

## Tale of two kinases: Protein kinase A and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II in pre-diabetic cardiomyopathy

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### Abstract

Metabolic syndrome is a pre-diabetic state characterized by several biochemical and physiological alterations, including insulin resistance, visceral fat accumulation, and dyslipidemias, which increase the risk for developing cardiovascular disease. Metabolic syndrome is associated with augmented sympathetic tone, which could account for the etiology of pre-diabetic cardiomyopathy. This review summarizes the current knowledge of the pathophysiological consequences of enhanced and sustained  $\beta$ -adrenergic response in pre-diabetes, focusing on cardiac dysfunction reported in diet-induced experimental models of pre-diabetic cardiomyopathy. The research reviewed indicates that both protein kinase A and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II play important roles in functional responses mediated by  $\beta_1$ -adrenoceptors; therefore, alterations in the expression or function of these kinases can be deleterious. This review also outlines recent information on the role of protein kinase A and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II in abnormal Ca<sup>2+</sup> handling by cardiomyocytes from diet-induced models of pre-diabetic cardiomyopathy.

**Key Words:** Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; Protein kinase A; Metabolic syndrome; Pre-diabetes; Pre-diabetic cardiomyopathy;  $\beta$ -Adrenoceptors

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**Core Tip:** Metabolic syndrome affects heart function leading to pre-diabetic cardiomyopathy. In an attempt to overcome contractility dysfunction, the activity of the

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sympathetic nervous system increases, but chronic stimulation of  $\beta$ -adrenoceptors leads to alterations in both protein kinase A and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II activity, the main effectors of the  $\beta$ -adrenergic response. This work recapitulates current evidence about the participation of protein kinase A and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II in experimental pre-diabetic cardiomyopathy, emphasizing the prevailing role of CaMKII in the development of cardiomyocyte  $\text{Ca}^{2+}$  mishandling and myocardial dysfunction associated with pre-diabetes.

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## INTRODUCTION

Pre-diabetes, a high-risk state for the development of type 2 diabetes mellitus (DM2), is a condition where glycemia is higher than normal but not yet high enough for DM2 diagnosis[1,2]. According to the American Diabetes Association this condition is identified by laboratory tests, including fasting blood glucose (FBG) values 100-125 mg/dL, glycated hemoglobin in the range of 5.7%-6.4% or 2 h blood glucose values 140-199 mg/dL (75-g oral glucose tolerance test)[2-4].

Metabolic syndrome (MetS) is considered a pre-diabetic state and currently represents a serious public health problem because of its increasing worldwide prevalence. MetS comprises a cluster of biochemical and physiological alterations that become risk factors for cardiovascular disease (CVD)[3]. Key components of MetS are central obesity, elevated triglyceride levels, low high-density lipoprotein cholesterol levels, high blood pressure, and dysglycemia. Insulin resistance (IR) is the critical factor underlying MetS, although the pathogenesis remains unclear. Furthermore, an important feature of MetS patients is the prevalence of a hyperadrenergic state that could account for the development of cardiac disease[5,6].

For DM2 patients, the term diabetic cardiomyopathy refers to the presence of  $\text{Ca}^{2+}$  mishandling, cardiomyocyte hypertrophy, apoptosis, and fibrosis, together with abnormal myocardial performance in the absence of hypertension, coronary artery disease, or valvular heart disease[7,8]. Although the clinical entity of pre-diabetic cardiomyopathy still lacks a universally accepted definition, studies have linked pre-diabetes to CVD. Each MetS component represents a risk factor for CVD; in combination, these components increase the rate and severity of CVD as it relates to several conditions including microvascular dysfunction, coronary atherosclerosis and calcification, and cardiac dysfunction, which lead to myocardial infarction and heart failure (HF)[3]. In animal models of pre-diabetes, obesity, IR, and other components of MetS can lead to cardiac dysfunction associated with structural and functional abnormalities (Table 1), implying cardiomyopathy mechanisms different from those of DM2. Furthermore, observational studies and large sample meta-analyses show that pre-diabetes, defined as impaired glucose tolerance, impaired FBG, or raised glycated hemoglobin, was associated with increased risk of CVD[9,10] and HF[11]. Moreover, meta-analysis of longitudinal studies indicates that MetS is linked to increased risk of myocardial infarction, stroke, and CVD, with the risk estimate being higher than that corresponding to its individual components[12,13]. A disturbing finding is that young pre-diabetic patients with evident impaired FBG levels show increased prevalence of left ventricular hypertrophy, reflecting that heart damage is already present at an early phase of glucose metabolism alteration[14]. Patients with obesity, dyslipidemia, or IR (MetS components) are likely to develop similar metabolism-related cardiomyopathy even in the absence of diabetes[15]. However, the mechanisms involved in the pathogenesis of what must be considered pre-diabetic cardiomyopathy remain poorly understood. For the purpose of this review, we will refer to IR-induced cardiomyopathy, obesity-related cardiomyopathy, or MetS-induced cardiomyopathy as 'pre-diabetic cardiomyopathy.'

