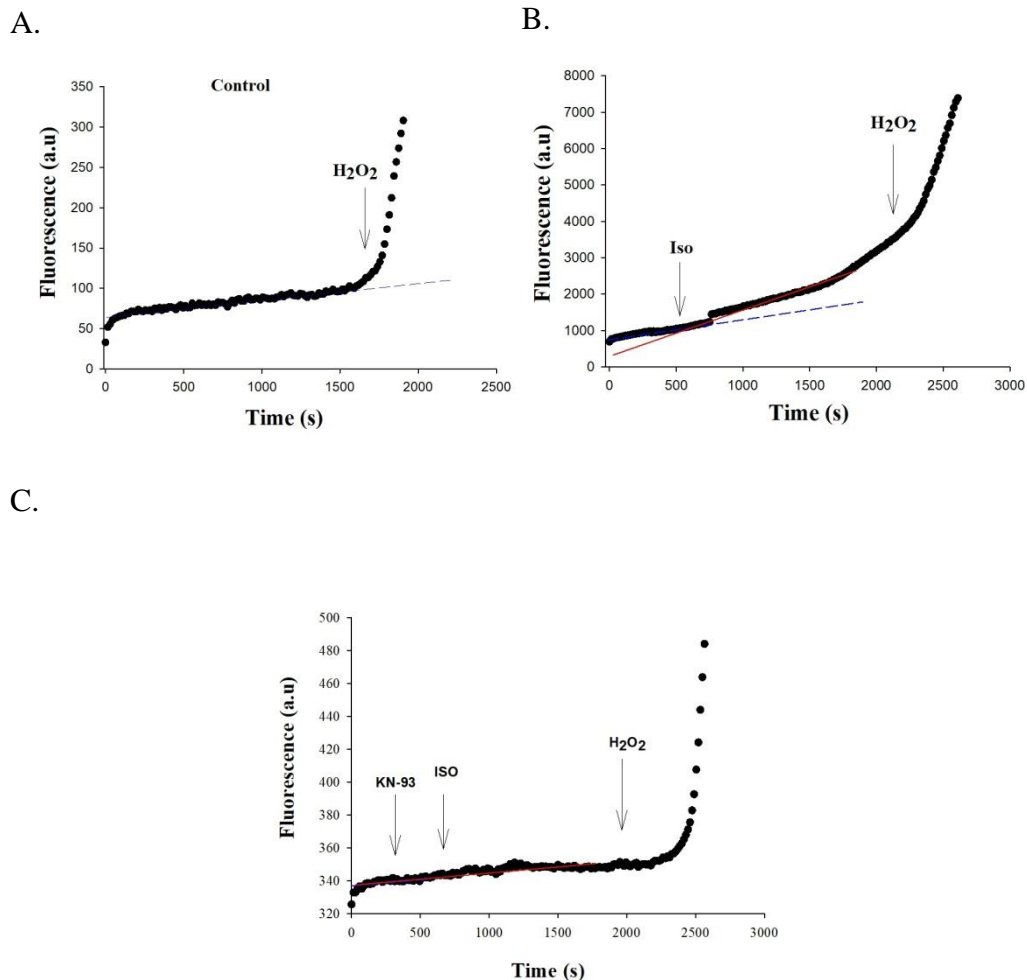
D.
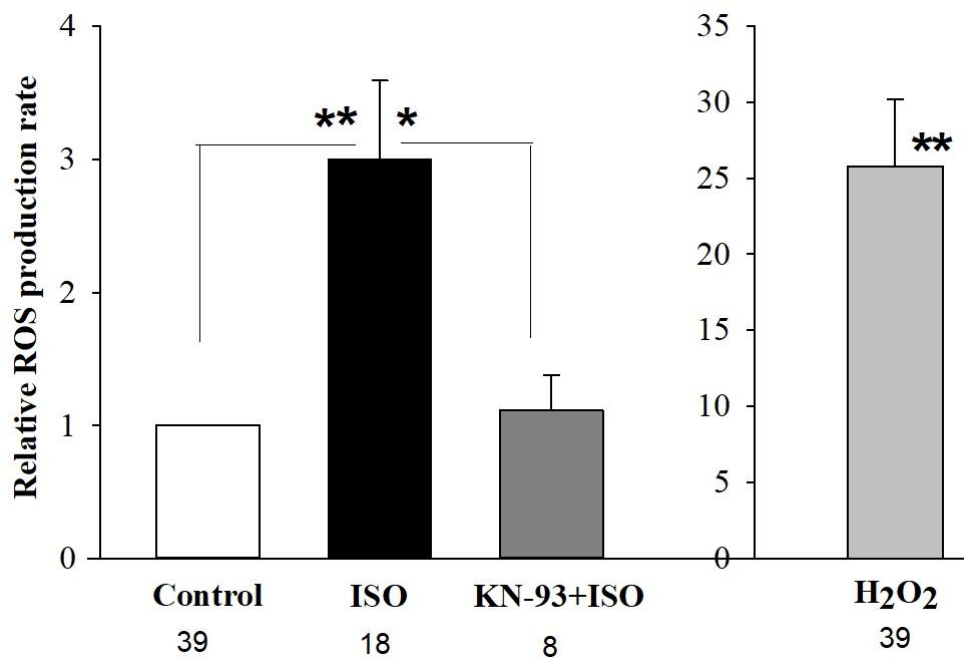
Figure.7.9. Effect of KN-93 on relative ROS production rate. A. ROS production over time from representative experiments. In B-C, ISO and H_2O_2 were applied at the time indicated (arrows). In C, KN-93 was applied 5 min before ISO. Blue dotted lines are best fit to data points prior to ISO. Red solid lines are the corresponding fits to data in the presence of ISO. D. Bar graphs showing the relative ROS production rate under indicated experimental conditions. Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, $*p < 0.05$, $**p < 0.01$.

7.10 KN-93 reduced the relative expression of pCREB

It is reported that ISO induced beta-adrenoreceptor-mediated cardiac hypertrophy involves cardiac oxidative stress. ISO induced ROS activate cardiac MAPK cascades including phosphorylation of CREB in 30 min of treatment (Zhang et al. 2005; Somvanshi at al. 2013). We observed an increase in CREB phosphorylation by ISO and it is reported that several upstream kinases phosphorylate CREB (Funakoshi et al. 2002). Hence, we tested the effect of KN-93 on pCREB levels to verify if CaMKII acts as an upstream kinase to phosphorylate CREB.

A.
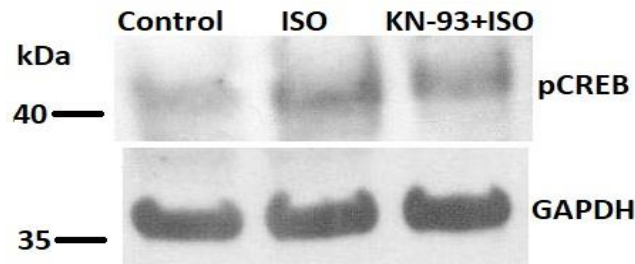
B.
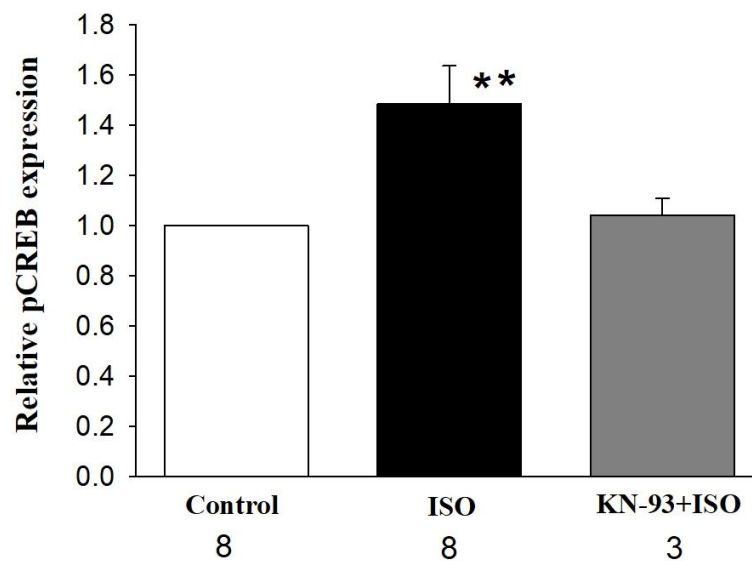
Figure.7.10. Effect of KN-93 on relative pCREB expression. A. Western blot showing the pCREB and GAPDH bands from control, ISO and KN-93+ISO treated cardiomyocytes. B. Bar graphs show the relative expression of pCREB under the indicated experimental conditions. Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, $**p < 0.01$ Vs control.

7.11 KN-93 does not inhibit miR-132 expression

miR-132 up-regulation is crucial for development of hypertrophy as we observed in ISO-treated cells and since KN-93 prevented the increase in ROS production rate by inhibiting CaMKII phosphorylation, a decrease in pCREB expression by KN-93 followed by reduction in miR-132 expression is expected. However, while we observed a significant up-regulation in miR-132 expression in ISO-treated cells, no reduction in miR-132 expression was observed in KN-93+ISO treated cells.

45
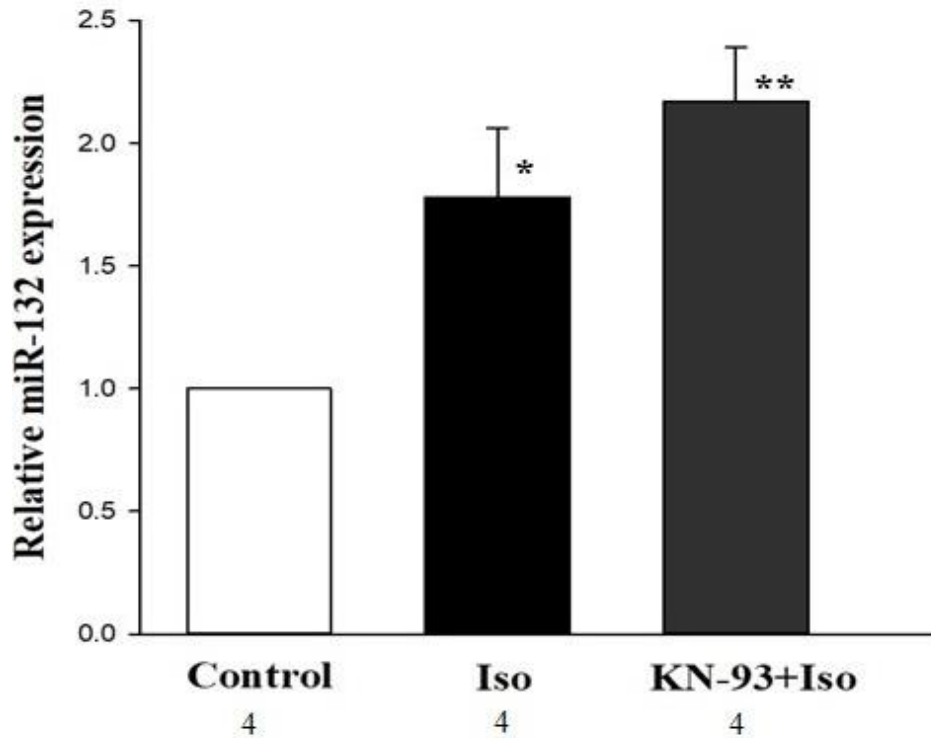
Figure.7.11. Effect of KN-93 on miR-132 expression. Bar graphs show the relative expression of miR-132 under indicated experimental condition. Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, $*p <$ 0.05, $**p < 0.01$ Vs control.

7.12 REST down-regulation by ISO was restored by Dzx but not by KN-93

We performed experiments to test the hypothesis that a transcription factor other than CREB, independent of ROS regulates miR-132 expression. Based on previous work we considered REST a likely candidate (Vo et al. 2005; Conaco et al. 2006; Wanet et al. 2012). We studied the expression of REST in control, ISO, Dzx+ISO and KN-93+ISO treated cells. Interestingly, we found that REST was down-regulated by ISO within 30 min of treatment. Pre-treatment with Dzx for 1 h restored the levels of REST in ISO-treated cells and KN-93 did not. This may explain our results since REST downregulation is expected to lead to an increase in miR-132 expression. It is reported that ectopic REST expression in adult rat ventricular myocytes prevents increased *Nppb* and *Nppa* mRNA levels (natriuretic peptide precursor A and natriuretic peptide precursor B) in response to endothelin-1 (Bingham et al. 2007; Kuwahara et al. 2001).

A.
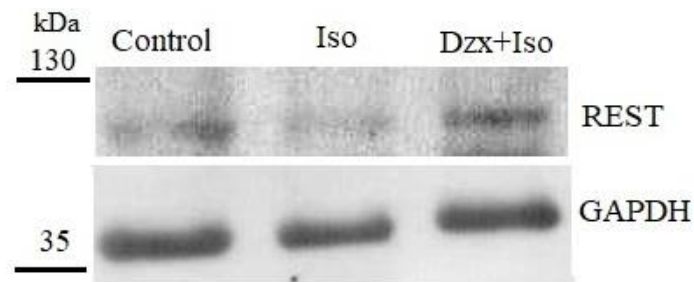
B.
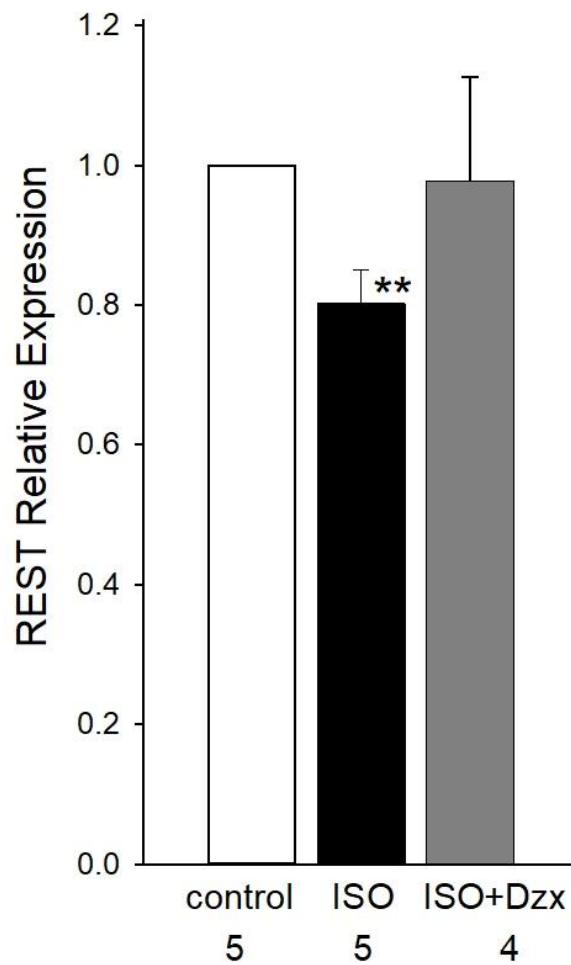
Figure 7.12. Effect of ISO on REST expression. A. Western blot showing the REST and GAPDH bands from control, ISO and ISO+Dzx treated cardiomyocytes. B. Bar graphs show the relative expression of REST under the indicated experimental conditions. Values represent mean $\pm$ SE, number indicated below each experimental condition represents the number of groups, $**p < 0.01$.

