Calmodulin-dependent protein kinase II activation promotes kidney mesangial expansion in streptozotocin-induced diabetic mice

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ABSTRACT

Calcium-calmodulin-dependent protein kinase II (CaMKII) is upregulated in diabetes mellitus (DM), leading to the overproduction of collagen in the myocardium. We hypothesized that CaMKII plays a role in the development of diabetic nephropathy (DN). Streptozotocin (STZ) injection into FVB wild-type mice led to mild mesangial matrix expansion, reproducing an essential feature of early human DN. Mesangial matrix measurements were performed on trichrome-stained paraffin sections using a trainable segmentation method based on WEKA (Waikato Environment for Knowledge Analysis) Image J-Fiji plugin (TWS plugin), and the electron micrographs of the whole glomeruli stitched from individual 4800x partial glomerular images. Both methods demonstrated that the statistically significant mesangial matrix expansion seen in the diabetic mice was prevented by chronic pretreatment with KN-93, a small molecule CaMKII inhibitor. This study indicates a role for CaMKII in the development of mesangial alterations in diabetes and suggests a possible new therapeutic target.

1. Introduction

Diabetes is a large global public health problem, a top ten cause of death, and one of the fastest-growing health challenges of the 21st century. Diabetic nephropathy, a serious complication of diabetes, leads to progressive decline in kidney function and presents a risk for kidney failure. Despite optimal glycemic control, the prevalence of DN remains high. There is no known effective mechanism-specific therapy available. Clearly, new therapeutic approaches are necessary to target the pathways driving DN progression.

Mesangial expansion is a hallmark of DN and a crucial structural change leading to the loss of renal function in diabetes mellitus (DM) [1]. The diabetic mesangial expansion has not been well characterized; even more difficult is the quantification of mild changes in the mesangium.

Mesangium forms the central region of the glomerular segments providing structural support for the glomerular capillaries and regulating the glomerular hemodynamics. It consists of mesangial cells that produce the mesangial matrix, containing, among other extracellular matrix proteins, Collagen IV and V. The composition and amount of mesangial matrix are tightly controlled in health [2]. As the mesangium is separated from the capillary lumen only by a layer of endothelium, events leading to the increase in endothelial permeability expose it to the circulating intracapillary content such as high glucose, cytokine, and growth factor levels seen in DM. Complex self-perpetuating mechanisms maintain the stability of the extracellular matrix alterations in DM. Studies performed on cultured mesangial cells showed that functioning calcium channels are necessary for the production of extracellular matrix [3]. Normal regulation of intracellular calcium is necessary for normal mesangial cell responsiveness [4]. Cytosolic calcium oscillations and CaMKII mediate urotensin II-induced extracellular protein synthesis and proliferation [5].

Ca\(^{2+}\) acts as an intracellular second messenger involved in cellular processes ranging from contraction to secretion to gene expression. CaMKII is a mediator of Ca\(^{2+}\) mobilizing extracellular stimuli and is involved in numerous calcium-dependent cellular processes. CaMKII, a multifunctional serine/threonine kinase, is a multigene family in which each of the four distinct classes of CaM kinase (\(\alpha, \beta, \gamma, \delta\)) is encoded by a separate gene. The \(\alpha\) and \(\beta\) classes are restricted to nervous tissue, whereas \(\gamma\) and \(\delta\) are found in most tissues. It has been shown that in a

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CaMKII is upregulated in DM by multiple mechanisms and significantly contributes to the disease progression [8, 9, 10]. For example, Ca²⁺ overload and oxidative stress, the known features of DM, activate CaMKII via Ca²⁺/CaM-dependent and oxidation-dependent pathways. CaMKII activation also can occur downstream of neurohormonal stimulation and may involve various posttranslational modifications such as O-GlcNAcylation, autophosphorylation, oxidation, and S-nitrosylation. CaMKII upregulation in DM leads to electrical, structural, and functional remodeling. Such upregulation is involved in the development of DM complications, e.g., cardiomyopathy, vascular dysfunction, and neuropathy. CaMKII in mesangial cells is activated by angiotensin II [11]. Angiotensin II also stimulates CaMKII-dependent collagen production in cardiac fibroblasts [12], and chronic CaMKII inhibition induces regression of diabetic cardiomyopathy in a mouse model [13]. Finally, excessive production of circulating catecholamines in DM can activate CaMKII via Ca²⁺/CaM-dependent and oxidation-dependent pathways. CaMKII activation also can occur downstream of neurohormonal stimulation and may involve various posttranslational modifications such as O-GlcNAcylation, autophosphorylation, oxidation, and S-nitrosylation. CaMKII upregulation in DM leads to electrical, structural, and functional remodeling. Such upregulation is involved in the development of DM complications, e.g., cardiomyopathy, vascular dysfunction, and neuropathy. CaMKII in mesangial cells is activated by angiotensin II [11]. Angiotensin II also stimulates CaMKII-dependent collagen production in cardiac fibroblasts [12], and chronic CaMKII inhibition induces regression of diabetic cardiomyopathy in a mouse model [13]. Finally, excessive production of circulating catecholamines in DM leads to chronic β-adrenoreceptor (β-AR) stimulation, which in turn promotes CaMKII activation and generation of reactive oxygen species. CaMKII plays a critical role in the pathophysiology of heart failure and itself can be an effective therapeutic target [14, 15, 16]. However, although the bidirectional link between the heart and the kidneys (cardiodrenal syndrome) is well recognized [17, 18], the impact of CaMKII inhibition on DN is unclear. The pathologic interplay between heart and kidney dysfunction in DM demands a more nuanced understanding of the underlying disruption of common cellular pathways in the myocyte and the kidney [18]. This study was to investigate the functional significance of CaMKII inhibition in DN progression. We evaluated the hypotheses that CaMKII plays an important role in the extracellular matrix production by mesangial cells, and its dysregulation contributes to mesangial expansion in DN.