Several reviews address the contribution of altered cardiac metabolism to dysfunction[7,15-18]; in this work we focus primarily on the possible link between pre-

**Table 1** Characteristics of experimental models of pre-diabetic cardiomyopathy

Animal model	MetS parameters						Cardiovascular dysfunction	Ref.
	BW	BP	BG	IR	TG	HDL-C		
<b>Dogs</b>								
HFD dogs (80% of calories from fat, 5 wk)	↑	↑	↔	+	ND	ND	↑ Heart rate; ↓ Myocardial oxygen delivery and metabolism; ↓ Cardiac index after exercising. ↑ Aortic pressure	Setty <i>et al</i> [51] and Dincer <i>et al</i> [73]
<b>Rats</b>								
Sucrose-fed Wistar rats (68%, 7-10 wk)	↔	ND	↔	+	ND	ND	↓ FS; + Systolic dysfunction	Dutta <i>et al</i> [68]
Sucrose-fed Sprague-Dawley rats (68%, 7-10 wk)	↔	ND	↔	+	ND	ND	↔ Heart hypertrophy; ↓ FS; + Systolic dysfunction	Hintz <i>et al</i> [67], and Hintz and Ren[72]
Sucrose-fed Wistar rats (30%, 17-24 wk)	↔	↑	ND	+	↑	↓	↔/↑ Heart rate; ↓ Ventricular pressure; ↑ Arrhythmia incidence after reperfusion	López-Acosta <i>et al</i> [56], and Carvajal and Baños [60]
Sucrose-fed Sprague-Dawley rats (32%, 10 wk)	↑	ND	↑	+	↑	ND	↔ Heart hypertrophy; ↓ FS and EF; ↑ Septum dimension	Vasanji <i>et al</i> [52]
Sucrose-fed Wistar rats (30%, 24 wk)	↑	↑	↔	ND	↑	↔	↔ Heart hypertrophy; + Systolic dysfunction; ↓ Cardiac cell contraction	Barrera-Lechuga <i>et al</i> [53] and Fernández-Miranda <i>et al</i> [70]
Sucrose-fed Wistar rats (30%, 35 wk)	↑	↔	ND	ND	ND	ND	↔ Heart rate; ↔ Heart hypertrophy; ↓ FS	Paulino <i>et al</i> [65]
Sucrose-fed Wistar rats (30%, 16-18 wk)	↔	↔	↔	+	↑	ND	+ Systolic dysfunction	Balderas-Villalobos <i>et al</i> [69]
Sucrose-fed Wistar rats (20%, 8 wk)	↑	ND	↔	+	↔	↓	↓ Heart rate; ↑ SAN rate variability; ↑ SAN fat deposits	Albarado-Ibañez <i>et al</i> [54]
Sucrose-fed Wistar rats (32%, 16 wk)	↑	↑	↑	+	↑	ND	↑ Heart rate; ↑ Heart hypertrophy; ↓ Heart contractility; ↑ Cardiomyocyte lipid deposits; ↑ Aortic pressure	Okatan <i>et al</i> [27,28]
Fructose-fed Wistar rats (10%, 3 wk)	↔	↔	↔	+	↑	↓	↓ Heart rate; ↑ Heart hypertrophy; ↓ FS; ↓ Heart contractility; + Systolic dysfunction; + Diastolic dysfunction; + LV hypertrophy; ↑ Arrhythmia incidence	Sommese <i>et al</i> [55]
HFD Long-Evans rats treated with STZ (40% lard, 21 wk)	↑	↔	↑	+	↔	↔	↔ Heart rate; ↔ Heart hypertrophy; ↔ FS; ↑ Lipid in the myocardium; + Diastolic dysfunction	Koncsos <i>et al</i> [62]
Sucrose-fed Wistar rats (30%, 4 mo)	↑	ND	↔	+	↑	↓	↑ Heart rate; ↔ Heart hypertrophy; + Diastolic dysfunction; ↑ Arrhythmia incidence	Romero-García <i>et al</i> [48] and Landa-Galvan <i>et al</i> [57]
<b>Mice</b>								
Fructose-fed C57bl/6 mice (10%, 3 wk)	↔	↔	↔	+	ND	ND	↓ FS; + LV hypertrophy; + Systolic dysfunction	Federico <i>et al</i> [71]
HFD C57bl/6 mice (60% of calories from fat, 8 wk)	↑	ND	↑	ND	↔	ND	↑ Heart rate; ↔ FS; ↑ Arrhythmia incidence	Sánchez <i>et al</i> [63]
HFD FVB-mice (45% of calories from fat, 5 mo)	↑	↔	ND	+	ND	ND	↔ Heart rate; ↑ Heart hypertrophy; ↓ FS; + Systolic dysfunction	Dong <i>et al</i> [66]

↔: No change; +: Presence; ↓: Decreased; ↑: Increased; BG: Blood glucose; BP: Blood pressure; BW: Body weight; EF: Ejection fraction; FS: Fractional shortening; HDL-C: High-density lipoprotein cholesterol; HFD: High-fat diet; IR: Insulin resistance; LV: Left ventricle; MetS: Metabolic syndrome; SAN: Sinus Atrial Node; STZ: Streptozotocin; TG: Triglycerides; ND: Not determined.

diabetic cardiomyopathy and alterations in the  $\beta$ -adrenergic system and two main downstream signaling effectors: cAMP-dependent protein kinase A (PKA) and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII).

In cardiac cells, the expression and activity of key  $\text{Ca}^{2+}$  handling proteins involved in excitation-contraction coupling (ECC) are altered in IR and diabetic cardiomyopathy [8]. Under physiological conditions, cardiac ECC begins with  $\text{Ca}^{2+}$  influx through L-type voltage-dependent  $\text{Ca}^{2+}$  channels. A small influx of  $\text{Ca}^{2+}$  activates the intracellular  $\text{Ca}^{2+}$  channel/ryanodine receptor (RyR) through a mechanism known as  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release, eliciting a transient  $\text{Ca}^{2+}$  increase in the cytoplasm of the cardiac cell that in turn activates the contractile machinery. Relaxation involves the clearance of intracellular  $\text{Ca}^{2+}$  by: (1) Re-uptake into the sarcoplasmic reticulum  $\text{Ca}^{2+}$  stores through the activity of the sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase; and (2)  $\text{Ca}^{2+}$  extrusion by the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger in the sarcolemma[19].

The  $\beta$ -adrenergic response is the main regulatory pathway of ECC, involving the activation of PKA and CaMKII. These kinases phosphorylate several  $\text{Ca}^{2+}$  handling proteins, including L-type voltage-dependent  $\text{Ca}^{2+}$  channels, RyRs, and phospholamban (PLN), thereby modifying their activity[20] (Figure 1). In this review, we summarize the recent evidence of alterations in the expression and/or activity of PKA and CaMKII in diet-induced animal models of pre-diabetic cardiomyopathy. This work also emphasizes the prevailing role of CaMKII in the development of myocardial dysfunction associated with pre-diabetes (Figure 1).