A.
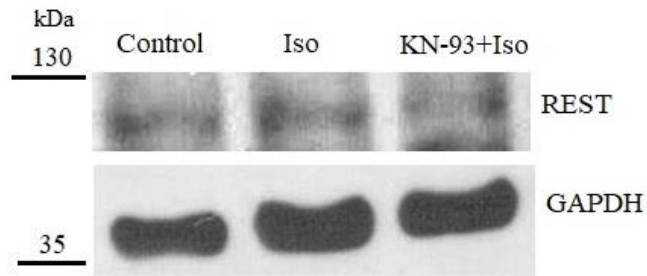
B.
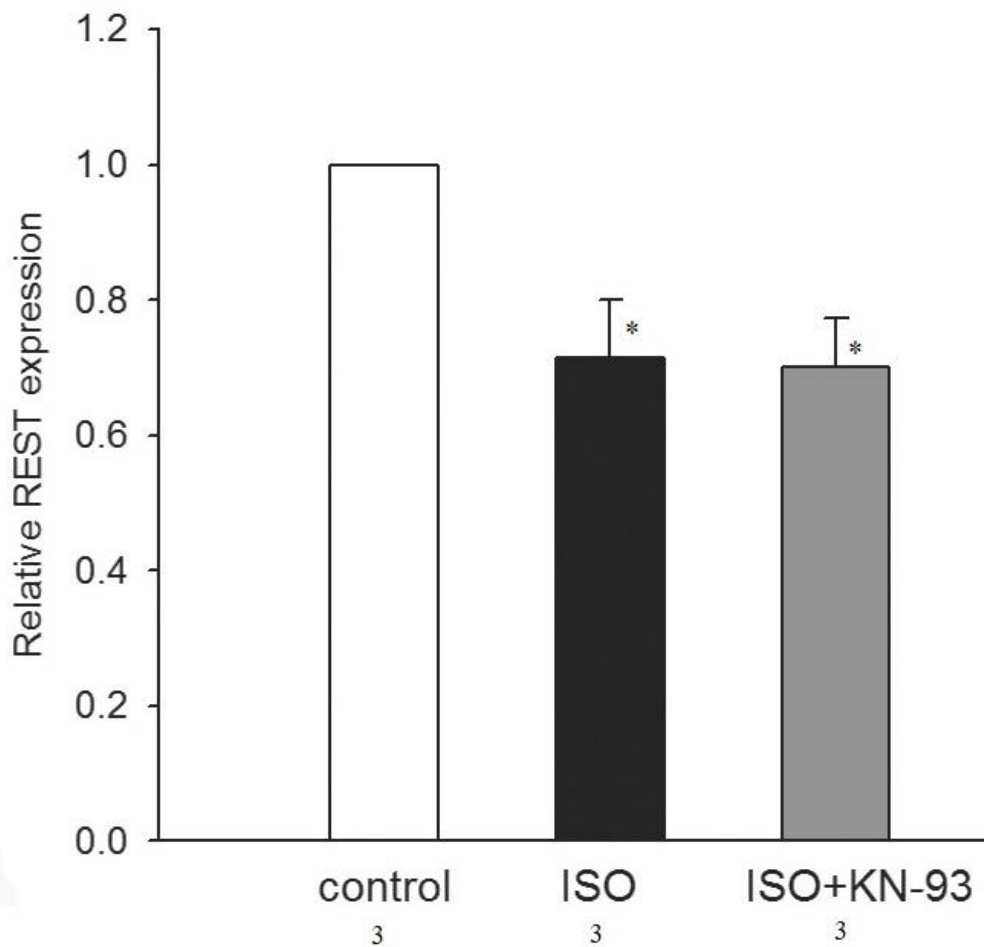
Figure 7.13. Effect of KN-93 on REST expression. A. Western blot showing the REST and GAPDH bands from control, ISO and ISO+KN-93 treated cardiomyocytes. B. Bar graphs show the relative expression of REST under the indicated experimental conditions. Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, $*p < 0.05$.

7.14 Mito-tempo (mitochondrial ROS scavenger) on miR-132 expression

Addition of the ROS scavenger mito-tempo in the presence of ISO did not reverse the expression of miR-132 to normal levels. This suggests the involvement of ROS-independent pathways in the regulation of miR-132.
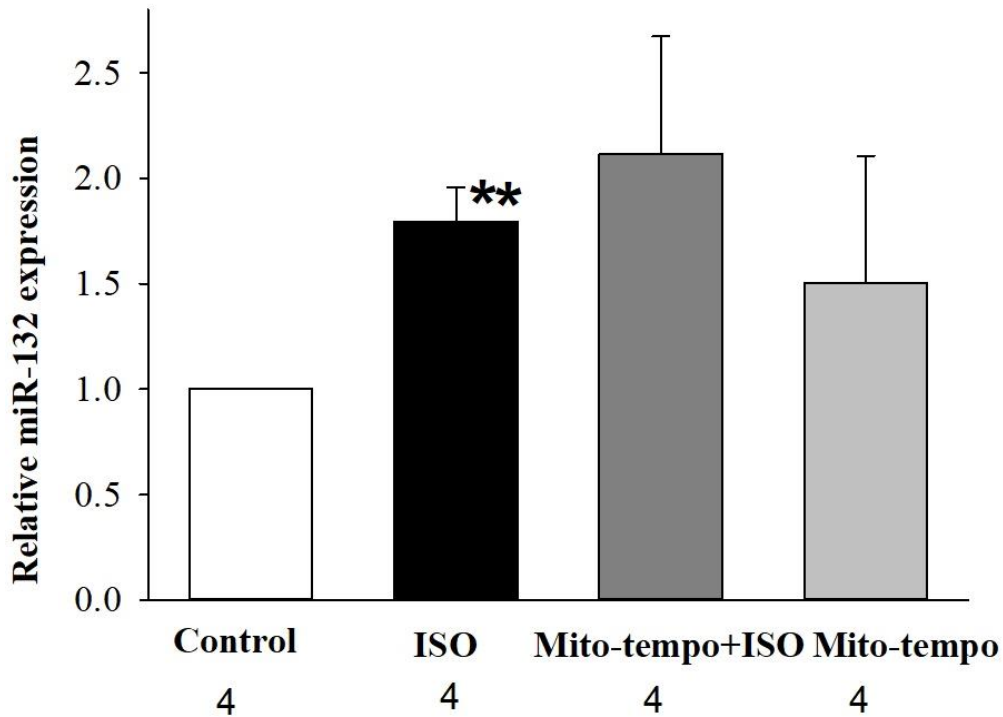
Figure 7.14. Effect of mito-tempo on miR-132 expression. Bar graphs showing the relative expression of miR-132 under indicated experimental condition. Values represent mean ± SE, number indicated below each experimental condition represents the number of groups, *$p <$ 0.01.

8. Discussion

8.1. ROS, miR-132 and hypertrophy

Arterial plasma noradrenaline predicts left ventricular mass in human patients who developed hypertrophy and high adrenergic activity is associated with cardiac hypertrophy (Strand et al. 2006). Several transcription factors are involved in development of hypertrophy and previous work has shown that catecholamines up-regulate calcineurin/NFAT and CaMKII/MEF-2 signaling to induce pathological cardiac hypertrophy (Sag et al. 2014). In addition to these signaling pathways, miR-132 has been shown to play a leading role. The expression of this microRNA is up-regulated by prolonged administration of ISO (Carrillo et al. 2011; Ucar et al. 2012) and overexpression of miR-132 leads to increased activation of calcineurin/NFAT signaling. Furthermore, antagomir injection of miR-132 rescues cardiac hypertrophy. miR-132 directly targets and down-regulates the anti-hypertrophic FoxO3 transcription factor (Ucar et al. 2012).

Hypertrophy is also associated with ROS production. The local elevation of ROS by a variety of sources has been shown to regulate hypertrophy-related signaling pathways (Sag et al, 2014) and we found in the present experiments that application of H_2O_2 brought about a significant increase in the expression of the pro-hypertrophic miR-132. H_2O_2 is one of the main ROS involved in redox signaling in cells (Burgoyne et al. 2012). To the best of our knowledge this is first demonstration that H_2O_2 up-regulates the expression of miR-132 in cardiac myocytes. We also found elevation in the rate of ROS production by ISO in myocytes that were externally paced. The increase in ROS production by activation of β-adrenergic receptors in the electrically stimulated heart cell was previously observed by Andersson et al. (2011) and it is not seen in the quiescent myocyte (Bovo et al, 2012). This is probably related to the role of L-type Ca^{2+} channels. Phosphorylation of several protein kinases in cardiac myocytes by H_2O_2 requires activation of L-type Ca^{2+} channels that normally open during action potentials (Sartoretto et al. 2012). Interactions between ROS and Ca^{2+} are reciprocal. Thus, the increase in the amplitude of L-type Ca^{2+} currents, Ca^{2+} transients and contraction produced by ISO are abrogated by the ROS scavenger N-acetylcysteine (NAC) (Andersson et al. 2011). NAC is a general antioxidant that reacts with hydroxyl radicals and with the non-radical hydrogen peroxide (Aruoma et al. 1989).

Given the role of ROS in the expression of miR-132 and hypertrophy it would be expected that drugs that decrease ROS production would have anti-hypertrophic effects. We found in

fact that diazoxide, a mitochondrial K_{ATP} channel opener blocked the increase in the rate of ROS production produced by ISO and it also blocked the increase in the expression of miR-132 produced by ISO but only partially prevented hypertrophy induced by this β-adrenergic agonist. Based on these observations we suggest that an increase in miR-132 expression is not strictly necessary for development of hypertrophy. A decrease in ROS production by diazoxide has been previously reported during ischemic and reperfusion conditions in Langerdorff-perfused rat hearts (Pasdois et al. 2008).

8.2. miR-132 and pCREB

Our experiments demonstrated an increase in the expression of the phosphorylated form of CREB by ISO with no changes in the levels of CREB. This suggests that ISO promotes phosphorylation of pre-existing CREB. It has been previously demonstrated that application of ISO to isolated cardiac myocytes increases the expression of pCREB within minutes (Goldspink and Russell 1994) and that phosphorylation of CREB increases its transcriptional activity (Mayr and Montminy 2001). Our experiments further demonstrated that H_2O_2 increases phosphorylation of CREB in the adult cardiomyocyte. Enhanced phosphorylation of CREB by H_2O_2 has been previously observed in neonatal myocytes (Ozgen et al. 2009). We also showed that diazoxide decreases ROS production and blocks up-regulation of pCREB by ISO. Taken together, our results are consistent with the view that phosphorylation of CREB by ROS results in enhanced transcription of miR-132 by pCREB. This conclusion is supported by the fact that the genomic features of the miR-132 locus of the mouse genome include four CREB binding sites which are highly conserved among the rat, mouse and human genomes (Wanet et al. 2012).

8.3. CaMKII

The multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has been implicated in cardiac hypertrophy and it is activated by β-adrenergic stimulation. Its nuclear form has been shown to regulate gene expression in neonatal cardiac myocytes (Ramirez et al. 1997).

In different cell types Ca^{2+} can bind to CaM which then translocates into the nucleus activating CaMK resulting in phosphorylation of the transcription factor CREB (cAMP response element binding protein) which promotes transcription of c-fos. CREB residue Ser-133 was the major site of phosphorylation by the CaM kinases in vitro and of

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phosphorylation after membrane depolarization in vivo. Interestingly, phosphorylation of Ser-142 seems to negatively regulate CREB-dependent transcription. CaM might also be associated with the nuclear envelope. In the heart, overexpression of CaM in transgenic mice causes severe cardiac hypertrophy, and results in higher CaMKII phosphorylation (and activity), and the expression of the hypertrophic marker atrial natriuretic factor (ANF). Interestingly, the CaM antagonist W-7 and the CaMKII antagonist KN-62 could prevent hypertrophy in cultured myocytes, further implicating Ca-CaM as a mediator of hypertrophic response. Ramirez et al. showed that specific activation of cardiac nuclear CaMKII-dB, but not the cytosolic form CaMKII-dC, in cultured neonatal myocytes was important in hypertrophic gene expression. Transgenic mice overexpressing nuclear CaMKII-dB also demonstrated cardiac hypertrophy and dilation.

Calmodulin-dependent kinase II (CaMKII) is a serine/threonine kinase that is regulated by the Ca^{2+}/calmodulin complex, ROS, and exchange proteins directly activated by cAMP (EPAC). Catecholamines bind to adrenergic receptors, which are classified as G-protein-coupled receptors, and signaling via these receptors activates CaMKII. Activation of the renin-angiotensin-aldosterone system increases the angiotensin II level, and directly induces cardiac hypertrophy via activation of CaMKII signaling. Studies performed in humans and rodents have shown that cardiac CaMKII activity is increased in heart failure. A number of transcriptional factors (e.g. NFAT, GATA4, MEF2, SRF, NF-κB and PGC1α) are involved in driving this hypertrophic program, and these factors in turn are under the control of a multitude of interacting signaling pathways. In the intact heart, chronic β-adrenergic stimulation of neonatal rat cardiomyocytes produces hypertrophy and brings about changes in gene expression patterns that mimic the fetal gene program observed in failing hearts. This fetal gene response is dependent of Ca^{2+}/calmodulin-protein kinase (CaMK) pathway (Sucharov et al. 2006). CaMKII is involved in the dynamic modulation of cellular Ca^{2+} regulation and has been implicated in the development of cardiac hypertrophy and heart failure (Zhang and Brown 2004).

8.4. Mitochondrial K$_{ATP}$ channel

K$_{ATP}$ channels were first discovered in the plasma membrane of cardiac myocytes (Noma 1983) and they play a relevant role as a link between excitability and metabolism in many cells (Foster and Coetzee 2016). K$_{ATP}$ channels have also been described in the inner membrane of mitochondria (Inoue et al. 1991). Roles of mitoK$_{ATP}$ channels include control of mitochondrial volume and the efficient energy transfer from mitochondria to cytosol (Liu et al. 2016; Foster and Coetzee 2016). In addition to their physiological roles, opening of mitoK$_{ATP}$ channels has been associated with protective effects during ischemic and pharmacological preconditioning (González et al. 2010). Moreover, opening of mitoK$_{ATP}$ channels has beneficial effects against hypertrophic cardiomyopathy, one of the leading causes of mortality and morbidity, but the underlying protective mechanism is unclear (Liu et al. 2016).

Unlike the indeterminacy of its structure, the basic function of mitoK$_{ATP}$ is relatively clear in the heart, though it is not completely understood. Under stress induced by multiple stimuli, efficient energy transfer from mitochondria to cytosol is guaranteed by mitoK$_{ATP}$ activation. Extrinsic stressful signals, including reactive oxygen species, transduced across the cytosol to the mitochondria, may induce the activation of mitoK$_{ATP}$, whose opening would decrease opening of the mitochondrial permeability transition (MPT) pore, which would result in myocyte death. In another study, the induction of hypertrophy in cultured ventricular myocytes by alpha1 adrenoceptor agonist phenylephrine (PE) was evidenced by increased cell size, elevated expression of myosin light chain-2 and atrial natriuretic peptide. Diazoxide, as one of the canonical mitoK$_{ATP}$ openers, almost completely prevented the hypertrophic inductive effects of PE. Of note, diazoxide, a K$_{ATP}$ opener, showed cardioprotective effects. After introduction of oxidative stress, the activity of mitoK$_{ATP}$ was upregulated according to a study by Brown et al. (2005) and Quindry et al. (2010, 2012). They concluded that K$_{ATP}$ acted as a molecular sensor for oxidative stress, whose activation helps to reduce free-radical generation in the mitochondrial respiratory chain. However, the study did assess to observe the activity of mitoK$_{ATP}$ during sustained and severe oxidative stress, which may have induced significant mitochondrial dysfunction, and the activity and expression of mitoK$_{ATP}$ may have been jeopardized under these conditions.

8.5. CaMKII and ROS

It is well known that CaMKII is activated by the elevation in intracellular calcium but more recently it`s activity has been shown to be modulated by ROS-dependent oxidation. It is involved in rapid-pacing induced cell death. KN-93, a pharmacological inhibitor of CaMKII, prevents the reduction in cell viability and the increase in apoptotic proteins such as caspase-3 activity and Bax-Bcl2 ratio induced by rapid pacing (Sepúlveda et al. 2013). In addition CaMKII activates several isoforms of NADPH oxidase (Sánchez et al. 2005; Yamamoto et al. 2006). KN-93 also prevents rapid pacing-induced ROS production indicating that the ROS produced during rapid pacing is CaMKII-dependent (Sepúlveda et al. 2013). An increase in intracellular calcium activated CaMKII which further led to up-regulation of NADPH oxidase (Nishio et al. 2012). Increased ROS production by CaMKII activation is cytosolic calcium-dependent (Odagiri et al. 2009). Also, CaMKII is activated by ROS-induced oxidation by mitochondrial membrane depolarization in a Ca^{2+} independent manner (Erickson et al. 2008). Hence, a positive feedback regulation of CaMKII activity is possible by ROS, increased in a Ca^{2+} dependent fashion. A recent report showed that CaMKII activation increased the activity of NHE (Na^+/H^+ exchanger) which may generate a vicious circle leading to ROS increase by mutual enhancement between CaMKII and NHE (Vila-Petroff et al. 2010).