We induced DM1 in FVB wild-type female mice by injecting a single high dose of STZ. This extensively characterized model [19] allows us to avoid the molecular bias inherent in genetically modified mouse lines, increasing the translational value of our study. We found that after a 10-week experiment, STZ-treated mice consistently developed mild mesangial expansion. To quantitate small changes in the mesangial matrix, we developed a digital algorithm to ensure consistent segmentation of this structure (see Materials and Methods). We found that DN manifested in FVB mice by mild mesangial expansion is almost entirely reversed by chronic inhibition of CaMKII.

2. Materials and methods

2.1. Experimental design

The current investigation was approved by the Animal Care and Use Committee of Atrium Health Wake Forest School of Medicine and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 8th Edition, updated 2011). We simultaneously assessed the effects of chronic CaMKII inhibition on the progression of diabetic cardiomyopathy and on the development of diabetic kidney mesangial expansion. We have shown that FVB wild-type mice develop established diabetic cardiomyopathy by 10 weeks after STZ induction [15, 20] (Details are available in the Online Supplemental Materials). The current report focuses on diabetic kidney mesangial expansion. We designed a 10-weeks study. Figure 1 illustrated the experimental procedures. Briefly, 29 age-matched (~4 months old) FVB female mice (Charles River Laboratories International, Inc.) were randomly divided into 2 groups: Control group (n = 8) and STZ-treatment group (n = 21). In the treatment group, DM1 was induced by STZ, 200 mg/kg intraperitoneally. 6 weeks after receiving STZ, animals were randomly divided into 3 groups: 1) DM1 group (n = 8), mice received 4 weeks vehicle only via an implanted mini-osmotic pump; 2) DM1/KN-93 group (n = 8), mice received 4 weeks treatment with KN-93, an antagonist of CaMKII (70 μg/kg/day) via mini-osmotic pump (Alzet [Cupertino, CA, model 1004]); and 3) DM1/KN-93 group, DM1 mice (n = 5) received KN-92 (70 μg/kg/day via mini-pump), an inactive KN-93 analog without CaM kinase inhibitory activity. The KN-92 group was a negative control to rule out off-target effects [14, 16].

During the 10-week study period, the general condition and food intake of animals were monitored daily; blood sugar was measured twice a week. Three sets of experiments were conducted to determine the effects of chronic CaMKII inhibition on the progression of diabetic cardiomyopathy: (1) Left ventricle (LV) function and dimensions were evaluated in the subgroups (5/group) animals from Control, DM1 and DM1/KN-93 groups at the beginning of the study and monthly via transthoracic echocardiography (Echo) as previously described [21, 22, 23]. (2) To assess the cellular basis of LV functional responses to chronic KN-93 treatment, LV myocyte functional performance was determined using the techniques well-established in our laboratory [21, 24]. Briefly, freshly isolated LV myocytes were placed in superfused dishes. Myocyte contraction was elicited by field-stimulation (0.5 Hz) and measured with the Fluorescence and Contractility System (IonOptix, Milton, MA). The percent shortening (SA), the peak velocity of shortening (dL/dtmax), the peak velocity of re-lengthening (dR/dtmax) and the peak systolic (Ca²⁺) transient ([Ca²⁺]i) at baseline and β-adrenergic reserve were obtained (see Supplemental Methods). (3) Analysis of gene expressions of LV myocytes from Control, DM1 and DM1/KN-93 groups was performed by quantitative real-time PCR, as previously described by our Laboratory [13, 25, 26, 27] (Supplemental Methods).