## **$\beta$ -ADRENERGIC RECEPTOR SIGNALING IN THE HEART: PKA AND CAMKII ACTIVATION**

The heart is innervated by parasympathetic and sympathetic fibers that regulate contractility rate and force. Sympathetic innervation of the atria and ventricles is provided by the stellate ganglion, whereas the vagus nerve provides parasympathetic fibers to the sinoatrial node, atrioventricular node, and atria[21].

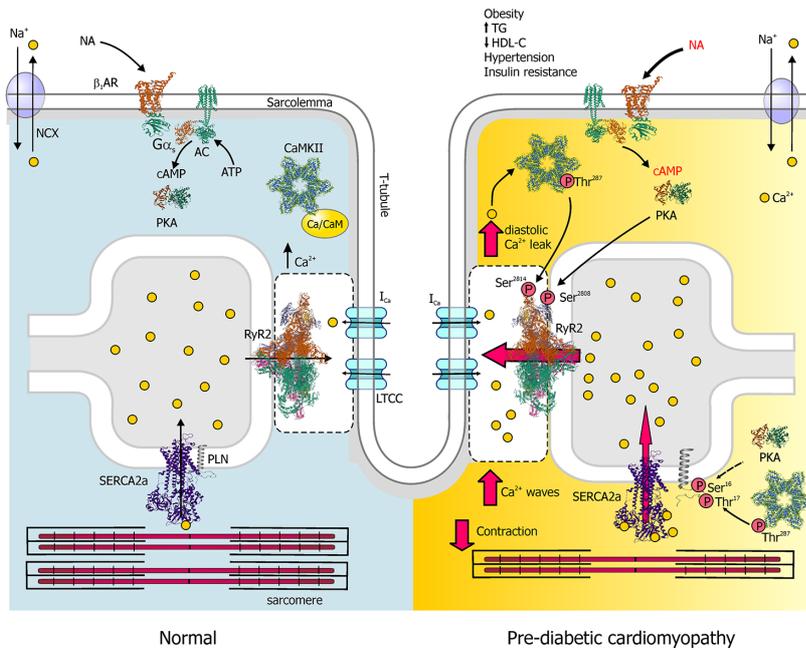
Sympathetic fibers synthesize and release noradrenaline (NA), while chromaffin cells located in the medulla of adrenal glands synthesize and release adrenaline (A) into the bloodstream. Both catecholamines exert their functional effects through the activation of selective receptors, called adrenoceptors (ARs)[22]. ARs are divided into three families:  $\alpha_1$ ,  $\alpha_2$ , and  $\beta$ . The  $\alpha_1$ -AR family is composed of  $\alpha_{1A}$ ,  $\alpha_{1B}$ , and  $\alpha_{1D}$  receptors, the  $\alpha_2$ -AR family by  $\alpha_{2A}$ ,  $\alpha_{2B}$ , and  $\alpha_{2C}$  subtypes, and the  $\beta$ -AR family comprises the  $\beta_1$ ,  $\beta_2$ , and  $\beta_3$  receptors. All three  $\alpha_1$ -AR subtypes couple predominantly to  $G_{\alpha_{q/11}}$  proteins; their activation leads to phospholipase C stimulation, activation of protein kinase C, and inositol 1,4,5-trisphosphate-mediated  $\text{Ca}^{2+}$  release from intracellular stores[23].  $\alpha_2$ -ARs couple to  $G_{\alpha_{i/o}}$  proteins, reducing cAMP formation, and inhibiting N- and P-type voltage-activated  $\text{Ca}^{2+}$  channels[24].  $\beta$ -ARs mainly couple to  $G_{\alpha_s}$  proteins, eliciting adenylyl cyclase (AC) activation and cAMP formation[22] (see below), although stimulation of  $\beta_2$ - and  $\beta_3$ -ARs also activates  $G_{\alpha_{i/o}}$  proteins[25,26].

The regulation of cardiac function by the sympathetic nervous system *via*  $\beta$ -ARs is of particular interest because dysregulation of this system has been reported in HF and metabolic disorders such as DM2 and MetS[27-29].

Radioligand binding assays with human heart preparations indicate that cardiac tissues express mainly  $\beta_1$ - and  $\beta_2$ -ARs, which represent 90% of all ARs and are expressed at an 8:2 ratio in both atria and ventricles[30]. There is also evidence for the expression of  $\beta_3$ -ARs in cardiomyocytes[26]; however, the  $\beta_1$  and  $\alpha_{1B}$  subtypes are the main ARs expressed in isolated mouse ventricular cardiomyocytes, with  $\beta_2$ - and  $\beta_3$ -ARs expressed by only 5% of cardiomyocytes but with high expression by endothelial cells[31]. These data support the notion that  $\beta$ -adrenergic responses in cardiomyocytes are primarily mediated by  $\beta_1$ -ARs.

Furthermore,  $\beta_1$ -ARs are located on the surface of all cardiomyocytes, whereas  $\beta_2$ -ARs are expressed exclusively at T-tubules. However, in HF,  $\beta$ -AR expression is redistributed so that  $\beta_2$ -ARs co-localize with  $\beta_1$ -ARs[32], suggesting that  $\beta$ -ARs participate in the cardiac remodeling that underlies the pathogenesis of cardiac diseases.

