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Role of CaMKII in free fatty acid/hyperlipidemia-induced cardiac remodeling both in vitro and in vivo

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ABSTRACT

Rationale: The cellular mechanisms of obesity/hyperlipidemia-induced cardiac remodeling are many and not completely elucidated. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a multifunctional serine/threonine kinase, has been reported to be involved in a variety of cardiovascular diseases. However, its role in obesity/hyperlipidemia-induced cardiac remodeling is still unknown.
Objective: The objective of this study was to demonstrate the role of CaMKII in the pathogenesis of obesity/hyperlipidemia-induced cardiac remodeling both in vitro and in vivo.
Methods and results: In cardiac-derived H9C2 cells, palmitate treatment induced cell apoptosis coupled with activation of the mitochondrial apoptotic pathway, and cell hypertrophic and fibrotic responses. All of these alterations were inhibited by pharmacological inhibition of CaMKII with either of two specific inhibitors, Myr-AIP and KN93. In addition, an increased inflammatory response coupled with activation of the MAPKs and NF-κB signaling pathway, exaggerated oxidative stress, ER stress and autophagy were also observed in palmitate-treated H9C2 cells, while pretreatment with CaMKII inhibitors decreased these pathological signals. Furthermore, we also demonstrated that TLR4 is upstream signal of CaMKII in palmitate-treated H9C2 cells. In $APOE^{-/-}$ mice fed a high-fat diet (HFD) for 16 weeks, serum lipid profiles (FFAs, TG, TC) and blood glucose levels were significantly increased compared with mice fed a normal diet. In addition, apparent cardiac hypertrophy, fibrosis and apoptosis associated with increased inflammation, ER stress, and autophagy were also observed in the hearts of HFD-fed mice. However, all these changes were reversed by 8-weeks of KN93 peritoneal injections. KN93 also increased antioxidant defense as evidenced by increased expression of the Nrf2 system in the hearts of HFD-fed mice.
Conclusions: Taken together, our results demonstrate a critical role of CaMKII in the pathogenesis of obesity/hyperlipidemia-induced cardiac remodeling. Also, TLR4 may be an upstream signal of cardiac CaMKII under hyperlipidemia conditions. These results suggest that CaMKII has the potential to be a therapeutic target in the prevention of obesity/hyperlipidemia-induced cardiac remodeling.

1. Introduction

Obesity/hyperlipidemia is considered the main contributing factor in the development of metabolic syndromes and is strongly associated with structural and functional changes in the heart in both humans and animal models [1]. Cardiac consequences of obesity/hyperlipidemia include cardiac hypertrophy, cardiac fibrosis, cardiac apoptosis and subclinical impairment of LV systolic and diastolic function [2,3]. Work from the last few decades has improved our understanding of the pathophysiological mechanisms contributing to obesity/hyperlipidemia-related cardiac remodeling. In particular, inflammation, oxidative stress, endoplasmic reticulum stress, and autophagy appear to play important roles in the development and persistence of cardiomyopathy [2]. Despite the plethora of proposed mechanisms, the detailed molecular mechanism still needs to be elucidated.

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine, with CaMKIIδ being the predominant isoform in the heart [4]. Increasing evidence has demonstrated that CaMKII is activated and plays a critical role during different cardiac pathological processes such as heart failure, myocardial infarction, myocardial ischemia/reperfusion injury, and arrhythmogenesis of different

Abbreviations: CaMKII, Ca2+/calmodulin-dependent protein kinase II; ROS, reactive oxygen species; ER, endoplasmic reticulum; MMP, mitochondrial membrane potential; MFI, median fluorescence intensity; CCCP, cyanide 3-chlorophenylhydrazone.

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etiologies [5]. Activation of CaMKII during cardiac stress has been reported to regulate a diverse array of proteins involved not only in excitation-contraction coupling (ECC) and relaxation, but also in cell death, transcriptional activation of hypertrophy, inflammation, and arrhythmias. CaMKII activity can be increased in multiple ways such as intracellular Ca^{2+} increase, oxidation, glycosylation, and nitrosylation at different sites in the regulatory domain of the kinase [6–8]. Thus, CaMKII may be activated in different ways in various cardiac diseases and may act as a nodal point for integrating different fundamental proteins involved in functional and transcriptional cardiac processes.

More recently, CaMKII was found to be activated in the liver and skeletal muscle of obese mice and was shown to be implicated in the development of insulin resistance [9,10]. In addition, free fatty acids (especially, palmitate) were demonstrated to activate CaMKII in hepatocytes in vitro. These results suggest that CaMKII can be activated in obesity or hyperlipidemia conditions and may have a role in mediating detrimental consequences of obesity. Thus, we hypothesize that CaMKII may also play an important role in the development of obesity/hyperlipidemia-induced cardiac remodeling. Therefore, our current study will evaluate the involvement of CaMKII in mediating cardiac injury under obesity or hyperlipidemia conditions, using a saturated fatty acid (palmitate)-stimulated cardiac-derived H9C2 cell model in vitro and a high fat diet (HFD)-fed APOE-deficient mouse model in vivo.