At the end of the experiment, each mouse was weighed, intracardiac blood and urine samples were taken. The kidneys were removed, weighed, and after collecting the electron microscopy samples, processed and embedded in paraffin (Figure 2). Sections were cut at 2.5 μm and stained with hematoxylin and eosin, Periodic Acid Schiff (PAS), and Masson trichrome stains (Trichrome, Masson, Aniline Blue staining kit, Newcomer Supply, Middleton, WI) on a slide stainer according to the manufacturer instructions. Mesangial expansion in diabetic kidneys was quantitated as described below.

2.2. Image processing and analysis

We have developed an automated process of mesangial matrix identification. Briefly, mesangial matrix measurements were performed on trichrome-stained paraffin sections using a trainable segmentation method based on the WEKA TWS plugin. A circumscribed, minimal convex polygon was traced to enclose the glomerular tuft (Figure 2A). Four segmentation classes were created: mesangial matrix, cell nuclei, cell cytoplasm, and background consisting of the remaining non-cellular components of the glomerulus and the red blood cells. Capillary walls were classified as background (Figure 2B). The segmentation result was displayed in square pixels and converted into the percent of the total glomerular area (mesangial matrix fractional area).

2.3. Electron microscopy

The biopsy tissue was processed for electron microscopy using standard clinical methods and analyzed at the AHWFSEM Electron Microscopy Core Facility. Whole glomerular profiles of 5 glomeruli per animal were obtained via sequential acquisition of 10% overlapping images for each glomerulus at magnification 4800x and stitched using the Image Composite Editor software (Microsoft, Inc., Redmond, WA). The subsequent image analysis steps based on the method of Weibel and Palade as described by Fioretto et al. [28] were performed with the Fiji software. Each glomerular profile was manually traced using the Fiji polygon tool to produce a circumscribed, minimal convex polygon enclosing the glomerular tuft and defining the glomerular tuft area for the subsequent
Figure 1. A schematization of the experimental procedures and experimental timeline.

Figure 2. Mesangial matrix measurements are illustrated. On trichrome-stained paraffin sections, a trainable segmentation method was performed based on the TWS plugin (A and B). A circumscribed, minimal convex polygon was traced to enclose the glomerular tuft (A, polygon in black). For trichrome-stained sections, four segmentation classes were created (B): mesangial matrix (red), cell nuclei (blue), cell cytoplasm (green), and background consisting of the non-cellular components of the glomerulus, red blood cells, and the extraglomerular components (yellow). Each glomerular profile from electron micrographs was manually traced using the Fiji polygon tool to produce a circumscribed, minimal convex polygon enclosing the glomerular tuft (C, polygon in blue), and a grid was overlayed on the glomerular image (C, yellow grid points).
measurements (Figure 2C). A grid was overlaid on the image with the density of 1 grid point per 18 μm² of the glomerular area, on an average of 290 points per glomerulus. The mesangial cell, mesangial matrix, and podocyte fractional areas were estimated by counting the number of grid points falling on these structures, as previously described [28].

2.4. Statistical analysis of data

All endpoints were expressed as mean ± SD or mean ± SE as indicated. For all endpoints, one-way ANOVA evaluated significant effects among the groups. When the ANOVA revealed significant differences, a Bonferroni adjustment was used to compare pairwise tests among each group. Treatment effects were determined by ANCOVA on the outcome measures adjusted for baseline values. In each animal, mesangial matrix fractional area, LV myocyte contraction, relaxation, and [Ca²⁺]_iT values were averaged and treated as a single data point. The mean differences in the fractional mesangial matrix area, cell dynamics, and the indo-1-AM fluorescence ratios between groups were calculated. Statistical significance was considered at p < 0.05.

3. Results

3.1. Characteristics of general features of STZ-Induced DM1

STZ mice developed sustained hyperglycemia (blood non-fasting glucose 517 ± 21 mg/dL, compared to 162.9 ± 13 mg/dL in Controls) from week 2, which persisted during the study period. Blood glucose greater than 400 mg/dL in mice provides a scenario close to the human DM1, with little interference from endogenous insulin while decreasing the likelihood of endogenous islet recovery [29]. In the diabetic mice, the urinary excretion of albumin was increased from 8 weeks of the experiment (6 times higher than in Controls). Water intake was also increased, but food (normal chow) intake was markedly reduced from 8 weeks on (Control: 3.1–3.5 g/day vs. DM1: 2.2–2.5 g/day), resulting in significantly decreased body weight (23%, DM1: 25.3 ± 2.5 g vs. Control: 33.2 ± 2.9 g). Even though the DM1/KN-93 mice were also severely hyperglycemic (blood glucose 476 ± 18 mg/dL), their body weight (29.5 ± 4.4 g) was not significantly different from that of the Controls. The DM1/KN-93 mice food intake and albuminuria were improved and close to control mice. In DM1 mice, kidneys were enlarged and weighed 76% more than Controls (DM1: 16.2 ± 4.9 g vs. Control: 9.2 ± 2.9 g) (p < 0.05). The DM1/KN-93 mice kidney sizes and weights were comparable to those in controls (DM1/KN-93: 8.7 ± 1.3 g).