$\beta$ -ARs are activated by both NA and A, but the subtypes show different affinity for the endogenous agonists, with rank order of potency:  $\beta_1$ -ARs,  $\text{NA} > \text{A}$ ;  $\beta_2$ -ARs,  $\text{A} > \text{NA}$ ; and  $\beta_3$ -ARs,  $\text{NA} \approx \text{A}$ [22]. As mentioned above, agonist-bound  $\beta$ -ARs stimulate AC activity *via*  $G_{\alpha_s}$  proteins. There are nine isoforms of membrane-integral ACs[33]; cardiac tissues primarily express the AC5 and AC6 isoforms[34]. ACs catalyze the synthesis of cAMP from ATP; cAMP directly activates PKA and the exchange protein directly activated by cAMP (Epac). These proteins participate in the activation of CaMKII *via* PKA-mediated increases in the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) and the Epac/phosphoinositide 3-kinase/Akt/n-nitric oxide synthase pathway,



**Figure 1  $\beta$ -Adrenergic stimulation in the normal heart and pre-diabetic cardiomyopathy.** Left panel:  $\beta$ -adrenergic stimulation in the normal heart. In physiological excitation-contraction coupling, membrane depolarization activates L-type voltage-dependent  $\text{Ca}^{2+}$  channels, inducing a small  $\text{Ca}^{2+}$  influx ( $I_{Ca}$ ) that triggers the activation of cardiac ryanodine receptors (RyR2, PDB accession code: 6W0V). This triggers the release of sufficient  $\text{Ca}^{2+}$  from the lumen of the sarcoplasmic reticulum to the cytoplasm to elicit contraction. During relaxation,  $\text{Ca}^{2+}$  is primarily removed from the cytoplasm by the sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (PDB accession code: 6HXB), which resequesters  $\text{Ca}^{2+}$  into the sarcoplasmic reticulum lumen.  $\text{Ca}^{2+}$  is also extruded by the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (PDB accession code: 3US9), while a small amount of  $\text{Ca}^{2+}$  is taken up by the mitochondrial calcium uniporter (PDB accession code: 6WDN). Noradrenaline activates  $\beta_1$ -adrenoceptors ( $\beta_1$ -ARs, PDB accession code: 6H70) located at the sarcolemma of cardiomyocytes; agonist-bound  $\beta_1$ -ARs stimulate  $G_s$  proteins and therefore one or more isoforms of adenylyl cyclase (PDB accession code: 6R3Q), leading to cAMP formation and the activation of the cAMP-dependent protein kinase (PDB accession code: 3FHI). Protein kinase A phosphorylates several  $\text{Ca}^{2+}$  handling proteins, including RyR2 at Ser<sup>2808</sup> and phospholamban (PDB accession code: 2LPF) at Ser<sup>16</sup>; the latter increases sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase pump activity.  $\text{Ca}^{2+}$  binds to calmodulin, and the complex Ca/calmodulin binds to and activates  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (PDB accession code: 3SOA), which phosphorylates RyR at Ser<sup>2814</sup> and phospholamban at Thr<sup>17</sup>. The exchange protein directly activated by cAMP (Epac) is also involved in  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II activation; however, its role in pre-diabetic cardiomyopathy has not yet been addressed; thus, it is not depicted in the figure. Right panel:  $\beta$ -adrenergic stimulation in pre-diabetic cardiomyopathy. In the presence of obesity, increased triglyceride levels, decreased high-density lipoprotein cholesterol, hypertension, and/or insulin resistance (all Metabolic Syndrome components), and abnormal  $\beta_1$ -AR activation (associated with either chronic sympathetic tone or changes in  $\beta$ -AR expression) dysregulates excitation-contraction coupling in cardiac cells. Pre-diabetic cardiomyopathy is characterized by abnormal diastolic  $\text{Ca}^{2+}$  leak (diastolic dysfunction) due to augmented RyR2 phosphorylation at Ser<sup>2808</sup> and Ser<sup>2814</sup> in the absence of adrenergic stimulation, generating spontaneous  $\text{Ca}^{2+}$  waves that may induce pro-arrhythmic events through altered  $\text{Na}^+/\text{Ca}^{2+}$  exchanger activity. In addition, phosphorylated phospholamban (at Ser<sup>16</sup> and Thr<sup>17</sup>) detaches from sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase 2a, augmenting its activity; finally,  $\text{Ca}^{2+}$  transient amplitude decreases and leads to impaired cell contraction. NA: Noradrenaline; AR: Adrenoceptors; NCX:  $\text{Na}^+/\text{Ca}^{2+}$  exchanger; AC: Adenylyl cyclase; PKA: Protein kinase A; CaMKII:  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II; CaM: Calmodulin; RyR: Ryanodine receptor; LTCC: L-type voltage-dependent  $\text{Ca}^{2+}$  channels; PLN: Phospholamban; HDL-C: High-density lipoprotein cholesterol; TG: Triglycerides; SERCA: Sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase.

respectively[35]. In turn, both PKA and CaMKII phosphorylate several proteins involved in cardiac ECC, such as L-type voltage-dependent  $\text{Ca}^{2+}$  channels, RyR2, and PLN, leading to increased heart rate and contractile force[19,36].

PKA is a serine/threonine kinase comprising two regulatory (R) and two catalytic (C) subunits. There are four isoforms of the catalytic subunit ( $C\alpha$ ,  $C\beta$ ,  $C\gamma$ ,  $C\delta$ ) and four isoforms of the regulatory subunit ( $RI\alpha$ ,  $RII\alpha$ ,  $RI\beta$ ,  $RII\beta$ [37]). The PKA complex is formed by two catalytic subunits and two regulatory subunits; the complexes are named according to the number of the regulatory subunit (*i.e.* PKA-I and PKA-II). The regulatory subunits contain two cAMP binding sites and a pseudo-substrate domain that binds to the active site of the catalytic subunit in the absence of cAMP. The binding of two cAMP molecules to each regulatory subunit induces a conformational change that promotes the dissociation of the catalytic subunits from the regulatory subunits[38].