It has been proposed that CaM (calmodulin) induced changes in mitochondrial Ca^{2+} homeostasis may lead to mitochondrial ROS production by affecting the oxidant buffering potential. Also, phosphorylation of CaMKII targets such as δ subunit of ATP-synthase and monoamine oxidase which are located in the outer membrane of mitochondria might have influenced the rate of respiration and further the production of ROS (Andreyev et al, 2005). Ca^{2+} dependent dehydrogenases that transfer more electrons to the respiratory chain and stimulate oxidative phosphorylation may be activated by CaM induced changes in mitochondrial Ca^{2+} (Odagiri et al. 2009). In another study, transient potential receptor melastatin-2 (TRPM-2), a Ca^{2+} permeable cation channel and CaMKII cascade were activated by oxidative stress to further induce intracellular ROS production. This TRPM2-Ca^{2+}-CaMKII-ROS signal loop led to mitochondria fragmentation and loss of mitochondrial membrane potential (Wang et al, 2017). We also studied the effect of KN-93 on ROS production in stimulated cardiomyocytes and observed a complete reduction in ROS as compared to ISO-treated cells indicating that the phosphorylation of CaMKII is essential for ROS production. Further studies on how CaMKII induces ROS and if the process is Ca^{2+}

dependent or independent have to be performed to unveil the regulation of ROS by CaMKII. Also, our experiments revealed that opening mitochondrial potassium ATP channel with diazoxide prevented phosphorylation of CaMKII induced by ISO. These observations provide clues on altered Ca^{2+} homeostasis and mitochondrial respiration rate possibly due to mitochondrial membrane depolarization. Calcium and electron transport changes in mitochondria by β-adrenergic stimulation might contribute to changes in ROS production. In addition KN-93 restored the levels of CREB induced by ISO implicating that CREB phosphorylation at Ser -133 residues is mediated by CaMKII.

8.6. REST and miR-132

REST is a transcriptional repressor that actively represses neuronal gene expression in non-neuronal cells (Qureshi and Mehler 2009). Upon neuronal differentiation REST is targeted to proteolysis by conjugating it to ubiquitin by the E3 ubiquitin ligase $SCF^{\beta TRCP}$ enabling REST target genes to express (Westbrook et al. 2008). REST is involved in the expression of miR-132 which is demonstrated as an increased miR-132 expression in mouse embryonic fibroblasts expressing a dominant negative form of REST (Conaco et al. 2006). Entire miR-212/132 locus is under the control of REST as both miR-212 and miR-132 are derived from the same primary transcript (Wanet et al. 2012). In our studies we observed that REST is down-regulated in ISO treated cells within 30 min. As REST negatively regulates miR-132, we might relate this effect to the enhancement in miR-132 expression by ISO. Consistent with the observation that levels of REST were restored by Dzx in the presence of ISO we found a significant reduction in miR-132 expression by Dzx, in addition to its effects on p-CREB. Therefore, Dzx regulates the expression of miR-132 through two different pathways: pCREB and REST, unlike KN-93 neither restored the levels of REST in the presence of ISO nor brought the levels of miR-132 back to normal values in ISO- treated cells.

Stability of REST is regulated by the $SCF^{\beta TRCP}$, an E3 ubiquitin ligase which leads to ubiquitination and subsequent proteasomal degradation in both non-neuronal and neuronal cells (Guardavaccaro et al. 2008; Westbrook et al. 2008). REST is phosphorylated and targeted for β-Trcp dependent, ubiquitin-based proteasomal degradation by its association with an upstream serine/threonine kinase, Casein kinase 1 (Kaneko et al. 2014). Hence, studies using inhibitors for the proteasome such as lactacystin or MG132 or casein kinase 1 inhibitor, D4476 need to be performed to verify their actions on miR-132 expression and hypertrophy. Although a detailed understanding on the mechanism of REST involvement in

β-adrenergic stimulated cardiac hypertrophy has to be elucidated, we observed the involvement of REST in a ROS-independent pathway in the regulation of miR-132 in hypertrophy.

9. Conclusion

- The expression of miR-132 is regulated by ROS-dependent and ROS-independent pathways.
- CaMKII plays an early role in the ROS-dependent phosphorylation of CREB.
- Both CREB and REST regulate miR-132 expression in ISO-induced cardiac hypertrophy.
- Diazoxide reduced miR-132 expression by targeting both CREB and REST expression.
- Diazoxide reduced cardiac hypertrophy induced by ISO.

10. Perspectives

Diazoxide provides anti-hypertrophic effects by down-regulating the expression of miR-132 in both ROS dependent and independent manners. A pharmacological agent that can target both CREB and REST transcription factors could prove effective in reducing miR-132 expression and hypertrophy. Since, miR-132 targets FoxO3, an anti-hypertrophic and pro-autophagic transcription factor, the actions of diazoxide on FoxO3 expression could be the successive aim for study. Delineating the mechanism by which diazoxide regulates miR-132 expression will provide insights on therapeutic strategies targeting factors involved in transcription for growth and differentiation and apoptosis.

Restoring REST levels using proteasome inhibitors to prevent its degradation and evaluating its effect on hypertrophic process could be an interesting outcome. The role of REST in the expression of miR-132 and development of hypertrophy is less explored. Thus, studies using inhibitors for CREB and REST on the factors involved in ROS-dependent and independent pathways will provide better understanding on the molecular mechanism of isoproterenol-induced cardiac hypertrophy.

11. Appendices

Appendix A

Mitochondrial K$_{ATP}$ channels in cardiac hypertrophy
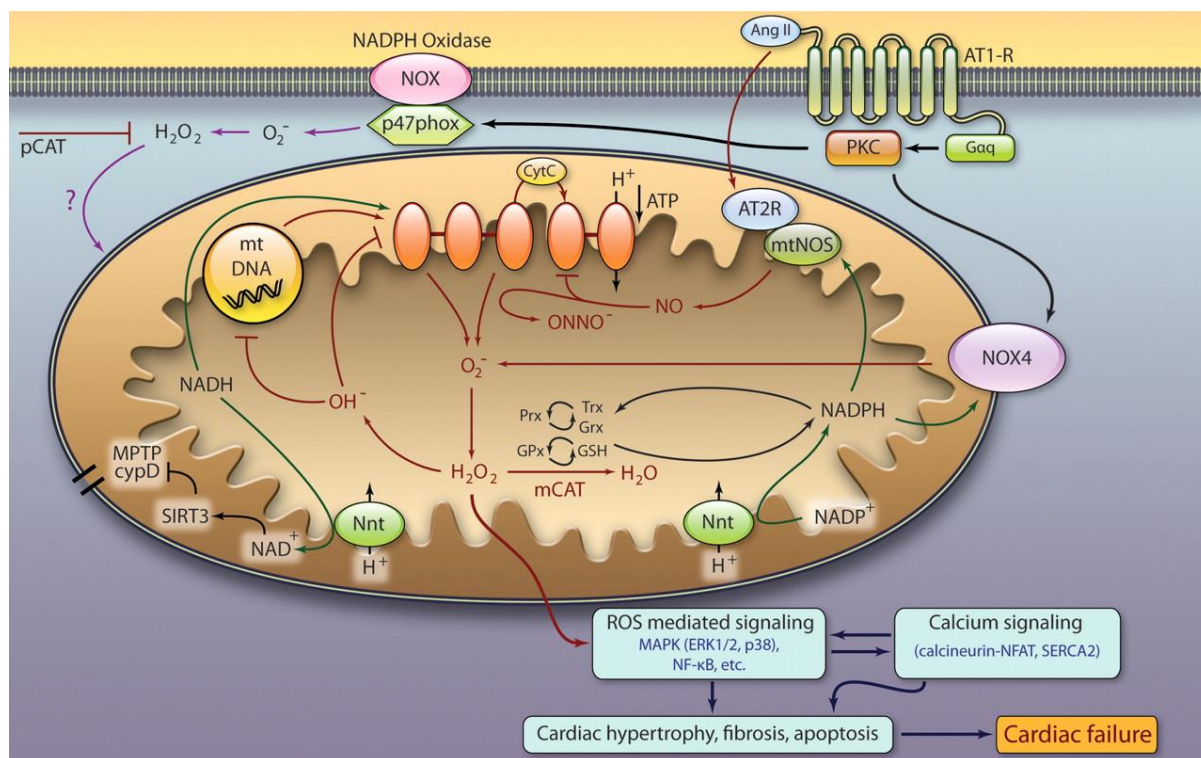
Figure A1. Proposed signaling mechanism of angiotensin/Gαq and mitochondrial ROS amplification in aging and cardiovascular diseases. AT1-R, angiotensin receptor-1; Nnt, nicotinamide nucleotide transhydrogenase. Modified from Dai et al, (2012).

Pharmacological interventions in the mitochondria during heart disease

Pharmacological agent	Mitochondria target and effect	Physiological/clinical implications	References
Metformin	Activation of AMPK signalling Activation of AMPK signalling, increased PGC-1α	Preservation of cardiac function, infarct size reduction Improved left ventricular function	(Benes et al. 2011; Yin et al. 2011; Gundewar et al. 2009)
Angiotensin receptor-neprilysin inhibitor (e.g., Sacubitril/Valsartan)	Currently unknown	Improved cardiac function and reduced fibrosis in rats, reduced cardiovascular death and heart failure hospitalization in humans	(Suematsu et al. 2016; Solomon et al. 2016)

Nonselective beta blocker/alpha-1 blocker (e.g.,Cardevilol)	Increased PGC-1α mediated by PKA-CREB	Elevated heart rate in patients with reduced ejection fraction	(DeVore et al. 2016)
Phosphodiesterases type 5 inhibitor	Upregulation of PGC-1α, increase in mtDNA content	Delayed heart failure progression and reversed cardiac remodelling	(Schwartz et al. 2012)
Tetrahydrobiopterin (BH4)	Prevention of eNOS uncoupling	Reduced left ventricular hypertrophy, cardiac dysfunction, and fibrosis due to pressure overload (mouse model)	(Moens et al. 2011, 2008)
Reseveratrol	Activation of PI3K/AKT/eNOS signalling pathway Activation of SIRT1 and mitochondrial biogenesis marker	Decreased left atrial fibrosis, regulated variation in ion channels Ameliorated Ang-II induced cardiac remodelling	(Chong et al. 2015; Biala et al. 2010)

Table A1. A summary of selected common and recent mitochondrial targeting pharmacologic drugs used in patients with heart failure. AMPK, adenosine monophosphate-activated kinase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1α; PKA, protein kinase A; CREB, cAMP response element binding protein; eNOS, endothelial nitric oxide synthase; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; SIRT1, silent mating type information regulation 2 homolog 1; Ang II, angiotensin II (Marquez et al. 2006).

Appendix B

Reactive oxygen species in cardiac hypertrophy

Activation of many signal transduction protein kinases, transcription factors, stimulation of DNA synthesis and expression of growth-related genes and regulation of myocardial excitation-contraction coupling involves redox signalling processes (Finkel 1999; Li and Shah 2004; Gao and Keene 1996). Myocardial infarction and rapid-pacing induced heart failure involve ROS derived from mitochondria (Ide et al. 2001). Activation of mitogen-activated protein kinases and transcription factor nuclear factor-κB is mediated by ROS produced by hypertrophic agonists. Norepinephrine, endothelin-1, angiotensin II, tumor necrosis factor α or pulsatile mechanical stretch induce cardiac hypertrophy in cultured cardiomyocytes and involve ROS production as evidenced by inhibition with antioxidants

(Cave et al. 2005). In angiotensin II induced left ventricular hypertrophy model, there is an increase in atrial natriuretic factor expression, a marker of hypertrophy which is inhibited in a Nox-2 knockout mice. This indicated that hypertrophy induced by angiotensin II is dependent on Nox-2 (Bendall et al. 2002). Pressure overload left ventricular hypertrophy development is due to ROS production following uncoupling of nitric oxide synthase indicating that Nox-2 is central to development of hypertrophy (Takimoto et al. 2005).

Alpha-adrenergic receptor hypertrophic signalling in adult rat ventricular myocytes is dependent on a small GTPase RAS in a redox-sensitive manner. Thiols on RAS are post translationally modified by thioredoxin-1 (Kuster et al. 2005). MEK1/2, ERK1/2 and p90ASK are phosphorylated within 5 min after acute activation of RAS in cardiomyocytes. NADPH oxidase activation is mediated by RAS to generate intracellular ROS. Superoxide production is inhibited by dominant negative Ras or Rac 1 in fibroblasts transformed with constitutively active isoform of p21Ras and H-Ras[v12] (Irani et al. 1997).
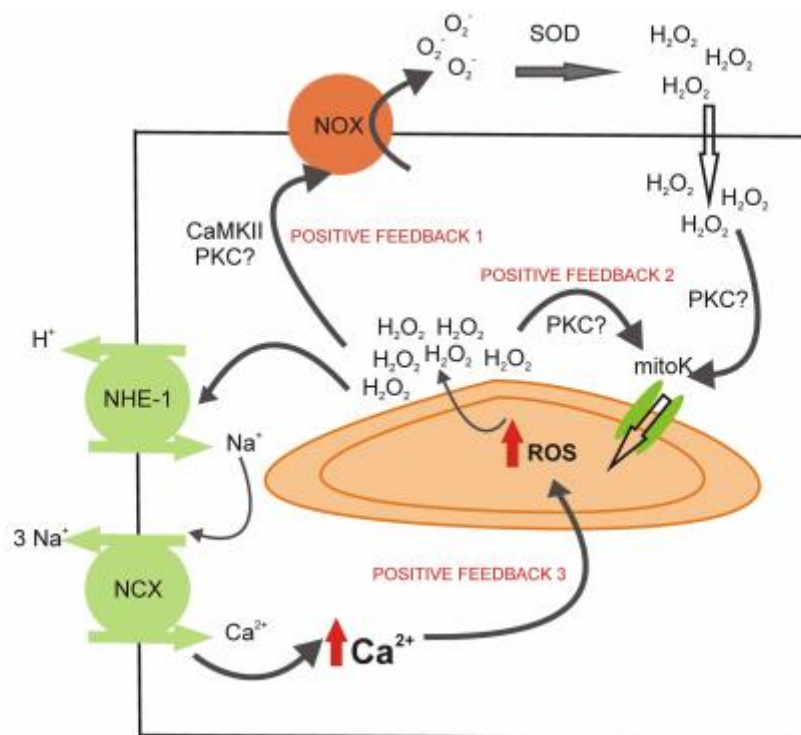
Figure B1. Potential sites of positive feedback mechanisms involved in mitochondrial ROS production during activation of RAAS. H_2O_2 released by mitochondria during the ROS-induced ROS-release mechanism can activate NOX (possibly via CaMKII or PKC activation), cycling the mitochondrial ROS production (positive feedback 1). Mitochondrial ROS contributes maintain the opening of mitoK$_{ATP}$ channels (positive

feedback 2), perhaps through activation of PKC. Finally, intracellular Ca^{2+} augmentation after NHE-1 and NCX reverse mode stimulation induces mitochondrial Ca^{2+} load and further generation of ROS (positive feedback 3) (De Giusti et al. 2013). Mitochondrial ROS contribute to maintain the opening of mitoK_{ATP} channels (positive feedback 2), perhaps through the activation of PKC (feedback 3).

Appendix C

Calcium and its regulatory proteins

Myocyte`s contractile function is tightly regulated by the myocyte`s components of excitation-contraction (E-C) coupling. In myocytes calcium release and uptake are regulated during the contraction process and excessive calcium can lead to pathological conditions. During excitation-contraction coupling the interaction between L-type calcium channel and the sarcoplasmic reticulum calcium release channel, ryanodine receptor 2 (RyR2) leads to a large amount of calcium through a process called calcium induced calcium release (CICR), activating the myofilaments and leading to contraction (Bers DM, 2008). Several calcium handling pumps, kinases, transporters and channels regulate calcium entry, extrusion and storage and modifications in calcium handling leading to calcium dysregulation contributes to pathological hypertrophy and heart failure. Severe contractile dysfunction features have been reported in an animal model of pressure-overload cardiac hypertrophy in transition to failure, in which SERCA2a protein levels and activity are decreased. Both systolic and diastolic functions are restored by overexpression of SERCA2a by gene transfer *in vivo* (Miyamoto et al. 2000; del Monte et al. 2001).