3.2. Cardiomyopathy of STZ-induced T1DM: effects of CaMKII inhibition

Consistent with past reports by our laboratory and others [30], DM1 caused both LV and cardiomyocyte dysfunction [13]. In this study, FVB wild-type mice developed established diabetic cardiomyopathy by 10 weeks after STZ induction. Echocardiography was performed in Control, DM1, and DM1/KN-93 mice (n = 5/group). Only DM1 mice showed markedly impaired LV diastolic function as evidenced by the significant decrease in the myocardial relaxation (e', defined by tissue Doppler-derived myocardial annular descent) (DM1: 2.4 ± 0.19 vs Control: 3.5 ± 0.22 cm/s, P < 0.05), and reduced LV filling pressure, defined by early transmitral flow velocity-to-mitral annular velocity ratio (or E/e') (32.6 ± 3.4 vs 17.8 ± 2.5%, p < 0.05). DM1 mice also exhibited marked LV systolic dysfunction as indicated by a significantly reduced percent fractional shortening (%FS) (DM1: 28.2 ± 4.1 vs Control: 39.4 ± 3.2, p < 0.05). There are no significant differences in heart rate and blood pressure among these groups. Of importance, there were intrinsic defects of LV myocyte relaxation and force-generating capacity [20]. LV myocyte functional performance from Control, DM1, and DM1/KN-93 groups (n = 8/group), as well as DM1/KN-92 (n = 5), were compared as detailed in the Online Supplement Results. The myocyte lengths (DM1: 148.7 ± 4.2 vs Control: 115.5 ± 2.5 μm, p < 0.01) and the ratio of length-width were significantly increased in DM1 mice. Cell contraction (dL/dtmax 76.4 ± 6.1 vs 143.1 ± 5.4 μm/s, relaxation (dR/dtmax, 60.3 ± 4.2 vs 122.4 ± 4.8 μm/s) ([Ca²⁺]_iT (0.18 ± 0.01 vs 0.21 ± 0.02) (p < 0.05) were significantly reduced in DM1 myocytes. Isoproterenol (ISO, 10⁻⁸ M)-stimulated increases in dL/dtmax (32 ± 3% vs 60 ± 5%), dR/dtmax and ([Ca²⁺]_iT (16 ± 1% vs 31 ± 2%) (P < 0.05) were also significantly reduced (Supplemental Figures 1 and 2). These findings documented an established cardiomyopathy. Notably, as shown in Supplemental Figure 3, DM1 myocytes had significantly increased protein levels of CaMKII (DM1: 0.56 ± 0.03 vs Control: 0.41 ± 0.06, p < 0.05). PCaMKII (0.65 ± 0.04 vs 0.34 ± 0.09, p < 0.05) with significantly decreased SERCA2a (0.41 ± 0.05 vs 0.98 ± 0.07), phosphorylated phospholamban (P/LPB/PLB (0.46 ± 0.08 vs 0.77 ± 0.05) (Supplemental Figure 4), and β1-AR (0.18 ± 0.04 vs 0.55 ± 0.05). But significantly increased β2-AR (0.48 ± 0.07 vs 0.16 ± 0.02), inducible nitric oxide synthase (iNOS) (0.77 ± 0.07 vs 0.36 ± 0.06) and nitrotyrosine (NT) (an indicator or marker of cell damage and inflammation) (0.14 ± 0.23 vs 0.80 ± 0.13) (p < 0.05).

Importantly, in DM1/KN-93 mice, LV %FS (36.8 ± 3.7%) and E/e' (17.8 ± 2.9%) were restored close to the values in the control animals. In DM1/KN-93 myocytes, the cell length and the length-width ratio were comparable to those in Controls (Supplemental Figure 1). DM1/KN-93 myocytes had similar protein levels of CaMKII (0.36 ± 0.08) and PCaMKII (0.41 ± 0.03), SERCA2a (1.29 ± 0.13), P-LPB/PLB (0.77 ± 0.09) (Supplemental Figures 3 and 4), β1-AR (0.48 ± 0.04), β2-AR (0.16 ± 0.03), iNOS (0.40 ± 0.09) and NT (0.29 ± 0.03), with preserved normal basal cell function of dL/dtmax (146 ± 6.9 μm/s), dR/dtmax (126.1 ± 10.8) and ([Ca²⁺]_iT (0.18 ± 0.02). ISO-caused increases in dL/dtmax (61 ± 2%), and ([Ca²⁺]_iT (32 ± 2%) were also close to control values [20] (Supplemental Figures 1 and 2). By contrary, in DM1/KN-92 myocytes, impaired basal and β-AR-stimulated cell function persisted with significantly low dL/dtmax (82.6 ± 7.1 μm/s), dR/dtmax (69.8 ± 5.7) and ([Ca²⁺]_iT (201 ± 0.22). Acute ISO infusion produced much less increase in dL/dtmax (34 ± 2%) and ([Ca²⁺]_iT (18 ± 1%) (Supplemental Figures 1 and 2). These findings indicate that the beneficial action of KN-93 is due to the inhibition of CaMKII.