Cardiomyocytes express the four isoforms of the PKA regulatory subunits, with the  $\alpha$ -isoforms being more abundant than the  $\beta$ -isoforms[39,40]. By using fluorescence resonance energy transfer-based cAMP reporters, Di Benedetto *et al*[41] showed that PKA-I and PKA-II are compartmentalized in cardiomyocytes through their binding to specific A-kinase anchoring proteins. PKA-I is expressed in a tightly striated manner that overlies the sarcomere Z and M lines, whereas PKA-II is strongly expressed in M lines and only slightly in Z lines.  $\beta$ -AR activation with the non-selective agonist

isoproterenol increases cAMP levels primarily in the PKA-II domain, leading to phosphorylation of the regulatory proteins troponin I and PLN as well as RyR2 at Serine 2808 (Ser<sup>2808</sup>), among other residues. The effect of the latter results in increased RyR2 open probability, although the exact impact on channel function is not clear[41-43]. Together, these findings suggest that PKA-II, rather than PKA-I, underlies the functional responses mediated by  $\beta_1$ -ARs.

CaMKII also phosphorylates proteins involved in cardiac ECC[36]. Four CaMKII isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) have been reported; CaMKII- $\delta$  is the dominant isoform in cardiomyocytes[44]. CaMKII is a multimer complex of 12 monomers assembled in two hexameric rings; each monomer consists of an N-terminal domain, an autoinhibitory regulatory region, and a C-terminal domain. Transient increases in  $[Ca^{2+}]_i$  are sensed by calmodulin, leading to the assembly of a  $Ca^{2+}$ /calmodulin complex, which binds to the CaMKII autoinhibitory regulatory domain and induces conformational changes that result in kinase activation and under some pathological conditions in CaMKII autophosphorylation at Thr<sup>287</sup>[45]. In addition, several other post-translational modifications promote autonomous CaMKII activity, such as oxidation at Met<sup>281/282</sup>, O-GlcNAcylation at Ser<sup>280</sup>, and S-nitrosylation at Cys<sup>290</sup>[35].

Emerging evidence supports a relevant role for Epac as a mediator of cAMP signaling in the heart. There are two Epac isoforms in mammals, Epac1 and Epac2; both contain an N-terminal regulatory domain and a C-terminal catalytic region. Upon cAMP binding, Epac proteins activate the Ras superfamily small GTPases Rap1 and Rap2[46]. CaMKII can also be activated by Epac2; in rat myocytes, the activation of  $\beta_1$ -ARs, but not  $\beta_2$ -ARs, lead to Epac2-dependent CaMKII- $\delta$  stimulation, which results in RyR2 phosphorylation at Ser<sup>2814</sup>. This effect is abolished in CaMKII- $\delta$ -KO mice, supporting a key role for this CaMKII isoform in cardiac responses mediated by  $\beta_1$ -ARs[47].

The research reviewed above suggests that both PKA and CaMKII- $\delta$  play important roles in  $\beta_1$ -AR-mediated responses and that alterations in the expression or function of these kinases can therefore be deleterious. Moreover, enhanced and sustained  $\beta$ -adrenergic stimulation contributes to the development of such pathological conditions as HF[29]; these alterations may also extend to diabetic and pre-diabetic cardiomyopathy[28,35,48]. A recent study showed that incubation of isolated mouse cardiomyocytes in high extracellular glucose (30 mmol/L) to mimic acute hyperglycemia leads to O-GlcNAcylation at CaMKII Ser<sup>280</sup> and enhanced kinase activity, resulting in RyR2 phosphorylation and pro-arrhythmogenic activity[45]. Despite the availability of several MetS experimental models, pre-diabetic cardiomyopathy has been less studied (see below); the role of PKA and CaMKII in this pathology remains to be elucidated.

## ANIMAL MODELS OF PRE-DIABETIC CARDIOMYOPATHY

Very few articles have considered MetS-associated cardiac alterations as pre-diabetic cardiomyopathy[49], most likely due to the lack of an accepted definition. Based on the graded effect of impaired glucose metabolism on diastolic function, it has been proposed that a morphological intermediate state between normal and diabetic states underlies pre-diabetic heart dysfunction[50]. One feature that perhaps differentiates pre-diabetic from diabetic cardiomyopathy is the absence of overt structural changes in the heart in the former, although this interpretation is under discussion[14].

Due to the multifactorial nature of cardiometabolic disease associated with obesity, IR, high blood pressure, high glycemic levels, and hypertriglyceridemia, the selection of an appropriate experimental model bearing the features of diet-induced pre-diabetic cardiomyopathy in humans has proven difficult. Most studies addressing diet-induced cardiometabolic alterations have been performed with laboratory animals under either carbohydrate- or fat and carbohydrate-enriched diets to emulate the Western diet, characterized by the ingestion of refined sugar and high caloric food. However, not all models – indeed, only eight[27,51-57] of those considered in this work – fulfill the requirement of at least three of the aforementioned criteria to be considered experimental models of MetS (Table 1).

Rats and mice are the most used animals for MetS models based on dietary manipulation; there are comprehensive reviews on this topic[58,59]. In this review, we focus on animal models with diet-induced pre-diabetic cardiomyopathy. Because the incidence of MetS in human populations is increasing, the establishment of MetS animal models is key to understanding the molecular mechanisms that are altered during the onset of myocardial disease. Although these diet-based experimental

models represent a critical milestone for pre-diabetic cardiomyopathy research, their utility is hampered by discrepancies in biochemical and corporal parameters, along with dissimilar outcomes that might be associated with the type and length of the diet. For instance, for 16 diet-induced models of pre-diabetic cardiomyopathy considered in this review, 10 showed a significant increase in body weight (Table 1), while only four models developed high blood pressure[28,51,53,60]. Also, in good agreement with a seminal report by Reaven[61], a hallmark feature of pre-diabetic cardiomyopathy models is the presence of IR. FBG levels were evaluated in 13 models, but only 4 reported altered values[28,52,62,63]. For dyslipidemia, high blood triglyceride levels were reported for seven models, and only four showed decreased blood high-density lipoprotein cholesterol levels[54,55,57,64] (Table 1).