In human and animal models of hypertrophy and heart failure the less abundant IP$_3$R increased many fold particularly in the dyadic junction implicating that these channels may be associated with pathological signalling (Higazi et al. 2009; Kockskämper et al. 2008). Activation of IP$_3$R is linked to α1 adrenergic receptor (α1AR)-induced Ca^{2+} spark rate and global Ca^{2+} oscillations and catecholamine-induced cardiomyocyte hypertrophic growth in neonatal rat ventricular myocytes (Luo et al. 2007). Hypertrophic agonist stimulated cardiac hypertrophy remodelling *in vivo* is enhanced by genetic manipulations of IP$_3$R signalling (Nakayama et al. 2010). Also, calcium dependent transcription factors are activated by increased IP$_3$-induced calcium release in the perinuclear region leading to expression of hypertrophic genes (X. Wu 2006; Harzheim et al. 2009).
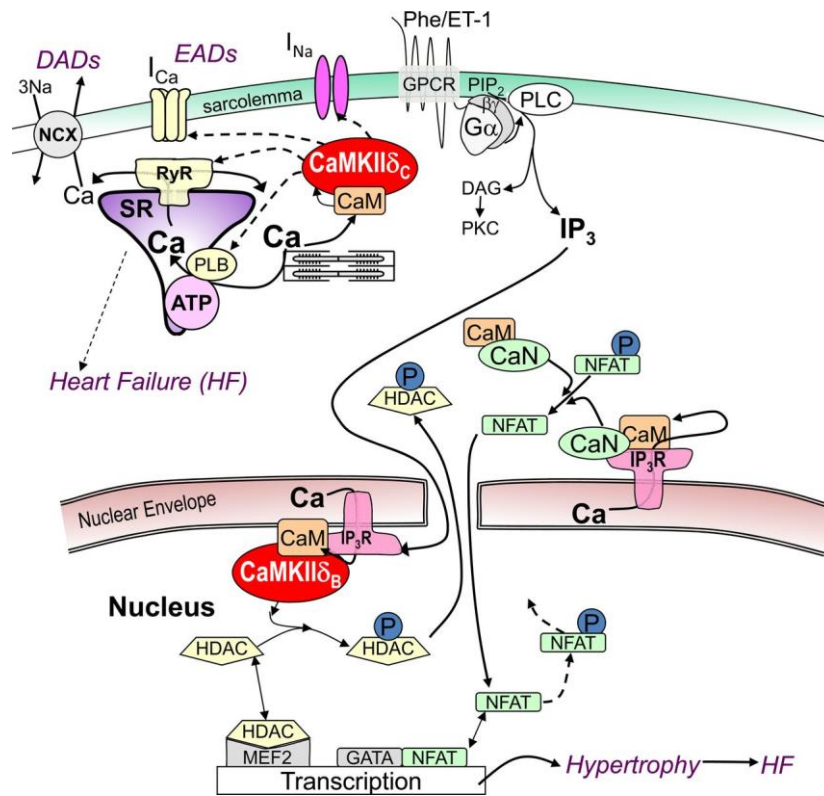
Figure C1. Ca^{2+}-dependent signaling in excitation-transcription coupling via Ca-CaM. CaMKIIδ can acutely regulate ion channels (that carry I_{Na} and I_{Ca}) and Ca^{2+} handling proteins (RyR2, IP3R, PLN), contributing to trigger arrhythmias such as early and delayed afterdepolarization (EADs and DADs). G protein-coupled receptor (GPCR) agonists endothelin-1 (ET-1) and phenylephrine (PE) activate Gαq/βγ and phospholipase C (PLC) to produce diacylglycerol (DAG), which can activate protein kinases C and D. PKC, CaMKII, and PKD can phosphorylate (P) HDAC, and calcineurin (CaN) can dephosphorylate nuclear factor of activated T cells (NFAT), altering nuclear MEF2- and GATA-dependent transcription (Bers 2011).

Gene/splicing variant	Type	Strategy	Cardiac phenotype	Location	References
CaMKIIδ*B*	Gain of function	α*MHC*-driven transgene	Cardiac hypertrophy	Nucleus	Zhang et al., 2002b
CaMKIIδ*C*	Gain of function	α*MHC*-driven transgene	Dilated cardiomyopathy	Cytosol	Zhang et al., 2003
CaMKIIδ	Loss of function	Global knockout exons 9–11	Protection from fibrosis, dysfunction, and late hypertrophy	Nucleus/Cytosol	Ling et al., 2009
CaMKIIδ	Loss of function	Global knockout exons 1–2	Protection from early hypertrophy and fibrosis	Nucleus/Cytosol	Backs et al., 2009
CaMKIIγ	Loss of function	Global knockout exons 1–2	Not investigated	Nucleus/Cytosol	Backs et al., 2010

Cardiomyocyte-specific transgenic overexpression of CaMKIIδ (splice variants B and C) are driven by the αMHC promoter. Global knockout models for CaMKIIδ were generated by two labs. The second cardiac CaMKII isoform, CaMKIIγ, has so far not been investigated with regard to cardiac stress situations.

Table C1. Genetic mouse models for CaMKIIδ and γ (Kreusser and Backs 2014).

Myocyte enhancer factor 2 (MEF2) proteins are responsive to calcium-controlled signalling pathways such as CaMK and calcineurin (McKinsey et al, 2002). They are an important target for several hypertrophic signalling pathways. Their basal activity is essential for cardiomyocytes homeostasis maintenance via regulation of genes responsible for it (Azzouzi et al. 2010). Pro-hypertrophic calcium dependent pathways such as calcineurin and CaMKII, PKD, PKC, MAPK-1, and p38- MAPK have been reported to upregulate the activity of MEF2. These pathways have shown to decrease the expression of NADH dehydrogenase subunit 6 (ND6) which is a part of complex 1 of oxidative phosphorylation system serving as the main source of energy in cardiac muscle. Reduction in ND6 expression leads to an increase in reactive oxygen species production resulting in cell death followed by pressure overload (Azzouzi et al. 2010).

Appendix D

Micro RNAs in cardiac hypertrophy

Thoracic aortic banding or constitutively activated calcineurin signalling induced cardiac hypertrophy revealed that several microRNAs are both up and down-regulated. miR-1, one of the most abundant cardiac microRNA negatively regulated the progression of cardiac hypertrophy in a transverse aortic constriction (TAC) rodent model. miR-1 targets several hypertrophic genes such as cyclin-dependent kinase 9 (cdk9), Ras GTPase-activating protein (RasGAP), Ras homolog enriched in brain (Rheb) and fibronectin (Sayed et al. 2007). Also, miR-133 regulated cardiac hypertrophy by targeting multiple anti-hypertrophic genes including Rhoa, signal transduction kinase cell divison control protein 42 (Cdc42), guanosine

triphosphate-guanosine diphosphate (GDP-GTP) exchange protein and the nuclear factor, negative elongation factor complex member A (Nelfa/Whsc 2) (Wang et al. 2016).

The first characterized microRNA involved in inducing cardiac hypertrophy is miR-195. This microRNA was up-regulated both in human and mouse hypertrophied hearts. miR-195 overexpression in cultured neonatal rat cardiomyocytes is sufficient to induce cardiac hypertrophy (van Rooij et al. 2006). miR-195 is a pro-hypertrophic factor that leads to dilated cardiomyopathy and heart dysfunction *in vivo* (Harris et al. 2006). miR-208a, miR-208b and miR-499 are a family of microRNAs called myomiRs that are encoded by introns of the separate myosin heavy chain genes and are located within the *Myh6, Myh7 and Myh7b* genes respectively (van Rooij et al. 2009). miR-208a overexpression is sufficient to induce cardiac hypertrophy by upregulating Myh7, resulting in cardiac systolic dysfunction (Callis et al. 2009).

miRNAs Pro-hypertrophic	Targets	miRNA-mRNA interaction	Models	References
miR-155	Tp53inp1	p53	NMCFs and AMI mouse	(He et al. 2016)
miR-199a	Gsk-3β	PI3k-Akt	NRCMs and TG mouse	(Li et al. 2017)
miR-199b	Dyrk1a	calcineurin/NFAT	NRCMs and TAC mouse	(da Costa Martins et al. 2010)
miR-19a/b	Atrogin1	calcineurin/NFAT	NRCMs and TAC mouse	(Song et al. 2014)
	Murf1	PKC	NRCMs and TAC mouse	
miR-208a	Thrap1	Thyroid hormone	NRCMs, TAC and TG mouse	(Callis et al. 2009)
	Myostatin1	Cell growth/differentiation	NRCMs, TAC and TG mouse	
miR-21	Pten	PI3k-AKT	Human glomerular mesanglial cells	(Dey et al. 2012)

miR-21-3p	Hdac8	AKT/Gsk3β	TAC mouse	(Yan et al. 2015)
miR-132/212 family	Foxo3	PI3K-Akt	H9c2 cells and TAC mouse	(Ucar et al. 2012)
miR-22	Sirt 1	AMPK	NRCMs, miR-22 KO mice	(Huang et al. 2013)
	Hdac4	AMPK	NRCMs, miR-22 KO mice	
	Pten	PI3K-AKT	NRCMs	(Xu et al. 2012)
miR-221	p27	PI3K-AKT	NRCM, TAC mouse	(Wang et al. 2012)
miR-23a	Foxo3a	PI3K-AKT	NMCMs, TAC and TG mouse	(Wang et al. 2012)
	Lpa1	PI3K-AKT	NRCMs	(Yang et al. 2013)
miR-27b	Pparγ	PPAR	NRCMs, TAC and TG mouse	(Wang et al. 2012)
miR-30a	Beclin 1	Autophagy	NRCMs, TAAC rat	(Pan et al. 2013)
miR-328	Serca2a	cGMP-PKG	NRVCs, TAC and TG mouse	(Li et al. 2014)
miR-350	Mapk11/14	MAPK	H9c2 cells and TAC rats	(Ge et al. 2013)
	Mapk8/9	MAPK	H9c2 cells and TAC rats	

Table D1. Summary of reported miRNAs and their targets in cardiac hypertrophy (Wang et al. 2016).

Appendix E

E. Results

E 1. pCREB expression is higher in the nucleus of ISO-treated cells

A.   **B.**
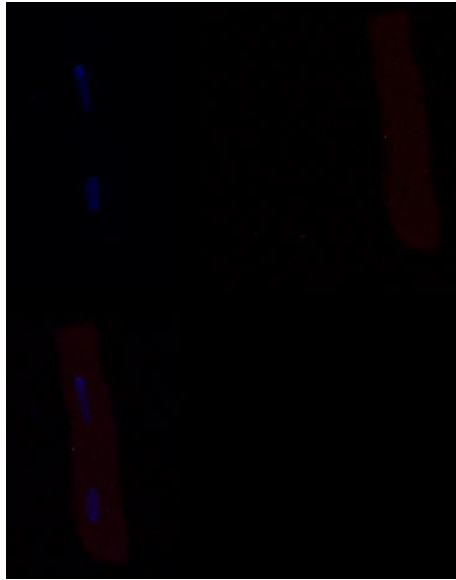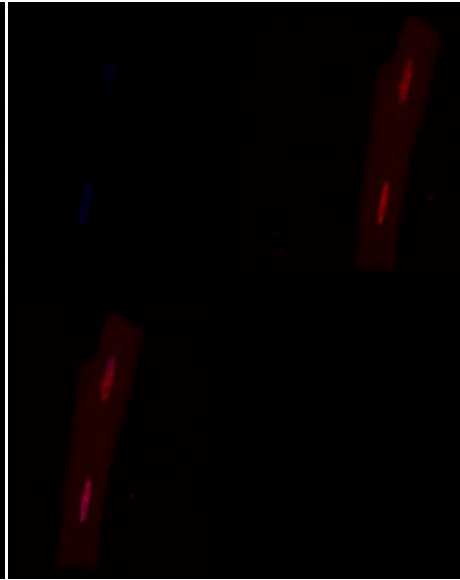
Control   **ISO**

C.
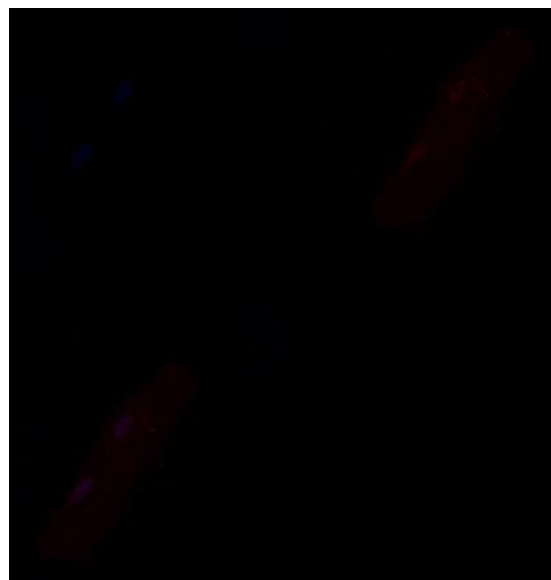
Dzx+ISO

Figure E1. pCREB expression is higher in the nucleus of ISO-treated cells. Confocal images of adult rat ventricular myocytes that were treated under indicated experimental

conditions. Images above show Hoechst stained nuclei (blue; upper left), pCREB expression (Red; upper right) and merge image in A. Control cells, B. ISO-treated cells and C. Dzx+ISO-treated cells.

12. References

Ago, T., Tong L., Peiyong Z., Wei C., Hong L., Jeffery D. M., Stephen F. V., and Junichi S. 2008. "A Redox-Dependent Pathway for Regulating Class II HDACs and Cardiac Hypertrophy." *Cell* 133 (6):978–93. https://doi.org/10.1016/j.cell.2008.04.041.

Amin, J. K, Lei X., David R P., Patrick J P., Singh K., Douglas B. S., and Wilson S. C., 2001. "Reactive Oxygen Species Mediate Alpha-Adrenergic Receptor-Stimulated Hypertrophy in Adult Rat Ventricular Myocytes." *Journal of Molecular and Cellular Cardiology* 33 (1):131–39. https://doi.org/10.1006/jmcc.2000.1285.

Andersson, D. C., Jérémy F., Takashi Y., Alain L., Shi-Jin Z., Abram K., and Håkan W. 2011. "Mitochondrial Production of Reactive Oxygen Species Contributes to the β-Adrenergic Stimulation of Mouse Cardiomycytes: ROS Increase Cardiomyocyte Ca $^{2+}$ and Contraction." *The Journal of Physiology* 589 (7):1791–1801. https://doi.org/10.1113/jphysiol.2010.202838.

Andreyev, A. Y., Yu E. K., and Starkov, A. A. 2005. "Mitochondrial Metabolism of Reactive Oxygen Species." *Biochemistry. Biokhimiia* 70 (2):200–214.

Aruoma, O. I., B. Halliwell, B. M. H., and Butler, J. 1989. "The Antioxidant Action of N-Acetylcysteine: Its Reaction with Hydrogen Peroxide, Hydroxyl Radical, Superoxide, and Hypochlorous Acid." *Free Radical Biology & Medicine* 6 (6):593–97.

Azzouzi, H. el., Ralph J. van O., Roel van der N., Wim S., Martin W. B., and Leon J. De W. 2010. "MEF2 Transcriptional Activity Maintains Mitochondrial Adaptation in Cardiac Pressure Overload." *European Journal of Heart Failure* 12 (1):4–12. https://doi.org/10.1093/eurjhf/hfp165.

Bendall, J. K., Alison C. C., Christophe H., Nicholas G., and Ajay M. S. 2002. "Pivotal Role of a Gp91(Phox)-Containing NADPH Oxidase in Angiotensin II-Induced Cardiac Hypertrophy in Mice." *Circulation* 105 (3):293–96.

Benes, Jan, Ludmila K., Zdenek D., Josef H., Dasa M., Jan K., Nikola K. et al. 2011. "Effect of Metformin Therapy on Cardiac Function and Survival in a Volume-Overload Model of Heart Failure in Rats." *Clinical Science (London, England: 1979)* 121 (1):29–41. https://doi.org/10.1042/CS20100527.

Berlo, Jop H. van, Marjorie M., and Jeffery D. M. 2013. "Signaling Effectors Underlying Pathologic Growth and Remodeling of the Heart." *The Journal of Clinical Investigation* 123 (1):37–45. https://doi.org/10.1172/JCI62839.

Bers, Donald M. 2011. "Ca^{2+} Calmodulin-Dependent Protein Kinase II Regulation of Cardiac Excitation-Transcription Coupling." *Heart Rhythm* 8 (7):1101–4. https://doi.org/10.1016/j.hrthm.2011.01.030.

Biala, Agnieszka, Eveliina T., Antti S., Jin S., Saara M., Marjut L., Essi M., Piet F., Dominik N. M., and Eero M. 2010. "Resveratrol Induces Mitochondrial Biogenesis and Ameliorates Ang II-Induced Cardiac Remodeling in Transgenic Rats Harboring Human Renin and Angiotensinogen Genes." *Blood Pressure* 19 (3):196–205. https://doi.org/10.3109/08037051.2010.481808.

Bingham, A. J., Ooi, L., Kozera, L., White, E., and Wood, I.C. 2007. "The Repressor Element 1-Silencing Transcription Factor Regulates Heart-Specific Gene Expression Using Multiple Chromatin-Modifying Complexes." *Molecular and Cellular Biology* 27 (11):4082–92. https://doi.org/10.1128/MCB.00269-07.