2. Methods

2.1. Cell culture

An H9C2 embryonic rat heart-derived cell line was obtained from the Shanghai Institute of Biochemistry and cell Biology (Shanghai, China) and cultured in DMEM/F12 medium (Gibico) supplemented with 5% FBS, 100 U/ml of penicillin, and 100 mg/ml of streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Animal studies

Male APOE-deficient mice on a C57BL/6 background weighing 18-22 g, were used in this study. The animals were purchased from HFK Bio-Technology Company (Beijing, China). All the animal experiment protocols were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University and were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Twenty-four mice were housed in an environmentally controlled room at 22 ± 2.0 °C and 50% ±5% humidity with a 12-h:12-h light/dark cycle and fed food and water ad libitum. Eight mice were fed standard animal chow and served as a control group (Ctrl). The remaining 16 mice were fed with a high fat diet (45% fat; Beijing HFK Bio-Technology, Beijing, China) for 8 weeks. After 8 weeks of feeding, HFD-fed mice were further divided into two groups: a HFD group (n = 8) and a HFD group treated with KN93 (n = 8). KN93 was given by peritoneal injection at 10 mg/kg/2 days for 8 weeks. Mice in the Ctrl and HFD groups were injected with vehicle only. At the end of the experiment, blood samples were taken from the jugular vein after an overnight fast and centrifuged at 4 °C for 10 min in order to collect serum. Animals were then sacrificed by an overdose injection of barbiturate. The heart was excised aseptically, immediately frozen in liquid nitrogen, and either stored at −80 °C or fixed in 4% paraformaldehyde for further analysis.

2.3. Statistical analysis

Each experiment was performed in a group size of 3 independent samples. Data were presented as means ± SEMs. The statistical significance of differences between groups was obtained by ANOVA multiple comparisons in GraphPad Pro5.0 (GraphPad, San Diego, CA). Differences were considered to be significant at $P < 0.05$.

An expanded Methods section describing all procedures and protocols can be found online (Supplement methods).

3. Results

3.1. CaMKII mediates palmitate-induced cell apoptosis through activation of the mitochondrial apoptotic pathway in H9C2 cells

Cell apoptosis is one of the characteristics of obesity-related cardiac remodeling, and free fatty acids (especially palmitate) have been reported to induce apoptosis in cardiac cells. Therefore, we tested the involvement of CaMKII in cell apoptosis using cardiac-derived H9C2 cells stimulated with palmitate in the presence or absence of two CaMKII inhibitors, KN93 or Myr-AIP, a membrane-permeable myristoylated-autocamtide-2-related inhibitory peptide. To choose a proper dosage, we first tested the dose dependent effects of KN93 on cell survival in H9C2 cells using a CCK8 assay. As shown from Fig. S1, a dosage of KN93 < 10 μmol/L has no effect on 24 h–cells survival. KN93 at the dosage of 10 μmol/L has been reported to show off-target effects [11,12], so we selected 5 μmol/L as the proper concentration for KN93. Myr-AIP is a specific peptide inhibitor of CaMKII and has also been widely utilized in various cell culture experiments in doses ranging from 1 μM to 30 μM, suggesting safe and effective properties of this agent. For comparative analysis, we used the same dosage as KN93 at 5 μM. In addition, the stimulated concentration of palmitate (PA) at 300 μmol was adopted according to previously published papers that showed this dosage was widely used to induce pathological injuries in cardiac cells [13–15]. We then tested the effects of CaMKII inhibition on palmitate-induced cell apoptosis. As shown in Fig. 1A, increased Annexin V-positive cells were observed in palmitate-treated cells, which was significantly decreased in the presence of either Myr-AIP or KN93, suggesting that CaMKII plays a role in palmitate-induced cardiac cell apoptosis.

Activation of CaMKII has been linked to mitochondrial dysfunction, which has been demonstrated to contribute to cell apoptosis [16,17]. The mitochondrial apoptosis pathway was examined, including the loss of mitochondrial membrane potential (MMP), release of cytochrome C from mitochondria to the cytosol, and activation of caspase9 and caspase3. MMP was tested by using JC-1 fluorescent dye, which is a two-component dye that accumulates and aggregates in mitochondria, driven by the electrochemical gradient. Once taken up into the mitochondria, JC-1 forms aggregates and fluoresces red; in the cytoplasm, JC-1 remains in the monomeric form and fluoresces green. Therefore, a decrease in red fluorescence indicates a loss of mitochondrial membrane potential. In addition, we also used CCCP, a mitochondrial uncoupler as a positive control. As shown in Fig. 1B, untreated control cells exhibited numerous brightly stained mitochondria that emitted reddish and orange fluorescence, representing a normally hyperpolarized membrane potential, while CCCP-treated cells emitted bright green fluorescence, indicating loss of MMP. We also observed an increase in the number of cells with loss of MMP in palmitate-treated cells, while this was markedly reversed by pretreatment with Myr-AIP or KN93 (Fig. 1B). In untreated cells, cytochrome C immunoreactivity was co-localized in the mitochondria. After exposure of H9C2 cells to palmitate for 24 h, the cells exhibited cytochrome C diffusely distributed throughout the cytoplasm and a decrease in mitochondrial cytochrome C, indicating that cytochrome C was released from mitochondria to the cytoplasm, while this was inhibited by pretreatment with Myr-AIP or KN93 (Fig. 1C). The effect of KN93 inhibition on cytochrome C release was further confirmed by Western blot analysis of the level of cytochrome C in both cytoplasmic and mitochondria fractions (Fig. 1D).

The damaged mitochondria morphology was further confirmed by transmission electron micrographs (TEM) showing swollen mitochondria with disruption of mitochondrial cristae structure and swollen vesicular matrices in palmitate-treated cells (Fig. 1E). Caspase9 is critical for cytochrome C dependent apoptosis and it resides inside mitochondria in non-apoptotic cardiomyocytes [18], so we also examined the release of caspase9. As shown in Fig. 1F, caspase9 in the cytosolic fraction