Importantly, despite the discrepancies in metabolic alterations all these animal models developed pre-diabetic cardiomyopathy, characterized by several cardiac alterations. For instance, increased heart rate was reported in five models[27,48,51,56,63]; however, other studies in which this parameter was evaluated did not report changes[60,62,65,66]. Systolic dysfunction has also been observed, including decreased heart contractility, ventricular pressure, and intracellular Ca<sup>2+</sup> transient amplitude[27,55,60,67-71], along with reduced fractional shortening[55,65-67,70-72] (Table 1). Diastolic dysfunction is also manifested by increased diastolic Ca<sup>2+</sup> leak in the form of Ca<sup>2+</sup> waves, without altering cytoplasmic Ca<sup>2+</sup> levels[48,55,62]. To compensate for compromised cardiac output, the heart grows; however, few studies have documented either heart hypertrophy[27,55,66] or left ventricle hypertrophy[55,71]. Interestingly, several pre-diabetic cardiomyopathy models develop increased aortic pressure[27,28,51,73] and high arrhythmia incidence under basal or stressful conditions[48,55,56,63] (Table 1). Of note, rats and mice are the most common animal models for inducing pre-diabetic cardiomyopathy, although pigs and dogs have also been employed because of their greater degree of similarity to human cardiac physiology, including ionic currents that contribute to the cardiac action potential[74], Ca<sup>2+</sup> removal mechanisms, and ECC regulatory mechanisms[19]. It is thus essential to select the appropriate experimental model considering the objectives of the study to be performed.

## β-AR/AC/cAMP/PKA AXIS IN PRE-DIABETIC CARDIOMYOPATHY

As mentioned above, β-AR activation modulates ECC; cardiac dysfunction can therefore develop following alterations in the signaling pathways triggered by β-AR activation. Several studies have focused on PKA and CaMKII function (Table 2), which are effectors of β-adrenergic responses and the main topic of this review. However, the mechanisms by which the β-adrenergic pathway is disturbed in MetS are not yet clear; thus, it is important to understand how the βAR/AC/cAMP/PKA axis is affected, and how these changes originate or exacerbate cardiac dysfunction. In this section, we will describe the alterations in this signaling pathway reported in MetS and compare them with previous results found in DM.

Pre-diabetic cardiomyopathy can involve over-activation of the β-AR response. Indeed, patients with MetS show increased sympathetic activation, as measured by microneurography[75]; further, a cross-sectional and longitudinal study reported that MetS is associated with increased resting heart rate[76]. Both studies suggest over-activation of sympathetic activity by MetS, and we recently reported increased basal heart rate in the rat sucrose-induced MetS model[48]. Moreover, following the administration of an arrhythmogenic cocktail (caffeine 80 mg/kg and epinephrine 2 mg/kg; intravenously), 80% of the animals developed ventricular fibrillation, which suggests altered β-AR-mediated responses.

The reported alterations could also be related to changes in β-AR expression. For example, two studies in streptozotocin-induced diabetic rats (an experimental model of type 1 DM) reported a reduction in β<sub>1</sub>-AR mRNA levels, but increased levels of both β<sub>2</sub>- and β<sub>3</sub>-AR mRNA. Conversely, the protein content of β<sub>1</sub>- and β<sub>2</sub>-ARs was reduced but that of β<sub>3</sub>-ARs was increased[27,77], suggesting β-AR expression remodeling in the diabetic heart. However, β<sub>1</sub>- and β<sub>2</sub>-AR protein levels were not affected in a rat model of obesity with IR and hypertriglyceridemia[78] or a diet-induced MetS mouse model [79]. Of note, the study by Okatan *et al*[27] also evaluated β-AR expression in rats with MetS. The authors reported unaltered mRNA levels but diminished protein levels of β<sub>1</sub>- and β<sub>2</sub>-ARs, accompanied by normal cardiac function (as evaluated by left ventricle developed pressure following stimulation with NA)[27]. These findings suggest that an increased β-AR-mediated response compensates for the reduction in β<sub>1</sub>- and β<sub>2</sub>-AR

**Table 2 Alterations in protein kinase A in experimental models of pre-diabetes induced by diet**

Experimental model	Kinase modification	Functional effects	Ref.
HFD dogs (80% of calories from fat, 5 wk)	ND	↑ RyR2- Ser <sup>2809</sup> phosphorylation	Dincer <i>et al</i> [73]
Sucrose-fed Sprague-Dawley rats (32%, 10 wk)	↑ PKA activity (kemptide phosphorylation)	↓ PLN-Ser <sup>16</sup> phosphorylation	Vasanji <i>et al</i> [52]
Sucrose-fed Wistar rats (30%, 35 wk)	↔ expression and activity	↔ PLN-Ser <sup>16</sup> phosphorylation; ↓ RyR2- Ser <sup>2808</sup> phosphorylation	Paulino <i>et al</i> [65]
Sucrose-fed Wistar rats (32%, 16 wk)	↑ PKA activity (Thr <sup>198</sup> phosphorylation)	↑ RyR2- Ser <sup>2808</sup> phosphorylation; ↓ PLN-Ser <sup>16</sup> phosphorylation	Okatan <i>et al</i> [28]
Fructose-fed Wistar rats(10%, 3 wk)	ND	↔ RyR2- Ser <sup>2808</sup> phosphorylation	Sommese <i>et al</i> [55]
HFD Long-Evans rats treated with STZ (40% lard, 21 wk)	ND	↔ PLN-Ser <sup>16</sup> phosphorylation	Koncsos <i>et al</i> [62]
HFD C57bl/6 mice (60% of calories from fat, 8 wk)	ND	↔ RyR2- Ser <sup>2808</sup> phosphorylation; ↔ PLN-Ser <sup>16</sup> phosphorylation	Sánchez <i>et al</i> [63]
Sucrose-fed Wistar rats (30%, 24 wk)	ND	↔ RyR2- Ser <sup>2808</sup> phosphorylation; ↔ PLN-Ser <sup>16</sup> phosphorylation	Fernández-Miranda <i>et al</i> [70]
Sucrose-fed Wistar rats (30%, 4 mo)	ND	↔ RyR2- Ser <sup>2808</sup> phosphorylation; ↔ PLN-Ser <sup>16</sup> phosphorylation	Romero-García <i>et al</i> [48]
HFD C57bl/6N mice (45% of total calories from fat, 8 wk)	ND	↔ PLN-Ser <sup>16</sup> phosphorylation	Llano-Diez <i>et al</i> [79]