Bovo, E., Stephen L. L., and Aleksey, V. Z. 2012. "Reactive Oxygen Species Contribute to the Development of Arrhythmogenic Ca^{2+} Waves during β-Adrenergic Receptor Stimulation in Rabbit Cardiomyocytes." *The Journal of Physiology* 590 (14):3291–3304. https://doi.org/10.1113/jphysiol.2012.230748.

Bradford, M. M. 1976. "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding." *Analytical Biochemistry* 72 (May):248–54.

Brower, G. L., Jason D. G., Mary F. F., David B. M., Tetyana V., Scott P. L., and Joseph S. J. 2006. "The Relationship between Myocardial Extracellular Matrix Remodeling and Ventricular Function☆." *European Journal of Cardio-Thoracic Surgery* 30 (4):604–10. https://doi.org/10.1016/j.ejcts.2006.07.006.

Brown, David A., Adam J. C., Korinne N. J., Micah S. J., Joshua M. L., Peter A. W., and Russell L. M. 2005. "Cardioprotection Afforded by Chronic Exercise Is Mediated by the Sarcolemmal, and Not the Mitochondrial, Isoform of the KATP Channel in the Rat." *The Journal of Physiology* 569 (Pt 3):913–24. https://doi.org/10.1113/jphysiol.2005.095729.

Brown, David I., and Kathy K. G. 2015. "Regulation of Signal Transduction by Reactive Oxygen Species in the Cardiovascular System." *Circulation Research* 116 (3):531–49. https://doi.org/10.1161/CIRCRESAHA.116.303584.

Burgoyne, Joseph, R., Héloïse M.D., Philip E., and Ajay M. S. 2012. "Redox Signaling in Cardiac Physiology and Pathology." *Circulation Research* 111 (8):1091–1106. https://doi.org/10.1161/CIRCRESAHA.111.255216.

Callis, Thomas E., Kumar P., Hee Y. S., Ru-H. T., Mariko T., Zhan-P. H., Jian, F. C., et al. 2009. "MicroRNA-208a Is a Regulator of Cardiac Hypertrophy and Conduction in Mice." *The Journal of Clinical Investigation* 119 (9):2772–86. https://doi.org/10.1172/JCI36154.

Carrillo, E. D., Escobar, Y., González, G., Hernández, A., Galindo, J.M., García,M.C., and Sánchez, J.A. 2011. "Posttranscriptional Regulation of the B2-Subunit of Cardiac L-Type Ca2+ Channels by MicroRNAs during Long-Term Exposure to Isoproterenol in Rats." *Journal of Cardiovascular Pharmacology* 58 (5):470–78. https://doi.org/10.1097/FJC.0b013e31822a789b.

Carrillo, E. D., Sampieri, R., Hernandez, A., Garcia, M.C., and Sanchez, J.A. 2015. "MiR-132 Regulates Rem Expression in Cardiomyocytes During Long-Term β-Adrenoceptor Agonism." *Cellular Physiology and Biochemistry* 36 (1):141–54. https://doi.org/10.1159/000374059.

Cave, A., D. Grieve, S. Johar, M. Zhang, and A. M. Shah. 2005. "NADPH Oxidase-Derived Reactive Oxygen Species in Cardiac Pathophysiology." *Philosophical Transactions of the Royal Society B: Biological Sciences* 360 (1464):2327–34. https://doi.org/10.1098/rstb.2005.1772.

Cheong, A., Andrew J. B., Jing L., Kumar, B., Sukumar, P., Munsch, C., Buckley, N.J. et al. 2005. "Downregulated REST Transcription Factor Is a Switch Enabling Critical Potassium Channel Expression and Cell Proliferation." *Molecular Cell* 20 (1):45–52. https://doi.org/10.1016/j.molcel.2005.08.030.

Chong, E., Shih-L. C., Ya-W. H., Singhal, R., Shuen-H. L., Trung L., Wen-Y. L., et al. 2015. "Resveratrol, a Red Wine Antioxidant, Reduces Atrial Fibrillation Susceptibility in the Failing Heart by PI3K/AKT/ENOS Signaling Pathway Activation." *Heart Rhythm* 12 (5):1046–56. https://doi.org/10.1016/j.hrthm.2015.01.044.

Cipolla, Gabriel A. 2014. "A Non-Canonical Landscape of the MicroRNA System." *Frontiers in Genetics* 5:337. https://doi.org/10.3389/fgene.2014.00337.

Conaco, Cecilia, Otto, S., Jong-J. H., and Gail M. 2006. "Reciprocal Actions of REST and a MicroRNA Promote Neuronal Identity." *Proceedings of the National Academy of Sciences of the United States of America* 103 (7):2422–27. https://doi.org/10.1073/pnas.0511041103.

Costa Martins, Paula A. da, Meriem Bourajjaj, Monika Gladka, Mara Kortland, Ralph J. van Oort, Yigal M. Pinto, Jeffery D. Molkentin, and Leon J. De Windt. 2008. "Conditional Dicer Gene Deletion in the Postnatal Myocardium Provokes Spontaneous Cardiac Remodeling." *Circulation* 118 (15):1567–76. https://doi.org/10.1161/CIRCULATIONAHA.108.769984.

Costa M., Paula A. da, Kanita S., Monika M. G., Anne-S. A., Stefanos L., Hamid el A., Arne H., et al. 2010. "MicroRNA-199b Targets the Nuclear Kinase Dyrk1a in an Auto-Amplification Loop Promoting Calcineurin/NFAT Signalling." *Nature Cell Biology* 12 (12):1220–27. https://doi.org/10.1038/ncb2126.

Dai, Dao-F., Luis F. S., Marc V., Tomazela, M.D., Mary J. E., Michael J. M., Katherine G., et al. 2009. "Overexpression of Catalase Targeted to Mitochondria Attenuates Murine Cardiac Aging." *Circulation* 119 (21):2789–97. https://doi.org/10.1161/CIRCULATIONAHA.108.822403.

Dai, D.-F., P. Rabinovitch, S., and Ungvari, Z. 2012. "Mitochondria and Cardiovascular Aging." *Circulation Research* 110 (8):1109–24. https://doi.org/10.1161/CIRCRESAHA.111.246140.

Date, Moto-o, Morita, T., Nobushige, Y., Nishida, K., Yamaguchi, O., Higuchi, Y., Hirotani, S. et al. 2002. "The Antioxidant N-2-Mercaptopropionyl Glycine Attenuates Left Ventricular Hypertrophy in in Vivo Murine Pressure-Overload Model." *Journal of the American College of Cardiology* 39 (5):907–12. https://doi.org/10.1016/S0735-1097 (01)01826-5.

Giusti D., Caldiz, V.C.C.I., Ennis, I.E., Pérez, N.G., Cingolani, H.E., and Aiello, A.E. 2013. "Mitochondrial Reactive Oxygen Species (ROS) as Signaling Molecules of Intracellular Pathways Triggered by the Cardiac Renin-Angiotensin II-Aldosterone System (RAAS)." *Frontiers in Physiology* 4. https://doi.org/10.3389/fphys.2013.00126.

DeVore, Adam D., Xiaojuan M., Robert J. Mentz, Gregg C. Fonarow, Melissa K. Van Dyke, Juan F. Maya, Hardy, N.C., Hammill B. G., and Hernandez, A.F. 2016. "Discharge Heart Rate and β-Blocker Dose in Patients Hospitalized with Heart Failure: Findings from the OPTIMIZE-HF Registry." *American Heart Journal* 173 (March):172–78. https://doi.org/10.1016/j.ahj.2015.10.026.

Dey, Nirmalya, Choudhury, N.G., Kasinath, B.S., and Choudhury, G.G. 2012. "TGFβ-Stimulated MicroRNA-21 Utilizes PTEN to Orchestrate AKT/MTORC1 Signaling for Mesangial Cell Hypertrophy and Matrix Expansion." Edited by Effie C. Tsilibary. *PLoS ONE* 7 (8):e42316. https://doi.org/10.1371/journal.pone.0042316.

Dhalla, A. K., Hill, M.F., and Singal, P.K. 1996. "Role of Oxidative Stress in Transition of Hypertrophy to Heart Failure." *Journal of the American College of Cardiology* 28 (2):506–14. https://doi.org/10.1016/0735-1097(96)00140-4.

Diedrichs, H., Hagemeister, J., Chi, M., Boelck, B., Müller-Ehmsen, J., and Schneider, C.A. 2007. "Activation of the Calcineurin/NFAT Signalling Cascade Starts Early in Human Hypertrophic Myocardium." *The Journal of International Medical Research* 35 (6):803–18. https://doi.org/10.1177/147323000703500609.

Dröse, Stefan, Peter J. Hanley, and Brandt, U. 2009. "Ambivalent Effects of Diazoxide on Mitochondrial ROS Production at Respiratory Chain Complexes I and III." *Biochimica Et Biophysica Acta* 1790 (6):558–65. https://doi.org/10.1016/j.bbagen.2009.01.011.

Erickson, Jeffrey R., Joiner, M.A., Guan, X., Kutschke, W., Yang, J., Oddis, C.V., Bartlett, R.K. et al. 2008. "A Dynamic Pathway for Calcium-Independent Activation of CaMKII by Methionine Oxidation." *Cell* 133 (3):462–74. https://doi.org/10.1016/j.cell.2008.02.048.

Farh, Kai-How, K., Grimson, A., Jan, C., Lewis, B.P., Johnston, W.K., Lim, L.P., Burge, C.B., and Bartel, D.P. 2005. "The Widespread Impact of Mammalian MicroRNAs on MRNA Repression and Evolution." *Science (New York, N.Y.)* 310 (5755):1817–21. https://doi.org/10.1126/science.1121158.

Finkel, T. 1999. "Signal Transduction by Reactive Oxygen Species in Non-Phagocytic Cells." *Journal of Leukocyte Biology* 65 (3):337–40.

Flagg, Thomas P., Enkvetchakul, D., Koster, J.C., and Nichols, C.G. 2010. "Muscle KATP Channels: Recent Insights to Energy Sensing and Myoprotection." *Physiological Reviews* 90 (3):799–829. https://doi.org/10.1152/physrev.00027.2009.

Foster, Monique N., and Coetzee, W.A. 2016. "KATP Channels in the Cardiovascular System." *Physiological Reviews* 96 (1):177–252. https://doi.org/10.1152/physrev.00003.2015.

Frey, N. 2004. "Hypertrophy of the Heart: A New Therapeutic Target?" *Circulation* 109 (13):1580–89. https://doi.org/10.1161/01.CIR.0000120390.68287.BB.

Funakoshi, Yuko, Ichiki, T., Takeda, K., Tokuno, T., Iino, N., and Takeshita, A. 2002. "Critical Role of CAMP-Response Element-Binding Protein for Angiotensin II-Induced Hypertrophy of Vascular Smooth Muscle Cells." *Journal of Biological Chemistry* 277 (21):18710–17. https://doi.org/10.1074/jbc.M110430200.

Gao, F. B., and Keene, J.D. 1996. "Hel-N1/Hel-N2 Proteins Are Bound to Poly(A)+ MRNA in Granular RNP Structures and Are Implicated in Neuronal Differentiation." *Journal of Cell Science* 109 (Pt 3) (March):579–89.

García, Maria C., Hernández, A., and Sánchez, J.A. 2009. "Role of Mitochondrial ATP-Sensitive Potassium Channels on Fatigue in Mouse Muscle Fibers." *Biochemical and Biophysical Research Communications* 385 (1):28–32. https://doi.org/10.1016/j.bbrc.2009.05.019.

Garlid, Keith D, Santos, P.D., Xie, Z.J., Costa, A.D.T., and Paucek, P. 2003. "Mitochondrial Potassium Transport: The Role of the Mitochondrial ATP-Sensitive K+ Channel in Cardiac Function and Cardioprotection." *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1606 (1–3):1–21. https://doi.org/10.1016/S0005-2728(03)00109-9.

Ge, Yuzhi, Pan, S., Guan, D., Yin, H., Fan, Y., Liu, J., Zhang, S. et al. 2013. "MicroRNA-350 Induces Pathological Heart Hypertrophy by Repressing Both P38 and JNK Pathways." *Biochimica Et Biophysica Acta* 1832 (1):1–10. https://doi.org/10.1016/j.bbadis.2012.09.004.

George, Alfred, L. 2013. "Molecular and Genetic Basis of Sudden Cardiac Death." *Journal of Clinical Investigation* 123 (1):75–83. https://doi.org/10.1172/JCI62928.

Ghosh, S. 2000. "Evidence for Mitochondrial KATP Channels as Effectors of Human Myocardial Preconditioning." *Cardiovascular Research* 45 (4):934–40. https://doi.org/10.1016/S0008-6363(99)00407-1.

Goldspink, P. H., and Russell, B. 1994. "The CAMP Response Element Binding Protein Is Expressed and Phosphorylated in Cardiac Myocytes." *Circulation Research* 74 (6):1042–49.

González, G., Zaldívar, D., Carrillo, E.D., Hernández, A., García, M.C., and Sánchez, J.A. 2010. "Pharmacological Preconditioning by Diazoxide Downregulates Cardiac L-Type Ca(2+) Channels." *British Journal of Pharmacology* 161 (5):1172–85. https://doi.org/10.1111/j.1476-5381.2010.00960.x.

Gordon, Joseph W., Pagiatakis, C., Salma, J., Du, M., Andreucci, J.J., Zhao, J., Hou, G. et al. 2009. "Protein Kinase A-Regulated Assembly of a MEF2{middle Dot}HDAC4 Repressor Complex Controls c-Jun Expression in Vascular Smooth Muscle Cells." *The Journal of Biological Chemistry* 284 (28):19027–42. https://doi.org/10.1074/jbc.M109.000539.

Griffiths, Eric R., Friehs, I., Scherr, E., Poutias, D., McGowan, F.X., and Nido, P.J. 2010. "Electron Transport Chain Dysfunction in Neonatal Pressure-Overload Hypertrophy Precedes Cardiomyocyte Apoptosis Independent of Oxidative Stress." *The Journal of Thoracic and Cardiovascular Surgery* 139 (6):1609–17. https://doi.org/10.1016/j.jtcvs.2009.08.060.

Guardavaccaro, Daniele, Frescas, D., Dorrello, N.V., Peschiaroli, A., Multani, A.S., Cardozo, T., Lasorella, A. et al. 2008. "Control of Chromosome Stability by the Beta-TrCP-REST-Mad2 Axis." *Nature* 452 (7185):365–69. https://doi.org/10.1038/nature06641.

Gundewar, Susheel, Calvert, J.W., Jha, S., Toedt-Pingel, I., Ji, S.Y., Nunez, D., Ramachandran, A., Anaya-Cisneros, M., Tian, R., and Lefer, D.J. 2009. "Activation of AMP-Activated Protein Kinase by Metformin Improves Left Ventricular Function and Survival in Heart Failure." *Circulation Research* 104 (3):403–11. https://doi.org/10.1161/CIRCRESAHA.108.190918.

Gusterson, Rosalind J., Jazrawi, E., Adcock, I.A., and Latchman, D.S. 2003. "The Transcriptional Co-Activators CREB-Binding Protein (CBP) and P300 Play a Critical Role in Cardiac Hypertrophy That Is Dependent on Their Histone Acetyltransferase Activity." *Journal of Biological Chemistry* 278 (9):6838–47. https://doi.org/10.1074/jbc.M211762200.

Harris, Kelley S., Zhang, Z., McManus, M.T., Harfe, B.D., and Sun, X. 2006. "Dicer Function Is Essential for Lung Epithelium Morphogenesis." *Proceedings of the National Academy of Sciences of the United States of America* 103 (7):2208–13. https://doi.org/10.1073/pnas.0510839103.

Harsdorf, von, R., Edwards, J.G., Shen, Y.T., Kudej, R.K., Dietz, R., Leinwand, L.A., Nadal-Ginard, B., and Vatner, S.F. 1997. "Identification of a Cis-Acting Regulatory Element Conferring Inducibility of the Atrial Natriuretic Factor Gene in Acute Pressure Overload." *Journal of Clinical Investigation* 100 (5):1294–1304. https://doi.org/10.1172/JCI119643.