3.3. Kidney mesangial expansion of STZ-induced T1DM: effects of CaMKII inhibition

Glomerular capillary tufts were enlarged in DM1 mice but not significantly (capillary tuft area DM1: 3298 ± 693 μm²; Control: 2924 ± 1067 μm²; and DM1/KN-93: 2976 ± 1352 μm²).

In the light microscopy analysis of kidney sections, compared with normal (Figure 3A), DM1 mice revealed a mild diffuse and global increase in the mesangial matrix, evident in the PAS (the mesangial matrix is PAS-positive) and trichrome (the mesangial matrix is blue) stained sections (Figure 3B). Other light microscopy features of human DN (arteriolar hyalinosis, thickening of the Bowman's capsule and the tubular basement membranes, etc.) were not detected. This mesangial matrix increase was not seen in the KN-93-treated DM1 mice (Figure 3C). However, a similar mild diffuse and global increase in the mesangial matrix was also present in the KN-92-treated AD1 mice (Figure 3D).

After appropriate training (see Methods), the TWS algorithm accurately classified the glomerular trichrome-blue pixels as belonging to the mesangial matrix or nuclei; capillary walls were classified as "background" (Figure 2B). Misclassification was minor. We could not achieve a reliable separation of the cell cytoplasm and nuclei in all glomerular tuft profiles using this method, which was not important for the goal of the experiment.

As shown in Figure 4A, the kidney mesangial matrix fractional area in DM1 mice increased by 81% (DM1: 9.4 ± 1% vs Control: 5.2 ± 2.8%). Inhibition of the CaMKII activity by KN-93 prevented this increase, resulting in mesangial areas slightly larger but statistically indistinguishable from Controls (DM1/KN-93: 6.2 ± 2.3%). Conversely, in KN-92 treated DM1 mice, the kidney mesangial matrix fractional area remains markedly increased (8.8 ± 0.4%) compared to controls (Figure 4A).
In the electron microscopy study, compared to controls (Figure 3E), the mesangial matrix increased in DM1 glomeruli (Figure 3F) in the absence of other ultrastructural changes of early diabetes (e.g., glomerular basement membrane thickening). This increase was prevented by KN-93 (Figure 3G), but this increase was persisted by KN-92-treated mice (Figure 3H). To quantitate the electron microscopy findings, the mesangial fractional area was measured using the classical grid overlay-point counting method. This method yielded a higher mesangial fractional area in both control and experimental groups than trichrome-stained sections. This discrepancy has likely resulted from the...
difference in resolution and from the difficulty in the correct classification of small dispersed collagen accumulations, which may stain lighter blue (Brightness above 180, HSV color model) or reddish shades of blue due to surrounding cell cytoplasm (Hue 200 and above, HSV color model). Still, as shown in Figure 4B, compared with Controls (Control 9.2 \( \pm 2.9\%\)), both DM1 (DM1:16.1 \( \pm 4.9\%\)) and DM1/KN-92 (14.3 \( \pm 3.9\%\)) presented with comparable mesangial matrix increases. This was not seen with KN-93 (DM1/KN-93: 8.7 \( \pm 1.3\%\) (Figure 4B). At the same time, the mesangial cell area did not increase significantly, and the fractional podocyte area was not different from that in Controls (Data not shown).

4. Discussion

In the current study, we have quantitated the mesangial expansion in early DN by using the automated process of the mesangial matrix identification approach we developed in a mouse model of DM1 with well-established cardiomyopathy. For the first time, we reported that chronic inhibition of CaMII reverses diabetic kidney mesangial expansion and also causes regression of diabetic cardiomyopathy. Our current observations support the view that the bidirectional interaction between the heart and kidneys sets up an important vicious circle. CaMII over-activation may be the common etiological mechanism in both organ systems. These data provide evidence and important insights that CaMII inhibition is able to reverse or reverse early diabetic nephropathy and restores normal cardiac functional performance in DM1, suggesting that chronic CaMII inhibition may also have promise as a therapeutic approach to the cardiorenal syndrome in patients with diabetes mellitus.