↔: No change; ↓: Decreased; ↑: Increased; ND: Not determined; HFD: High-fat diet; PKA: Protein kinase A; PLN: Phospholamban; RyR2: Ryanodine receptor type 2; STZ: Streptozotocin.

expression in MetS. Nevertheless, further research is required to fully elucidate the link between MetS,  $\beta_1$ -AR expression, signaling alterations, and cardiac dysfunction.

$\beta$ -AR stimulation results in AC activation *via*  $G_{\alpha_s}$  proteins. However, we found no studies that evaluated  $G_{\alpha_s}$  expression or activity in MetS experimental models, although decreased  $G_{\alpha_s}$  protein expression was reported for diabetic Yucatan minipigs [80]. Furthermore, AC activity was normal in ventricular preparations from obese rabbits[81], which would suggest that cAMP intracellular concentration is unchanged; however, AC activity has not been studied in MetS models.

PKA is activated by cAMP and contributes to enhanced heart rate and contractility by phosphorylating several proteins, including RyR2 and PLN. In streptozotocin-induced diabetic mice, both PKA activity and cytosolic PKA catalytic subunit content were reduced[82]. Further, in rat isolated cardiomyocytes, incubation in medium supplemented with high glucose (25.5 mmol/L) reduced PKA activity[83]. Finally, PKA activity diminished along with a reduction in the positive inotropic response induced by isoproterenol in obese diabetic Zucker rats[84]. Together, these studies indicate that hyperglycemic conditions affect PKA function.

Three studies have evaluated PKA activity in pre-diabetic models: Okatan *et al*[28] and Vasanji *et al*[52] reported increased kinase activity, but Paulino *et al*[65] did not detect significant changes (Table 2). PKA activity has also been studied indirectly by determining the phosphorylation levels of PLN (Ser<sup>16</sup>) or RyR2 (Ser<sup>2808</sup> in rats; Ser<sup>2809</sup> in dogs), with contradictory results (Table 2). Two studies reported increased RyR2 phosphorylation[28,73], one a decrease[65], and four lack of effect[48,55,63,70], while reduced PLN-Ser<sup>16</sup> phosphorylation was found in two studies[28,52], and six reported no change[48,62,63,65,70,79].

Furthermore, upregulated PKA expression was reported for a genetic MetS model, a double knock-out of LDL-receptor (LDLR<sup>-/-</sup>) and leptin-deficient (*ob/ob*) murine model, likely indicating that the genetic background contributes to the phenotype of the pathology[85]. Thus, the observed variations in PKA function could be due to the different conditions to which the animals were exposed, for example, diet composition and length (Table 2).

In summary, several studies found alterations in heart function or cardiomyocyte contraction in diet-induced models of pre-diabetes, which could be associated with altered PKA activity (Table 2). Because only three studies directly evaluated kinase activity[28,52,65] and reported contradictory results, more work is needed to determine the precise role of PKA in pre-diabetic cardiomyopathy. Together, the information reviewed suggests modification of the  $\beta$ -AR/AC/cAMP/PKA signaling

pathway upstream of PKA or disruption of other effectors of the  $\beta$ -adrenergic response, such as CaMKII, which are not yet broadly studied in MetS. We found no data on PKA alterations in diabetic or pre-diabetic patients; clearly studies addressing this issue would provide valuable information on the pathophysiology of MetS- and diabetes-induced cardiomyopathy.

## CAMKII AS A NOVEL TARGET IN PRE-DIABETIC CARDIOMYOPATHY

CaMKII has been proposed as a key contributor to the deleterious effects of chronic  $\beta$ -AR activation in diabetic cardiomyopathy, primarily by exacerbating RyR2-mediated diastolic  $\text{Ca}^{2+}$  leak[86,87]. Studies in experimental models of IR and fructose fed-induced pre-diabetic cardiomyopathy have unveiled the role of hyperglycemia and reactive oxygen species in inducing abnormal CaMKII phosphorylation at Thr<sup>287</sup> and activation, altering cardiomyocyte intracellular  $\text{Ca}^{2+}$  handling and promoting cardiac arrhythmic events[55,71,88]. Hyperglycemia leads to CaMKII glycosylation, increasing RyR2-mediated  $\text{Ca}^{2+}$  leak and reducing sarcoplasmic reticulum  $\text{Ca}^{2+}$  load in cardiac cells[88]. However, in pre-diabetic cardiomyopathy hyperglycemia is not overt[48]; thus, abnormal CaMKII activation relies on additional mechanisms[48,55]. The length of CaMKII activation relies on the frequency of  $\text{Ca}^{2+}$  release events, and extended CaMKII activation is also related to autophosphorylation at Thr<sup>287</sup>, which prevents CaMKII auto-inhibition[87]. In animal models of pre-diabetes, cardiac CaMKII remains active even when the  $[\text{Ca}^{2+}]_i$  declines, constituting a mechanism for anomalous CaMKII activation (Table 3)[48,55,87]. CaMKII phosphorylates RyR2 at Ser<sup>2814</sup>; CaMKII abnormal activation can therefore induce higher activity of RyRs even at diastolic  $\text{Ca}^{2+}$  levels, leading to increased spontaneous  $\text{Ca}^{2+}$  wave frequency and propensity to spontaneous cardiomyocyte contraction and arrhythmias[48,55]. Of note, CaMKII activity was determined only in one study[52] (Table 3); therefore, further studies are needed.