Harzheim, D., Movassagh, M., Foo, R.S.Y., Ritter, O.,, Tashfeen, A., Conway, S.J., Bootman, M.D., and Roderick, H.L. 2009. "Increased InsP3Rs in the Junctional Sarcoplasmic Reticulum Augment Ca2+ Transients and Arrhythmias Associated with Cardiac Hypertrophy." *Proceedings of the National Academy of Sciences* 106 (27):11406–11. https://doi.org/10.1073/pnas.0905485106.

He, Julie, B., Joiner, M.A., Singh, M.V., Luczak, E.D., Swaminathan, P.D., Koval, O.M., Kutschke, W. et al. 2011. "Oxidation of CaMKII Determines the Cardiotoxic Effects of Aldosterone." *Nature Medicine* 17 (12):1610–18. https://doi.org/10.1038/nm.2506.

He, Lin, and Hannon, G.J. 2004. "MicroRNAs: Small RNAs with a Big Role in Gene Regulation." *Nature Reviews. Genetics* 5 (7):522–31. https://doi.org/10.1038/nrg1379.

He, Wangwei, Huang. H., Xie. Q., Wang. Z, Fan, Y., Kong, B., Huang, D., and Xiao, Y. 2016. "MiR-155 Knockout in Fibroblasts Improves Cardiac Remodeling by Targeting Tumor Protein P53-Inducible Nuclear Protein 1." *Journal of Cardiovascular Pharmacology and Therapeutics* 21 (4):423–35. https://doi.org/10.1177/1074248415616188.

Higazi, Daniel R., Claire J. Fearnley, Drawnel, F.M., Talasila, A., Corps, E.M., Ritter, O., McDonald, F., Mikoshiba, K., Bootman, M.D., and Roderick, H.L. 2009. "Endothelin-1-Stimulated InsP3-Induced Ca2+ Release Is a Nexus for Hypertrophic Signaling in Cardiac Myocytes." *Molecular Cell* 33 (4):472–82. https://doi.org/10.1016/j.molcel.2009.02.005.

Hirotani, S. 2002. "Involvement of Nuclear Factor-KappaB and Apoptosis Signal-Regulating Kinase 1 in G-Protein-Coupled Receptor Agonist-Induced Cardiomyocyte Hypertrophy." *Circulation* 105 (4):509–15. https://doi.org/10.1161/hc0402.102863.

Hong, Whan, K., Kim, K.Y., Lee, J.H., Shin, H.K., Kwak, Y.G., Kim, S.O., Lim, H., and Yoo, S. 2002. "Neuroprotective Effect of (2S,3S,4R)-N"-Cyano-N-(6-Amino-3, 4-Dihydro-3-Hydroxy-2-Methyl-2-Dimethoxymethyl-2H-Benzopyran-4-Yl)-N'-Benzylguanidine (KR-31378), a Benzopyran Analog, against Focal Ischemic Brain Damage in Rats." *The Journal of Pharmacology and Experimental Therapeutics* 301 (1):210–16.

Huang, Zhan-Peng, Chen, J., Seok, H.Y., Zhang, Z., Kataoka, M., Hu, X., and Wang, D. 2013. "MicroRNA-22 Regulates Cardiac Hypertrophy and Remodeling in Response to Stress." *Circulation Research* 112 (9):1234–43. https://doi.org/10.1161/CIRCRESAHA.112.300682.

Hwang, Shil, G., Oh, k., Koo, H., Seo, H.W., You, K., and Lee, B.H. 2006. "Effects of KR-31378, a Novel ATP-Sensitive Potassium Channel Activator, on Hypertrophy of H9c2 Cells and on Cardiac Dysfunction in Rats with Congestive Heart Failure." *European Journal of Pharmacology* 540 (1–3):131–38. https://doi.org/10.1016/j.ejphar.2006.04.031.

Ide, T., Tsutsui, H., Hayashidani. S, Kang, D., Suematsu, N., Nakamura, K., Utsumi, H., Hamasaki, N., and Takeshita, A. 2001. "Mitochondrial DNA Damage and Dysfunction Associated with Oxidative Stress in Failing Hearts after Myocardial Infarction." *Circulation Research* 88 (5):529–35.

Inoue, Isao, Nagase, H., Kishi, K., and Higuti, T. 1991. "ATP-Sensitive K+ Channel in the Mitochondrial Inner Membrane." *Nature* 352 (6332):244–47. https://doi.org/10.1038/352244a0.

Irani, K. 2000. "Oxidant Signaling in Vascular Cell Growth, Death, and Survival : A Review of the Roles of Reactive Oxygen Species in Smooth Muscle and Endothelial Cell Mitogenic and Apoptotic Signaling." *Circulation Research* 87 (3):179–83.

Irani, K., Xia, Y., Zweier, J.L., Sollott, S.J., Der, C.J., Fearon, E.R., Sundaresan, M., Finkel, T., and Goldschmidt-Clermont, P.J. 1997. "Mitogenic Signaling Mediated by Oxidants in Ras-Transformed Fibroblasts." *Science (New York, N.Y.)* 275 (5306):1649–52.

Jabůrek, M., Yarov-Yarovoy, V., Paucek, P., and Garlid, K.D. 1998. "State-Dependent Inhibition of the Mitochondrial KATP Channel by Glyburide and 5-Hydroxydecanoate." *The Journal of Biological Chemistry* 273 (22):13578–82.

Jackson, Richard J., and Standart, N. 2007. "How Do MicroRNAs Regulate Gene Expression?" *Science's STKE: Signal Transduction Knowledge Environment* 2007 (367):re1. https://doi.org/10.1126/stke.3672007re1.

Joiner, Mei-ling A., Koval, O.M., Li, J.,, He, B.J., Allamargot, C., Gao, Z., Luczak, E.D. et al. 2012. "CaMKII Determines Mitochondrial Stress Responses in Heart." *Nature* 491 (7423):269–73. https://doi.org/10.1038/nature11444.

Joladarashi, D., Thandavarayan, R., Babu, S., and Krishnamurthy, P. 2014. "Small Engine, Big Power: MicroRNAs as Regulators of Cardiac Diseases and Regeneration." *International Journal of Molecular Sciences* 15 (9):15891–911. https://doi.org/10.3390/ijms150915891.

Kaneko, N., Hwang, J.Y., Gertner, M., Pontarelli, F., and Zukin, R.S. 2014. "Casein Kinase 1 Suppresses Activation of REST in Insulted Hippocampal Neurons and Halts Ischemia-Induced Neuronal Death." *Journal of Neuroscience* 34 (17):6030–39. https://doi.org/10.1523/JNEUROSCI.4045-13.2014.

Kim, Sun-Ok, Cho, I.S., Gu, H.K., Lee, D.H., Lim, H., and Yoo, S. 2004. "KR-31378 Protects Neurons from Ischemia-Reperfusion Brain Injury by Attenuating Lipid Peroxidation and Glutathione Loss." *European Journal of Pharmacology* 487 (1–3):81–91. https://doi.org/10.1016/j.ejphar.2003.12.037.

Kiriakidou, Marianthi, Nelson, P.T., Kouranov, A., Fitziev, P., Bouyioukos, C., Mourelatos, Z., and Hatzigeorgiou, A. 2004. "A Combined Computational-Experimental Approach Predicts Human MicroRNA Targets." *Genes & Development* 18 (10):1165–78. https://doi.org/10.1101/gad.1184704.

Kockskämper, Jens, Zima, A.V., Roderick, H.L., Pieske, B., Blatter, L.A., and Bootman, M.D. 2008. "Emerging Roles of Inositol 1,4,5-Trisphosphate Signaling in Cardiac Myocytes." *Journal of Molecular and Cellular Cardiology* 45 (2):128–47. https://doi.org/10.1016/j.yjmcc.2008.05.014.

Koyama, Hirofumi, Nojiri, H., Kawakami, S., Sunagawa, T., Shirasawa, T., and Shimizu, T. 2013. "Antioxidants Improve the Phenotypes of Dilated Cardiomyopathy and Muscle Fatigue in Mitochondrial Superoxide Dismutase-Deficient Mice." *Molecules* 18 (2):1383–93. https://doi.org/10.3390/molecules18021383.

Kreusser, Michael M., and Backs, J. 2014. "Integrated Mechanisms of CaMKII-Dependent Ventricular Remodeling." *Frontiers in Pharmacology* 5 (March). https://doi.org/10.3389/fphar.2014.00036.

Kuratomi, Shinobu, Kuratomi, A., Kuwahara, K., Ishii, T.M., Nakao, K., Saito, Y., and Takano, M. 2007. "NRSF Regulates the Developmental and Hypertrophic Changes of HCN4 Transcription in Rat Cardiac Myocytes." *Biochemical and Biophysical Research Communications* 353 (1):67–73. https://doi.org/10.1016/j.bbrc.2006.11.119.

Kuroda, J., Matsushima, T.A.S., Zhai, P., Schneider, M.D., and Sadoshima, J. 2010. "NADPH Oxidase 4 (Nox4) Is a Major Source of Oxidative Stress in the Failing Heart." *Proceedings of the National Academy of Sciences* 107 (35):15565–70. https://doi.org/10.1073/pnas.1002178107.

Kuster, Gabriela M., Pimentel, D.R., Adachi, T., Ido, Y., Brenner, D.A., Cohen, R.A., Liao, R., Siwik, D.A., and Colucci, S.W. 2005. "Alpha-Adrenergic Receptor-Stimulated Hypertrophy in Adult Rat Ventricular Myocytes Is Mediated via Thioredoxin-1-Sensitive Oxidative Modification of Thiols on Ras." *Circulation* 111 (9):1192–98. https://doi.org/10.1161/01.CIR.0000157148.59308.F5.

Kuwahara, K., Saito, Y., Ogawa, E., Takahashi, N., Nakagawa, Y., Naruse, Y., Harada, M. et al. 2001. "The Neuron-Restrictive Silencer Element-Neuron-Restrictive Silencer Factor System Regulates Basal and Endothelin 1-Inducible Atrial Natriuretic Peptide Gene Expression in Ventricular Myocytes." *Molecular and Cellular Biology* 21 (6):2085–97. https://doi.org/10.1128/MCB.21.6.2085-2097.2001.

Kuwahara, Koichiro, Saito, Y., Takano, M., Arai, Y., Yasuno, S., Nakagawa, Y., Takahashi, N. et al. 2003. "NRSF Regulates the Fetal Cardiac Gene Program and Maintains Normal Cardiac Structure and Function." *The EMBO Journal* 22 (23):6310–21. https://doi.org/10.1093/emboj/cdg601.

Kwon, S. 2003. "H2O2 Regulates Cardiac Myocyte Phenotype via Concentration-Dependent Activation of Distinct Kinase Pathways." *Journal of Molecular and Cellular Cardiology* 35 (6):615–21. https://doi.org/10.1016/S0022-2828(03)00084-1.

Lee, Ho, B., Seo, H.W., Yoo, S., Kim, S., Lim, H., and Shin, H.S. 2001. "Differential Action of KR-31378, a Novel Potassium Channel Activator, on Cardioprotective and Hemodynamic Effects." *Drug Development Research* 54 (4):182–90. https://doi.org/10.1002/ddr.10028.

Lee, Tsung-Ming, Lin, M., and Chang, N. 2008. "Effect of ATP-Sensitive Potassium Channel Agonists on Ventricular Remodeling in Healed Rat Infarcts." *Journal of the American College of Cardiology* 51 (13):1309–18. https://doi.org/10.1016/j.jacc.2007.11.067.

Lemos C., Rodrigo, F., Leite, L.M.R., Filgueiras, A.B.T., Júnior, I.L.F., Marques de Sousa, T.A.G., Martins, P.R., Kowaltowski, A.J., and Facundo, H.T.F. 2015. "Mitochondrial ATP-Sensitive Potassium Channel Opening Inhibits Isoproterenol-Induced Cardiac Hypertrophy by Preventing Oxidative Damage." *Journal of Cardiovascular Pharmacology* 65 (4):393–97. https://doi.org/10.1097/FJC.0000000000000210.

Li, Cui, Li, X., Gao, X., Zhang, R., Zhang, Y., Liang, H., Xu, C. et al. 2014. "MicroRNA-328 as a Regulator of Cardiac Hypertrophy." *International Journal of Cardiology* 173 (2):268–76. https://doi.org/10.1016/j.ijcard.2014.02.035.

Li, Jian-Mei, and Shah, A.M. 2004. "Endothelial Cell Superoxide Generation: Regulation and Relevance for Cardiovascular Pathophysiology." *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 287 (5):R1014-1030. https://doi.org/10.1152/ajpregu.00124.2004.

Li, Z., Song, Y., Liu, L., Hou, N., An, x., Zhan, D., Li, Y. et al. 2017. "MiR-199a Impairs Autophagy and Induces Cardiac Hypertrophy through MTOR Activation." *Cell Death and Differentiation* 24 (7):1205–13. https://doi.org/10.1038/cdd.2015.95.

Lin, Ying, Liu, X., Cheng, Y., Yang, J., Huo, Y., and Zhang, C. 2009. "Involvement of MicroRNAs in Hydrogen Peroxide-Mediated Gene Regulation and Cellular Injury Response in Vascular Smooth Muscle Cells." *Journal of Biological Chemistry* 284 (12):7903–13. https://doi.org/10.1074/jbc.M806920200.

Liu, Julia C., Liu, J., Holmström, K.M., Menazza, S., Parks, R.J., Fergusson, M.M., Yu, Z. et al. 2016. "MICU1 Serves as a Molecular Gatekeeper to Prevent In Vivo Mitochondrial Calcium Overload." *Cell Reports* 16 (6):1561–73. https://doi.org/10.1016/j.celrep.2016.07.011.

Liu, Yan-Li, Liu, B., Qu, Y., Chai, H., Li, R., and Zhang, L. 2013. "[Oxidative stress and calcium/calmodulin-dependent protein kinase II contribute to the development of sustained β adrenergic receptor-stimulated cardiac hypertrophy in rats]." *Sheng Li Xue Bao: [Acta Physiologica Sinica]* 65 (1):1–7.

Livak, K. J., and Schmittgen, T.D. 2001. "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method." *Methods (San Diego, Calif.)* 25 (4):402–8. https://doi.org/10.1006/meth.2001.1262.

Lo, Li-Wei, Chen, Y., Chen, Y-J., Wongcharoen, W., Lin, C-I., and Chen, S-A. 2007. "Calmodulin Kinase II Inhibition Prevents Arrhythmic Activity Induced by Alpha and Beta Adrenergic Agonists in Rabbit Pulmonary Veins." *European Journal of Pharmacology* 571 (2–3):197–208. https://doi.org/10.1016/j.ejphar.2007.05.066.

Lu, J., McKinsey, T.A., Nicol, R.L., and Olson, E.N. 2000. "Signal-Dependent Activation of the MEF2 Transcription Factor by Dissociation from Histone Deacetylases." *Proceedings of the National Academy of Sciences of the United States of America* 97 (8):4070–75. https://doi.org/10.1073/pnas.080064097.

Luo, Da-li, Gao, J., Fan, L-L., Tang, Y., Zhang, Y-Y., and Han, Q. 2007. "Receptor Subtype Involved in ? $_1$ -Adrenergic Receptor-Mediated Ca $^{2+}$ Signaling in Cardiomyocytes." *Acta Pharmacologica Sinica* 28 (7):968–74. https://doi.org/10.1111/j.1745-7254.2007.00605.x.

MacDonnell, Scott M., Thomas, J.W., Kubo, H., Hanscome, M., Liu, Q., Jaleel, N., Berretta, R. et al. 2009. "CaMKII Negatively Regulates Calcineurin-NFAT Signaling in Cardiac Myocytes." *Circulation Research* 105 (4):316–25. https://doi.org/10.1161/CIRCRESAHA.109.194035.

Madamanchi, Nageswara R., and Runge, M.S. 2013. "Redox Signaling in Cardiovascular Health and Disease." *Free Radical Biology & Medicine* 61 (August):473–501. https://doi.org/10.1016/j.freeradbiomed.2013.04.001.

Marks, Andrew R. 2013. "Calcium Cycling Proteins and Heart Failure: Mechanisms and Therapeutics." *Journal of Clinical Investigation* 123 (1):46–52. https://doi.org/10.1172/JCI62834.

Marquez, Jubert, Lee, S.R., Kim, N., and Han, J. 2006. "Rescue of Heart Failure by Mitochondrial Recovery." *International Neurourology Journal* 20 (1):5–12. https://doi.org/10.5213/inj.1632570.285.

Maulik, Kumar, S., and Kumar, S. 2012. "Oxidative Stress and Cardiac Hypertrophy: A Review." *Toxicology Mechanisms and Methods* 22 (5):359–66. https://doi.org/10.3109/15376516.2012.666650.