4.1. LV and myocyte function and chronic CaMII inhibition

In our study, FVB wild-type mice develop established diabetic cardiomyopathy by 10 weeks after STZ induction. Consistent with past reports by our laboratory and others [13, 20, 30], DM1 caused LV systolic and diastolic dysfunction [13, 20]. Importantly, LV chamber abnormalities occurred in parallel with dysfunction of LV myocyte. There were significantly depressed myocyte contractility (dL/dt\(_{\text{max}}\)) and [Ca\(^{2+}\)]\(_{\text{IT}}\). There was a maladaptive LV myocyte shape remodeling. After chronic KN-93 treatment, these changes were normalized, and myocyte \( \beta \)-AR desensitization reversed. By contrary, adverse myocyte remodeling, decreased myocyte basal and ISO-stimulated functional and [Ca\(^{2+}\)]\(_{\text{IT}}\) responses persisted in the KN-92 group.

Reversal of DM1 caused intrinsic defects of myocyte force-generating capacity and relaxation after chronic KN-93 treatment may be largely due to the normalization of basal and \( \beta \)-AR stimulated Ca\(^{2+}\) handling. Likewise, recovery of normal [Ca\(^{2+}\)]\(_{\text{IT}}\) regulation by chronic CaMII inhibition may be the key mechanism for the reversal of DM1 caused intrinsic defects of myocytes.

DM1 myocytes had significantly increased levels of CaMKII\(\delta\) and PCaMKII\(\delta\) proteins, demonstrating upregulation of cardiac CaMII (Supplemental Figure 3). Of note, we examined the phosphorylation of CaMII to estimate the CaMII activity. As shown in Supplemental Figure 3, Western blot analysis revealed significantly increased phosphorylation of CaMII at Thr287 in DM1. Diverse mechanisms contribute to excessive CaMII activation in DM1. For instance, Ca\(^{2+}\) overload and oxidative stress are general features of diabetic myocardium, which activate CaMII by Ca\(^{2+}\)/CaM-dependent and oxidation-dependent pathways. Recent evidence indicates that diabetic hyperglycemia induces both excessive ROS and CaMII activation by O-GlcNAcylation (OGN) and oxidation. CaMII acts on diverse downstream targets in cardiomyocytes. Abnormally elevated CaMII activity and CaMII-ROS-OGN pathway can cause dysfunction of several downstream events whose components are regulated by CaMII, such as E-C coupling, structural remodeling, and transcriptional activation of certain inflammatory proteins and apoptosis [31, 32, 33]. Consistently, we and others showed that in DM, excessive activation of cardiac CaMII contributes to abnormal myocyte Ca\(^{2+}\) homeostasis by altering key proteins involved in Ca\(^{2+}\) handling, excitation-contraction coupling, cardiomyocyte function, and structural remodeling. These key proteins include the ryanodine receptor, PLB, L-type Ca\(^{2+}\) channel, Na\(^+\)/Ca\(^{2+}\) exchanger, and SERCA2a. In the present study, chronic KN-93 treatment prevented DM1-induced upregulation of CaMII\(\delta\) (Supplemental Figure 3, Western blot analysis showing that KN-93 inhibits the phosphorylation of CaMII at Thr287), downregulation of cardiac \( \beta_{1}\)-AR and SERCA2a, and reversed the increased \( \beta_{3}\)-AR and nitrotyrosine, thus preventing LV and cardiomyocyte dysfunction, depressed [Ca\(^{2+}\)]\(_{\text{IT}}\), and reduced \( \beta\)-adrenergic reserve [13, 20]. The ability of CaMII inhibition to counteract pathologic oxidation may be another important mechanism by which KN-93 treatment could ameliorate diabetic cardiomyopathy. Our present findings support the view that in DM, CaMII is a master regulator of pathways important for cardiac remodeling and dysfunction.

4.2. Kidney mesangial expansion and chronic CaMII inhibition

4.2.1. Quantitative changes of kidney mesangial expansion in DM1

The earliest structural kidney change in human DM is gross enlargement and glomerular hypertrophy [34]. Kidneys were enlarged in our DM1 mice. The early characteristic features of human DN are the glomerular basement membrane thickening and mesangial expansion [1, 28, 35]. Semiquantitative light and quantitative electron microscopic measures of mesangial expansion strongly correlate with the clinical manifestations of DN [35, 36] and, to a large extent, determine its progression [36]. Thus, our DM1 mouse model reproduces a key feature of early human DN. Similar to the early human DN, the mesangial expansion occurred due to the accumulation of its extracellular matrix component.

To evaluate mild changes in the mesangium, we implemented a mesangial matrix segmentation method based on a Fiji image processing package and a trainable plugin (see Supplementary content). The average relative area of the total mesangium measured on the electron micrographs in nondiabetic humans was 14.2 \( \pm 4.1\%\), with the matrix and cellular components each averaging 7.1 \( \pm 2.4\%\) [35] to 18.6 \( \pm 3.6\%\) [37]. Our results obtained in control mice were also within this range.