In spontaneously hypertensive rats, which could be considered a genetic model of MetS, the knock-out of *Camk2n1* (*SHR-Camk21<sup>-/-</sup>*), a peptide that regulates the association of  $\text{Ca}^{2+}$ /calmodulin with CaMKII, reduced kinase activity in the heart, thereby improving cardiac function[89]. Interestingly, the effect of deleting CaMKII- in sucrose-induced cardiac dysfunction has not yet been evaluated.

In heart disease, CaMKII has been implicated in ECC disorders that lead to cardiac dysfunction[90]; in particular, CaMKII overactivation is associated with the appearance of arrhythmias linked to abnormal  $\text{Ca}^{2+}$  handling[91-93].

As mentioned above, phosphorylation at Ser<sup>2814</sup> by abnormal CaMKII activation induces RyR2 hyperactivity. Thus, preventing RyR phosphorylation by a point mutation (Ser2814Ala) that inactivates the phosphorylation site of CaMKII circumvents the development of HF induced by transverse aortic constriction in mice [94]. In contrast, a mutation that mimics RyR2 constitutive activation by CaMKII exacerbates arrhythmogenesis and sudden cardiac death in mice with HF[95]. Moreover, in mice with HF, knock-out of the CaMKII  $\gamma$ / isoforms protects against cardiac dysfunction and fibrosis induced by pressure overload and  $\beta$ -adrenergic stimulation[96,97]. Of note, in two diet-induced pre-diabetic cardiomyopathy models, pharmacological inhibition of CaMKII prevents  $\text{Ca}^{2+}$  mishandling and RyR dysregulation[48,55].

Notably, post-translational modifications (specifically, oxidation, O-glycosylation, and phosphorylation) of CaMKII are increased in heart samples of diabetic patients[88, 98,99], suggesting altered kinase activity. As for PKA, research on the possible role of CaMKII alterations in diabetic or pre-diabetic patients is required to increase our understanding of the pathophysiology of MetS- and diabetes-induced cardiomyopathy.

## CONCLUSION

MetS is a serious public health problem with increased risk for CVD and DM2, leading to cardiac dysfunction in the form of pre-diabetic cardiomyopathy. This, in turn, stimulates the  $\beta$ -adrenergic response with inotropic and chronotropic positive effects that initially compensate the deficient heart contraction but that eventually become deleterious in chronic disease.

**Table 3 Alterations in Ca<sup>2+</sup>/calmodulin-dependent protein kinase II in experimental models of pre-diabetes induced by diet**

Experimental model	CaMKII alterations	Functional effects	Ref.
Sucrose-fed Sprague-Dawley rats (32% 10 wk)	↑ CaMKII activity (autocamtide phosphorylation)	↓ PLN-Thr <sup>17</sup> phosphorylation	Vasanji <i>et al</i> [52]
Fructose-fed Wistar rats (10%, 3 wk)	↑ CaMKII oxidation; ↔ CaMKII expression	↑ RyR2-Ser <sup>2814</sup> phosphorylation	Sommese <i>et al</i> [55]
Sucrose-fed Wistar rats (30%, 4 mo)	↑ CaMKII-Thr <sup>287</sup> phosphorylation; ↔ CaMKII expression	↑ RyR2-Ser <sup>2814</sup> phosphorylation	Romero-García <i>et al</i> [48]
HFD Long-Evans rats treated with STZ (40% lard, 21 wk)	↔ CaMKII-Thr <sup>287</sup> phosphorylation; ↔ CaMKII expression	↔ PLN-Thr <sup>17</sup> phosphorylation	Koncsos <i>et al</i> [62]

↔: No change; ↓: Decreased; ↑: Increased; CaMKII: Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; HFD: High-fat diet; PLN: Phospholamban; RyR2: Ryanodine receptor type 2; STZ: Streptozotocin.

There is evidence supporting the hypothesis that MetS alters β-adrenergic signaling, but it is still not clear how β-adrenergic signaling is affected in diet-induced MetS models. β<sub>1</sub>-ARs are the more abundant isoform in cardiomyocytes and are the primary mediators of the β-adrenergic response under physiological conditions. However, the link between MetS, β<sub>1</sub>-AR expression and signaling alterations and cardiac dysfunction remains to be fully established.

β-AR stimulation leads to PKA and CaMKII activation, and MetS could involve kinase overactivation. For PKA, the available data indicate overactivation, no change, or reduced activity; further research is clearly needed. For CaMKII, the evidence suggests a critical role in the development of pre-diabetic cardiomyopathy; understanding the mechanisms that dysregulate CaMKII activity in MetS would therefore contribute importantly to elucidating the molecular basis of cardiac dysfunction.

Importantly, the majority of the information reported in this review was generated with small rodent models; further studies are required in animal models that more closely approximate human cardiac physiology.

### Future perspectives

Several issues remain to be addressed in investigating the possible effect of MetS on β-adrenergic signaling pathways in cardiomyocytes and actions on PKA and CaMKII activity. For instance, the role of Epac2, which is also activated by β<sub>1</sub>-AR stimulation, has not been elucidated in pre-diabetic cardiomyopathy. Furthermore, it is not well established whether MetS modifies β-AR expression by cardiomyocytes or what role receptor desensitization might play in the hyper-adrenergic state induced by the syndrome. An additional relevant question is whether MetS induces post-translational modifications in CaMKII that result in an altered activity. Additional knowledge would allow for laying the foundation for the rational design of targeted therapies to prevent or treat the development of pre-diabetic cardiomyopathy.

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