Mayr, B., and Montminy, M. 2001. "Transcriptional Regulation by the Phosphorylation-Dependent Factor CREB." *Nature Reviews. Molecular Cell Biology* 2 (8):599–609. https://doi.org/10.1038/35085068.

McKinsey, T. A., Zhang, C.L., Lu, J., and Olson, E.N. 2000. "Signal-Dependent Nuclear Export of a Histone Deacetylase Regulates Muscle Differentiation." *Nature* 408 (6808):106–11. https://doi.org/10.1038/35040593.

McKinsey, T. A., C. Zhang, L., and Olson, E.N. 2001. "Identification of a Signal-Responsive Nuclear Export Sequence in Class II Histone Deacetylases." *Molecular and Cellular Biology* 21 (18):6312–21.

McKinsey, Timothy A., Zhang, C., and Olson, E.N. 2002. "MEF2: A Calcium-Dependent Regulator of Cell Division, Differentiation and Death." *Trends in Biochemical Sciences* 27 (1):40–47.

McMullen, Julie R., and Jennings, G.L. 2007. "Differences between Pathological and Physiological Cardiac Hypertrophy: Novel Therapeutic Strategies to Treat Heart Failure." *Clinical and Experimental Pharmacology & Physiology* 34 (4):255–62. https://doi.org/10.1111/j.1440-1681.2007.04585.x.

Métrich, Mélanie, Laurent, A-C., Breckler, M., Duquesnes, N., Hmitou, I., Courillau, D., Blondeau, J-P., Crozatier, B., Lezoualc'h, F., and Morel, E. 2010. "Epac Activation Induces Histone Deacetylase Nuclear Export via a Ras-Dependent Signalling Pathway." *Cellular Signalling* 22 (10):1459–68. https://doi.org/10.1016/j.cellsig.2010.05.014.

Miyamoto, M. I., Monte, F., Schmidt, U., DiSalvo, T.S., Kang, Z.B., Matsui, T., Guerrero, J.L., Gwathmey, J.K., Rosenzweig, A., and Hajjar, R.J. 2000. "Adenoviral Gene Transfer of SERCA2a Improves Left-Ventricular Function in Aortic-Banded Rats in Transition to Heart Failure." *Proceedings of the National Academy of Sciences of the United States of America* 97 (2):793–98.

Moens, An L., Ketner, E.A., Takimoto, E., Schmidt, T.S., O'Neill, C.A., Wolin, M.S., Alp, N.J., Channon, K.M., and Kass, D.A. 2011. "Bi-Modal Dose-Dependent Cardiac Response to Tetrahydrobiopterin in Pressure-Overload Induced Hypertrophy and Heart Failure." *Journal of Molecular and Cellular Cardiology* 51 (4):564–69. https://doi.org/10.1016/j.yjmcc.2011.05.017.

Moens, An L., Takimoto, E., Tocchetti, C.G., Chakir, K., Bedja, D., Cormaci, G., Ketner, E.A. et al. 2008. "Reversal of Cardiac Hypertrophy and Fibrosis from Pressure Overload by Tetrahydrobiopterin: Efficacy of Recoupling Nitric Oxide Synthase as a Therapeutic Strategy." *Circulation* 117 (20):2626–36. https://doi.org/10.1161/CIRCULATIONAHA.107.737031.

Monte, Williams, F.E., Lebeche, D., Schmidt, U., Rosenzweig, A., Gwathmey, J.K., Lewandowski, E.D., and Hajjar, R.J. 2001. "Improvement in Survival and Cardiac Metabolism after Gene Transfer

of Sarcoplasmic Reticulum Ca(2+)-ATPase in a Rat Model of Heart Failure." *Circulation* 104 (12):1424–29.

Mukoyama, M., Nakao, K., Obata, k., Jougasaki, M., Yoshimura, M., Morita, E., Hosoda, K., Suga, S., Ogawa, Y., and Yasue, H. 1991. "Augmented Secretion of Brain Natriuretic Peptide in Acute Myocardial Infarction." *Biochemical and Biophysical Research Communications* 180 (1):431–36.

Nakagawa, Y, Kuwahara, K., Harada, M., Takahashi, N., Yasuno, S., Adachi, Y., Kawakami, R., Nakanishi, M., Tanimoto, K., and Usami, S. 2006. "Class II HDACs Mediate CaMK-Dependent Signaling to NRSF in Ventricular Myocytes." *Journal of Molecular and Cellular Cardiology* 41 (6):1010–22. https://doi.org/10.1016/j.yjmcc.2006.08.010.

Nakayama, H., Bodi, I., Maillet, M., DeSantiago, J., Domeier, T.L., Mikoshiba, K., Lorenz, J.N., Blatter, L.A., Bers, D.M., and Molkentin, J.D. 2010. "The IP3 Receptor Regulates Cardiac Hypertrophy in Response to Select Stimuli." *Circulation Research* 107 (5):659–66. https://doi.org/10.1161/CIRCRESAHA.110.220038.

Nishio, S., Teshima, Y., Takahashi, N., Thuc, L.C., Saito, S., Fukui, A., Kume, O. et al. 2012. "Activation of CaMKII as a Key Regulator of Reactive Oxygen Species Production in Diabetic Rat Heart." *Journal of Molecular and Cellular Cardiology* 52 (5):1103–11. https://doi.org/10.1016/j.yjmcc.2012.02.006.

Noma, A. 1983. "ATP-Regulated K+ Channels in Cardiac Muscle." *Nature* 305 (5930):147–48.

Odagiri, K., Katoh, H., Kawashima, H., Tanaka, T., Ohtani, H., Saotome, M., Urushida, T., Satoh, H., and Hayashi, H. 2009. "Local Control of Mitochondrial Membrane Potential, Permeability Transition Pore and Reactive Oxygen Species by Calcium and Calmodulin in Rat Ventricular Myocytes." *Journal of Molecular and Cellular Cardiology* 46 (6):989–97. https://doi.org/10.1016/j.yjmcc.2008.12.022.

Ogawa, Emiko, Saito, Y., Kuwahara, K., Harada, M., Miyamoto, Y., Hamanaka, I., Kajiyama, N. et al. 2002. "Fibronectin Signaling Stimulates BNP Gene Transcription by Inhibiting Neuron-Restrictive Silencer Element-Dependent Repression." *Cardiovascular Research* 53 (2):451–59.

Osterholt, M., Nguyen, T.D., Schwarzer, M., and Doenst, T. 2013. "Alterations in Mitochondrial Function in Cardiac Hypertrophy and Heart Failure." *Heart Failure Reviews* 18 (5):645–56. https://doi.org/10.1007/s10741-012-9346-7.

Ozgen, N., Guo, J., Gertsberg, Z., Danilo, P., Rosen, M.R., and Steinberg, S.F. 2009. "Reactive Oxygen Species Decrease CAMP Response Element Binding Protein Expression in Cardiomyocytes via a Protein Kinase D1-Dependent Mechanism That Does Not Require Ser133 Phosphorylation." *Molecular Pharmacology* 76 (4):896–902. https://doi.org/10.1124/mol.109.056473.

Pan, Wei, Zhong, Y., Cheng, C., Liu, B., Wang, L., Li, Xiong, L., and Liu, S. 2013. "MiR-30-Regulated Autophagy Mediates Angiotensin II-Induced Myocardial Hypertrophy." *PloS One* 8 (1):e53950. https://doi.org/10.1371/journal.pone.0053950.

Pasdois, Philippe, Beauvoit, B., Tariosse, L., Vinassa, B., Bonoron-Adèle, S., and Santos, P.D. 2008. "Effect of Diazoxide on Flavoprotein Oxidation and Reactive Oxygen Species Generation during Ischemia-Reperfusion: A Study on Langendorff-Perfused Rat Hearts Using Optic Fibers." *American Journal of Physiology. Heart and Circulatory Physiology* 294 (5):H2088-2097. https://doi.org/10.1152/ajpheart.01345.2007.

Passier, R., Zeng, H., Frey, N., Naya, F.J., R. L. Nicol, T. A. McKinsey, P. Overbeek, J. A. Richardson, S. R. Grant, and E. N. Olson. 2000. "CaM Kinase Signaling Induces Cardiac Hypertrophy and Activates the MEF2 Transcription Factor in Vivo." *The Journal of Clinical Investigation* 105 (10):1395–1406. https://doi.org/10.1172/JCI8551.

Pimentel, D. R., Amin, J.K., Xiao, L., Miller, T., Viereck, J., Oliver-Krasinski, J., Baliga, R. et al. 2001. "Reactive Oxygen Species Mediate Amplitude-Dependent Hypertrophic and Apoptotic Responses to Mechanical Stretch in Cardiac Myocytes." *Circulation Research* 89 (5):453–60.

Pousségur, Jacques, and Mechta-Grigoriou, F. 2006. "Redox Regulation of the Hypoxia-Inducible Factor." *Biological Chemistry* 387 (10–11):1337–46. https://doi.org/10.1515/BC.2006.167.

Quindry, John C., Miller. L, McGinnis, G., Kliszczewicz, B., Irwin, J.M., Landram, M., Urbiztondo, Z., Nanayakkara, G., and Amin, R. 2012. "Ischemia Reperfusion Injury, KATP Channels, and Exercise-Induced Cardioprotection against Apoptosis." *Journal of Applied Physiology (Bethesda, Md.: 1985)* 113 (3):498–506. https://doi.org/10.1152/japplphysiol.00957.2011.

Quindry, John C., Schreiber, L., Hosick, P., Wrieden, J., Irwin, J.M., and Hoyt, E. 2010. "Mitochondrial KATP Channel Inhibition Blunts Arrhythmia Protection in Ischemic Exercised Hearts." *American Journal of Physiology. Heart and Circulatory Physiology* 299 (1):H175-183. https://doi.org/10.1152/ajpheart.01211.2009.

Qureshi, Irfan A., and Mehler, M.F. 2009. "Regulation of Non-Coding RNA Networks in the Nervous System--What's the REST of the Story?" *Neuroscience Letters* 466 (2):73–80. https://doi.org/10.1016/j.neulet.2009.07.093.

Teresa, R.M., Sah, V.P., Zhao, X-L., Hunter, J.J., Chien, K.R., and Brown, J.H. 1997. "The MEKK-JNK Pathway Is Stimulated by α_1 -Adrenergic Receptor and Ras Activation and Is Associated with *in Vitro* and *in Vivo* Cardiac Hypertrophy." *Journal of Biological Chemistry* 272 (22):14057–61. https://doi.org/10.1074/jbc.272.22.14057.

Remenyi, Judit, Christopher J. Hunter, Cole, C., Ando, H., Impey, S., Monk, C.E., Martin, K.J., Barton, G.J., Hutvagner, G., and Arthur, J.S.C. 2010. "Regulation of the MiR-212/132 Locus by MSK1 and CREB in Response to Neurotrophins." *The Biochemical Journal* 428 (2):281–91. https://doi.org/10.1042/BJ20100024.

Rooij, Eva van, Quiat, D., Johnson, B.A., Sutherland, L.B., Qi, X., Richardson, J.A., Kelm, R.J., and Olson, E.N. 2009. "A Family of MicroRNAs Encoded by Myosin Genes Governs Myosin Expression and Muscle Performance." *Developmental Cell* 17 (5):662–73. https://doi.org/10.1016/j.devcel.2009.10.013.

Rooij, Eva van, Sutherland, L.B, Liu, N., Williams, A.H., McAnally, J., Gerard, R.D., Richardson, J.A., and Olson, E.N. 2006. "A Signature Pattern of Stress-Responsive MicroRNAs That Can Evoke Cardiac Hypertrophy and Heart Failure." *Proceedings of the National Academy of Sciences of the United States of America* 103 (48):18255–60. https://doi.org/10.1073/pnas.0608791103.

Sag, Can M., Santos, C.X.C., and Shah, A.M. 2014. "Redox Regulation of Cardiac Hypertrophy." *Journal of Molecular and Cellular Cardiology* 73 (August):103–11. https://doi.org/10.1016/j.yjmcc.2014.02.002.

Saito, Y., Nakao, K., Arai, H., Nishimura, K., Okumura, K., Obata, K., Takemura, G., Fujiwara, H., Sugawara, A., and Yamada, T. 1989. "Augmented Expression of Atrial Natriuretic Polypeptide Gene in Ventricle of Human Failing Heart." *The Journal of Clinical Investigation* 83 (1):298–305. https://doi.org/10.1172/JCI113872.

Sánchez, Gina, Pedrozo, Z., Domenech, R.J., Hidalgo, C., and Donoso, P. 2005. "Tachycardia Increases NADPH Oxidase Activity and RyR2 S-Glutathionylation in Ventricular Muscle." *Journal of Molecular and Cellular Cardiology* 39 (6):982–91. https://doi.org/10.1016/j.yjmcc.2005.08.010.

Sánchez, J.A., García, M.C., Sharma, V.K., Young, K.C., Matlib, M.A., and Sheu, S-S. 2001. "Mitochondria Regulate Inactivation of L-Type Ca $^{2+}$ Channels in Rat Heart." *The Journal of Physiology* 536 (2):387–96. https://doi.org/10.1111/j.1469-7793.2001.0387c.xd.

Sanna, Bastiano, Bueno, O.F., Dai, Y.S., Wilkins, B.J., and Molkentin, J.D. 2005. "Direct and Indirect Interactions between Calcineurin-NFAT and MEK1-Extracellular Signal-Regulated Kinase 1/2 Signaling Pathways Regulate Cardiac Gene Expression and Cellular Growth." *Molecular and Cellular Biology* 25 (3):865–78. https://doi.org/10.1128/MCB.25.3.865-878.2005.

Sartoretto, Juliano L., Kalwa, H., Shiroto, T., Sartoretto, S.M., Pluth, M.D., Lippard, S.J., and Michel, T. 2012. "Role of Ca2+ in the Control of H2O2-Modulated Phosphorylation Pathways Leading to ENOS Activation in Cardiac Myocytes." Edited by Gianfranco Pintus. *PLoS ONE* 7 (9):e44627. https://doi.org/10.1371/journal.pone.0044627.

Sayed, Danish, Hong, C., Chen, L-Y., Lypowy, J., and Abdellatif, M. 2007. "MicroRNAs Play an Essential Role in the Development of Cardiac Hypertrophy." *Circulation Research* 100 (3):416–24. https://doi.org/10.1161/01.RES.0000257913.42552.23.

Schaeffer, P. J., DeSantiago, J., Yang, J., Flagg, T.P., Kovacs, A., Weinheimer, C.J., Courtois, M. et al. 2009. "Impaired Contractile Function and Calcium Handling in Hearts of Cardiac-Specific Calcineurin B1-Deficient Mice." *AJP: Heart and Circulatory Physiology* 297 (4):H1263–73. https://doi.org/10.1152/ajpheart.00152.2009.

Schwartz, Bryan G., Laurence A. Levine, Comstock, G., Stecher, V.J., and Kloner, R.A. 2012. "Cardiac Uses of Phosphodiesterase-5 Inhibitors." *Journal of the American College of Cardiology* 59 (1):9–15. https://doi.org/10.1016/j.jacc.2011.07.051.

Sengupta, Arunima, Molkentin, J.D., and Yutzey, K.E. 2009. "FoxO Transcription Factors Promote Autophagy in Cardiomyocytes." *The Journal of Biological Chemistry* 284 (41):28319–31. https://doi.org/10.1074/jbc.M109.024406.

Sepúlveda, Marisa, Gonano, L.A., Back, T.G., Chen, S.R.W., and Petroff, M.W. 2013. "Role of CaMKII and ROS in Rapid Pacing-Induced Apoptosis." *Journal of Molecular and Cellular Cardiology* 63 (October):135–45. https://doi.org/10.1016/j.yjmcc.2013.07.013.

Seshiah, Puvi N., Weber, D.S., Rocic, P., Valppu, L., Taniyama, Y., and Griendling, K.K. 2002. "Angiotensin II Stimulation of NAD(P)H Oxidase Activity: Upstream Mediators." *Circulation Research* 91 (5):406–13.

Shimizu, Ippei, and Minamino, T. 2016. "Physiological and Pathological Cardiac Hypertrophy." *Journal of Molecular and Cellular Cardiology* 97 (August):245–62. https://doi.org/10.1016/j.yjmcc.2016.06.001.

Simpson, P. 1985. "Stimulation of Hypertrophy of Cultured Neonatal Rat Heart Cells through an Alpha 1-Adrenergic Receptor and Induction of Beating through an Alpha 1- and Beta 1-Adrenergic Receptor Interaction. Evidence for Independent Regulation of Growth and Beating." *Circulation Research* 56 (6):884–94. https://doi.org/10.1161/01.RES.56.6.884.