Glomerular structure measurements based on our deep learning segmentation method demonstrated a statistically significant expansion of the mesangial matrix in DM1 mice after a 10-week experiment. Chronic treatment with a CaMII inhibitor KN-93 for 4 weeks reversed this expansion. We confirmed this result on electron micrographs using the classical point counting method of Weibel and Palade. Even though the machine segmentation method was more conservative, the ratio between the experimental groups was the same. This suggests that the CaMII inhibitor has disrupted an important pathogenetic link in the development of early DN.

4.2.2. Possible mechanisms underlying the effect of CaMII inhibition on kidney mesangial expansion in DM1

Disrupted Ca\(^{2+}\) homeostasis is recognized as a major contributor to the diabetic nephropathy phenotype. Altered regulation of intracellular Ca\(^{2+}\) concentration plays a key role in multi-system kidney dysfunction. For example, mesangial cells use endocytosis to take up and degrade circulating immunoglobulin. This normal process stimulates mesangial cell proliferation and matrix deposition. Calcium influx regulates the endocytosis rate, and calmodulin is the calcium sensor of endocytosis [38]. As illustrated in Supplemental Figure 5, signaling molecules involved in the production of mesangial matrix and upregulated in DM induce mesangial collagen production via CaMII. High-glucose condition-induced changes contribute to the loss of contractility of mesangial cells with stimulations by vasoactive hormones [4]. Mesangial cell apoptosis and proliferation are regulated by intracellular Ca\(^{2+}\) signals [39]. Diabetes and high glucose enhance store-operated Ca\(^{2+}\) entry and increase expression of Ca\(^{2+}\) signaling proteins in mesangial cells. A
sustained elevation in the intracellular Ca\(^{2+}\) concentration may activate a number of harmful processes, including phenotype change, dysregulated cell proliferation, and cell injury and death.

The multifunctional CaMKII is a nodal point in the regulation of intracellular Ca\(^{2+}\) handling, ion channels, and gene transcription [40, 41, 42]. CaMKII is a ubiquitous enzyme that is present all cell types [43], functions as a mediator of extracellular calcium stimuli, and thus, is involved in calcium-dependent cellular processes in the kidney. In DM, increased hyperglycemic and oxidative stress and O-GlcNAcylation are chronically present. These mechanisms likely synergize to cause substantial chronic CaMKII activation and functional consequences in the kidneys. CaMKII is not only activated in the heart but also in the kidney tissues.

Although, in this study, CaMKII expression and activity were not measured in the kidneys, recent evidence suggests that DM causes increased CaMKII activation. Diabetic patients have a significantly greater proportion of oxidized to total CaMKII. Glucose-induced Ca\(^{2+}\) waves in cardiomyocytes depend on both CaMKII and OGN [10]. Mesangial proliferation and extracellular matrix protein accumulation induced by high glucose depend on activation of CaMKII and increased CaMKII phosphorylation [5]. Thus, inhibition of CaMKII by KN-93 likely counteracts the effects of DM-related stresses in the glomerular mesangium, reversing the mesangial expansion.

It is increasingly recognized that CaMKII has an impact on many organs, such as the heart, liver, pancreas, kidneys, eyes (retinopathy), and nervous system (neuropathy). CaMKII employs different mechanisms to achieve target specificity and efficiency in its role as a master regulator of many cellular processes. Although the signaling pathways connecting CaMKII activation to the proliferation of the mesangial matrix are not well characterized, emerging data highlights the importance of the cytokine transforming growth factor \(\beta\) (TGF-\(\beta\)) as a key factor in the development of renal hypertrophy and accumulation of extracellular matrix in DM. TGF-\(\beta\) elicits powerful fibrogenic actions by both stimulating the extracellular matrix synthesis and inhibition of matrix degradation. In human DM and animal models of DM, TGF-\(\beta\) mRNA and protein levels are significantly increased in the glomeruli [44]. The JNK-CaMKII-Fis1 pathway is involved in mesangial cell apoptosis and mitochondrial dysfunction in diabetic renal injury under high-glucose stress conditions [45]. In addition to Ca\(^{2+}\)/CaM binding, CaMKII activation also occurs downstream of neurohormonal stimulation (e.g., via G-protein coupled receptors). The sympathetic nervous system and renin-angiotensin II-aldosterone system (RAAS) are active in DM [46]. CaMKII is a nodal signal for multiple programmed cell death.