Siwik, D. A., Tzortzis, J.D., Pimental, D.R., Chang, D, L-F., Pagano, P.J., Singh, K., Sawyer, D.B., and Colucci, W.S. 1999. "Inhibition of Copper-Zinc Superoxide Dismutase Induces Cell Growth, Hypertrophic Phenotype, and Apoptosis in Neonatal Rat Cardiac Myocytes In Vitro." *Circulation Research* 85 (2):147–53. https://doi.org/10.1161/01.RES.85.2.147.

Somvanshi, R. K., Qiu, X., and Kumar, U. 2013. "Isoproterenol Induced Hypertrophy and Associated Signaling Pathways Are Modulated by Somatostatin in H9c2 Cells." *International Journal of Cardiology* 167 (3):1012–22. https://doi.org/10.1016/j.ijcard.2012.03.077.

Song, Woo, D., Ryu, J.Y., Kim, J, O., Kwon, E.J., and Kim, D.H. 2014. "The MiR-19a/b Family Positively Regulates Cardiomyocyte Hypertrophy by Targeting Atrogin-1 and MuRF-1." *The Biochemical Journal* 457 (1):151–62. https://doi.org/10.1042/BJ20130833.

Sprenkle, A. B., Murray, S.F., and Glembotski, C.C. 1995. "Involvement of Multiple Cis Elements in Basal- and Alpha-Adrenergic Agonist-Inducible Atrial Natriuretic Factor Transcription. Roles for Serum Response Elements and an SP-1-like Element." *Circulation Research* 77 (6):1060–69.

Strand, Arne H., Gudmundsdottir, H., Os, I., Smith, G., Westheim, A.S., Bjørnerheim, R., and Kjeldsen, S.E. 2006. "Arterial Plasma Noradrenaline Predicts Left Ventricular Mass Independently of Blood Pressure and Body Build in Men Who Develop Hypertension over 20 Years." *Journal of Hypertension* 24 (5):905–13. https://doi.org/10.1097/01.hjh.0000222761.07477.7b.

Sucharov, Carmen C., Mariner, P.D., Nunley, K.R., Long, C., Leinwand, L., and Bristow, M.R. 2006. "A Beta1-Adrenergic Receptor CaM Kinase II-Dependent Pathway Mediates Cardiac Myocyte Fetal Gene Induction." *American Journal of Physiology. Heart and Circulatory Physiology* 291 (3):H1299-1308. https://doi.org/10.1152/ajpheart.00017.2006.

Takimoto, Eiki, Champion, H.C., Li, M., Ren, S., Rodriguez, E.R., Tavazzi, B., Lazzarino, G. et al. 2005. "Oxidant Stress from Nitric Oxide Synthase-3 Uncoupling Stimulates Cardiac Pathologic

Remodeling from Chronic Pressure Load." *The Journal of Clinical Investigation* 115 (5):1221–31. https://doi.org/10.1172/JCI21968.

Tardiff, J. C. 2006. "Cardiac Hypertrophy: Stressing out the Heart." *Journal of Clinical Investigation* 116 (6):1467–70. https://doi.org/10.1172/JCI28884.

Terentyev, Dmitry, Györke, I., Belevych, A.E., Terentyeva, R., Sridhar, A., Nishijima, Y., Blanco, E.C. et al. 2008. "Redox Modification of Ryanodine Receptors Contributes to Sarcoplasmic Reticulum Ca2+ Leak in Chronic Heart Failure." *Circulation Research* 103 (12):1466–72. https://doi.org/10.1161/CIRCRESAHA.108.184457.

Testai, L., Rapposelli, S., Martelli, A., Breschi, M.C., and Calderone, V. 2015. "Mitochondrial Potassium Channels as Pharmacological Target for Cardioprotective Drugs: MITOCHONDRIAL POTASSIUM CHANNELS AS PHARMACOLOGICAL TARGET." *Medicinal Research Reviews* 35 (3):520–53. https://doi.org/10.1002/med.21332.

Toischer, Karl, Adam G. Rokita, Unsöld, B., Zhu, W., Kararigas, G., Sossalla, S., Reuter, S.P. et al. 2010. "Differential Cardiac Remodeling in Preload versus Afterload." *Circulation* 122 (10):993–1003. https://doi.org/10.1161/CIRCULATIONAHA.110.943431.

Touyz, R. M., Yao, G., and Schiffrin, E.L. 2003. "C-Src Induces Phosphorylation and Translocation of P47phox: Role in Superoxide Generation by Angiotensin II in Human Vascular Smooth Muscle Cells." *Arteriosclerosis, Thrombosis, and Vascular Biology* 23 (6):981–87. https://doi.org/10.1161/01.ATV.0000069236.27911.68.

Ucar, A, Gupta, S.K., Fiedler, J., Erikci, E., Kardasinski, M., Batkai, S., Dangwal, S. et al. 2012. "The MiRNA-212/132 Family Regulates Both Cardiac Hypertrophy and Cardiomyocyte Autophagy." *Nature Communications* 3:1078. https://doi.org/10.1038/ncomms2090.

Ucar, A, Vafaizadeh, V., Jarry, H., Fiedler, J., Klemmt, P.A.B., Thum, T., Groner, B., and Chowdhury, K. 2010. "MiR-212 and MiR-132 Are Required for Epithelial Stromal Interactions Necessary for Mouse Mammary Gland Development." *Nature Genetics* 42 (12):1101–8. https://doi.org/10.1038/ng.709.

Ushio-Fukai, M., Alexander, R.W., Akers, M., and Griendling, K.K. 1998. "P38 Mitogen-Activated Protein Kinase Is a Critical Component of the Redox-Sensitive Signaling Pathways Activated by Angiotensin II. Role in Vascular Smooth Muscle Cell Hypertrophy." *The Journal of Biological Chemistry* 273 (24):15022–29.

Vila-Petroff, Mundiña-Weilenmann, M.C., Lezcano, N., Snabaitis, A.K., Huergo, M.A., Valverde, C.A., Avkiran, M., and Mattiazzi, A. 2010. "Ca2+/Calmodulin-Dependent Protein Kinase II Contributes to Intracellular PH Recovery from Acidosis via Na+/H+ Exchanger Activation." *Journal of Molecular and Cellular Cardiology* 49 (1):106–12. https://doi.org/10.1016/j.yjmcc.2009.12.007.

Vo, N., Klein, M.E., Varlamova, O., Keller, D.M., Yamamoto, T., Goodman, R.H., and Impey, S. 2005. "A CAMP-Response Element Binding Protein-Induced MicroRNA Regulates Neuronal Morphogenesis." *Proceedings of the National Academy of Sciences of the United States of America* 102 (45):16426–31. https://doi.org/10.1073/pnas.0508448102.

Wanet, A., Tacheny, A., Arnould, T., and Renard, P. 2012. "MiR-212/132 Expression and Functions: Within and beyond the Neuronal Compartment." *Nucleic Acids Research* 40 (11):4742–53. https://doi.org/10.1093/nar/gks151.

Wang, C., Wang, S., Zhao, P., Wang, X., Wang, J., Wang, Y., Song, L., Zou, Y., and Hui, R. 2012. "MiR-221 Promotes Cardiac Hypertrophy in Vitro through the Modulation of P27 Expression." *Journal of Cellular Biochemistry* 113 (6):2040–46. https://doi.org/10.1002/jcb.24075.

Wang, H-J., Zhu, Y-C, and Yao, T. 2002. "Effects of *All - Trans* Retinoic Acid on Angiotensin II-Induced Myocyte Hypertrophy." *Journal of Applied Physiology* 92 (5):2162–68. https://doi.org/10.1152/japplphysiol.01192.2001.

Wang, J., Song, Y., Zhang, Y., Xiao, H., Sun, Q., Hou, N., Guo, S. et al. 2012. "Cardiomyocyte Overexpression of MiR-27b Induces Cardiac Hypertrophy and Dysfunction in Mice." *Cell Research* 22 (3):516–27. https://doi.org/10.1038/cr.2011.132.

Wang, J., Liew, O., Richards, A., and Chen, Y-T. 2016. "Overview of MicroRNAs in Cardiac Hypertrophy, Fibrosis, and Apoptosis." *International Journal of Molecular Sciences* 17 (5):749. https://doi.org/10.3390/ijms17050749.

Wang, K., Lin, Z-Q., Long, B., Li, J-H., Zhou, J., and Li, P-F. 2012. "Cardiac Hypertrophy Is Positively Regulated by MicroRNA MiR-23a." *The Journal of Biological Chemistry* 287 (1):589–99. https://doi.org/10.1074/jbc.M111.266940.

Wang, Q., Huang, L., and Yue, J. 2017. "Oxidative Stress Activates the TRPM2-Ca 2+ -CaMKII-ROS Signaling Loop to Induce Cell Death in Cancer Cells." *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1864 (6):957–67. https://doi.org/10.1016/j.bbamcr.2016.12.014.

Wang, X., Zhu, S., Pei, Z., Drozda, M., Stavrovskaya, I.G., Signore, S.J. Cormier, K. et al. 2008. "Inhibitors of Cytochrome c Release with Therapeutic Potential for Huntington's Disease." *Journal of Neuroscience* 28 (38):9473–85. https://doi.org/10.1523/JNEUROSCI.1867-08.2008.

Westbrook, T.F., Hu, G., Ang, X.L., Mulligan, P., Pavlova, N.N., Liang, A., Leng, Y. et al. 2008. "SCFbeta-TRCP Controls Oncogenic Transformation and Neural Differentiation through REST Degradation." *Nature* 452 (7185):370–74. https://doi.org/10.1038/nature06780.

Wu, J., and Xie, X. 2006. "Comparative Sequence Analysis Reveals an Intricate Network among REST, CREB and MiRNA in Mediating Neuronal Gene Expression." *Genome Biology* 7 (9):R85. https://doi.org/10.1186/gb-2006-7-9-r85.

Wu, X. 2006. "Local InsP3-Dependent Perinuclear Ca2+ Signaling in Cardiac Myocyte Excitation-Transcription Coupling." *Journal of Clinical Investigation* 116 (3):675–82. https://doi.org/10.1172/JCI27374.

Xia, Y., Rajapurohitam, V., Cook, M.A., and Karmazyn, M. 2004a. "Inhibition of Phenylephrine Induced Hypertrophy in Rat Neonatal Cardiomyocytes by the Mitochondrial KATP Channel Opener Diazoxide." *Journal of Molecular and Cellular Cardiology* 37 (5):1063–67. https://doi.org/10.1016/j.yjmcc.2004.07.002.

Xu, X.-D., Song, X-W., Li, Q., Wang, G-K., Jing, Q., and Qin, Y-W. 2012. "Attenuation of MicroRNA-22 Derepressed PTEN to Effectively Protect Rat Cardiomyocytes from Hypertrophy." *Journal of Cellular Physiology* 227 (4):1391–98. https://doi.org/10.1002/jcp.22852.

Yamada, S., Kane, G.C., Behfar, A., Liu, X-K., Dyer, R.B., Faustino, R.S., Miki, T., Seino, S., and Terzic, A. 2006. "Protection Conferred by Myocardial ATP-Sensitive K $^+$ Channels in Pressure Overload-Induced Congestive Heart Failure Revealed in *KCNJ11* Kir6.2-Null Mutant: Kir6.2 Knockout Precipitates Heart Failure." *The Journal of Physiology* 577 (3):1053–65. https://doi.org/10.1113/jphysiol.2006.119511.

Yamamoto, E., Lai, Z-F., Yamashita, T., Tanaka, T., Kataoka, K., Tokutomi, Y., Ito, T., Ogawa, H., and Kim-Mitsuyama, S. 2006. "Enhancement of Cardiac Oxidative Stress by Tachycardia and Its Critical Role in Cardiac Hypertrophy and Fibrosis." *Journal of Hypertension* 24 (10):2057–69. https://doi.org/10.1097/01.hjh.0000244956.47114.c1.

Yan, M., Chen, C., Gong, W., Yin, Z., Zhou, L., Chaugai, S., and Wang, D.W. 2015. "MiR-21-3p Regulates Cardiac Hypertrophic Response by Targeting Histone Deacetylase-8." *Cardiovascular Research* 105 (3):340–52. https://doi.org/10.1093/cvr/cvu254.

Yang, J., Nie, Y., Wang, F., Hou, J., Cong, X., Hu, S., and Chen, X. 2013. "Reciprocal Regulation of MiR-23a and Lysophosphatidic Acid Receptor Signaling in Cardiomyocyte Hypertrophy." *Biochimica Et Biophysica Acta* 1831 (8):1386–94. https://doi.org/10.1016/j.bbalip.2013.05.005.

Yin, M., van der Horst, I.C.C., van Melle, J.P., Qian, C., van Gilst, W.H., Silljé, H.H.W., and Boer, R.A. 2011. "Metformin Improves Cardiac Function in a Nondiabetic Rat Model of Post-MI Heart Failure." *American Journal of Physiology. Heart and Circulatory Physiology* 301 (2):H459-468. https://doi.org/10.1152/ajpheart.00054.2011.

Yoo, S.-e., Yi, K.Y., Lee, S., Suh, J., Kim, N., Lee, B.H., Seo, H.W. et al. 2001. "A Novel Anti-Ischemic ATP-Sensitive Potassium Channel (*K* ATP) Opener without Vasorelaxation: *N* -(6-

Aminobenzopyranyl)- *N′* -Benzyl- *N′ ′* -Cyanoguanidine Analogue." *Journal of Medicinal Chemistry* 44 (24):4207–15. https://doi.org/10.1021/jm010183f.

Zechner, D., Thuerauf, D.J., Hanford, D.S., McDonough, P.M., and Glembotski, C.C. 1997. "A Role for the P38 Mitogen-Activated Protein Kinase Pathway in Myocardial Cell Growth, Sarcomeric Organization, and Cardiac-Specific Gene Expression." *The Journal of Cell Biology* 139 (1):115–27.

Zelko, Igor N., Mariani, T.J., and Folz, R.J. 2002. "Superoxide Dismutase Multigene Family: A Comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) Gene Structures, Evolution, and Expression." *Free Radical Biology & Medicine* 33 (3):337–49.

Zhang, C. L., McKinsey, T.A., Chang, S., Antos, C.L., Hill, J.A., and Olson, E.N. 2002. "Class II Histone Deacetylases Act as Signal-Responsive Repressors of Cardiac Hypertrophy." *Cell* 110 (4):479–88.

Zhang, G, Kimura, S., Nishiyama, A., Shokoji, T., Rahman, M., Yao, L., Nagai, Y., Fujisawa, Y., Miyatake, A., and Abe, Y. 2005. "Cardiac Oxidative Stress in Acute and Chronic Isoproterenol-Infused Rats." *Cardiovascular Research* 65 (1):230–38. https://doi.org/10.1016/j.cardiores.2004.08.013.

Zhang, M., Takimoto, E., Lee, D-i., Santos, C.X.C., Nakamura, T., Hsu, S., Jiang, A. et al. 2012. "Pathological Cardiac Hypertrophy Alters Intracellular Targeting of Phosphodiesterase Type 5 From Nitric Oxide Synthase-3 to Natriuretic Peptide Signaling." *Circulation* 126 (8):942–51. https://doi.org/10.1161/CIRCULATIONAHA.112.090977.

Zhang, T., and Brown, J.H. 2004. "Role of Ca2+/Calmodulin-Dependent Protein Kinase II in Cardiac Hypertrophy and Heart Failure." *Cardiovascular Research* 63 (3):476–86. https://doi.org/10.1016/j.cardiores.2004.04.026.

Zhang, Z-Y., Liu, X-H, Hu, W-C, Rong, F and Wu, X-D. 2010. "The Calcineurin-Myocyte Enhancer Factor 2c Pathway Mediates Cardiac Hypertrophy Induced by Endoplasmic Reticulum Stress in Neonatal Rat Cardiomyocytes." *American Journal of Physiology. Heart and Circulatory Physiology* 298 (5):H1499-1509. https://doi.org/10.1152/ajpheart.00980.2009.

Zhao, Zhenghang, Fefelova, N., Shanmugam, M., Bishara, P., Babu, G.J., and Xie, L-H. 2011. "Angiotensin II Induces Afterdepolarizations via Reactive Oxygen Species and Calmodulin Kinase II Signaling." *Journal of Molecular and Cellular Cardiology* 50 (1):128–36. https://doi.org/10.1016/j.yjmcc.2010.11.001.