Of particular significance are the coexistence of diabetic kidney mesangial expansion and diabetic cardiomyopathy in this model. Chronic CaMKII inhibition is sufficient to ameliorate or even reverse this cardio-renal syndrome. It is well known that DM and heart failure (HF) can cause diabetic kidney disease (DKD), and DKD can intensify HF. In this model, kidney injury is likely not secondary to HF. Diabetes is the cause of kidney injury and cardiomyopathy, although cardiomyopathy may place extra burdens on renal function. Thus, heart and kidney dysfunction may act synergistically, and each worsens the prognosis of the other, forming vicious circles. DN and cardiomyopathy may share mechanisms (such as CaMKII overactivation) common to injury of both organ systems. At present, treatment options for DM, CKD, and HF do not necessary overlap [47]. CaMKII inhibition may target important common therapeutic goals. CaMKII is configured to coordinate and transduce upstream Ca\(^{2+}\) and ROS signals into physiological and pathophysiological downstream responses. Hence, in contrast to a relatively developed knowledge of the role of CaMKII in cardiovascular disease, the first indicators of involvement of CaMKII in diabetic renal injury are only emerging. Much more work needs to be done to understand the precise interaction and pathophysiological circles of this cardio-renal connection in DM patients.

The results of our study are supported by previous work in STZ-treated rats [5] showing that increased concentration of urotensin II and urotensin II receptor in mesangial cells exposed to a high glucose concentration leads to increased collagen IV and fibronectin production via a calcium-sensitive CaMKII-dependent pathway that can be disrupted by KN-93. Urotensin II increases CaMK II phosphorylation in mouse glomerular mesangial cells and upregulates TGF-\(\beta\)-\(\beta\) via a calcium-dependent pathway. In addition, UII-induced glomerular mesangial cell proliferation is dependent on CaMKII activation [5].

However, our results differ from a previous study reporting that CaM Kinase II-\(\delta\) is required for the development of diabetic hyperglycemia and retinopathy but not for nephropathy [48]. Several factors may contribute to this difference. First, different animal models were used. Our genetically intact DM1 mice received KN-93 to block all subtypes of CaMKII. On the other hand, Chen and co-workers crossed leptin receptor mutant mice (Leprdb/db) (a model of DM2) into CaMKII\(\beta\)-deleted mice to study the role of CaMKII in DN. Because the Leprdb/db/CaMKII\(\beta\) deleted mutant animal progresses normally through development without CaM kinase, there are likely compensatory mechanisms confounding data interpretation. The loss of CaM\(\delta\) kinase may alter subcellular targeting or regulatory function of CaM residual isoforms, thus likely modifying glucose metabolic pathways and altering substrate utilization. Optimal glycemic control is insufficient for the prevention and treatment of diabetic nephropathy. Our observations support the view that CaMKII is a master regulator of pathways involved in the development and progression of diabetic nephropathy.

### 4.2.2.1. Potential study limitations

Some limitations should be considered in interpreting our data. First, we used a mouse model of STZ-induced DM1. There are well-documented differences between mouse and human diabetes. Although pathologic changes in STZ-treated mice mimic many functional, structural, and neurohormonal changes of clinical DM1, our results may not necessarily be applicable to humans. Second, we utilized the most widely used CAMKII inhibitor KN-93 to study cellular and in vivo functions [49]. But KN-93 also inhibits L-type Ca\(^{2+}\) current (\(I_{\text{ca,L}}\)) and voltage-dependent K\(^+\) (\(I_{\text{Kv}}\)), which may be independent of CAMKII actions [16, 50]. KN-92 similarly blocks the K\(^+\) channel and is therefore useful in excluding channel-specific effects [16, 50]. Comparing the effects of KN-93 and KN-92 appears to address the most known off-target effects of KN-93 [16] [14,51]. Clearly, an effort has to be taken to develop more potent, CaMKII-specific inhibitors. Third, the current study was conducted only in age-matched groups of female mice. Because both diabetic cardiomyopathy and diabetic nephropathy risks are significantly higher in female diabetics, these diabetic complications are less studied in female diabetics. It has been reported that diabetic women are five times more likely to develop heart failure compared with two-fold for men [52]. There is a rapid onset of cardiomyopathy in STZ-induced female diabetic mice [30]. Female sex in diabetes was associated with a 35% increased risk of DN compared with male sex in adjusted analyses [53]. However, it is vital to conduct studies in both genders to reveal the underlying mechanisms responsible for this female disadvantage. Whether male and female diabetes may cause distinct types of diabetic cardiomyopathy or DN with different progression remain to be determined. More insights will be gained from ongoing investigations in our laboratory of a time-course study of age- and sex-matched mice in the early, middle, and late stages of diabetes. Finally, we did not measure CaMKII expression and activity in the kidneys and did not address molecular mechanisms underlying the preventive and/or therapeutic actions of chronic inhibition of CaMKII on the kidney mesangial expansion.

### 5. Conclusion

STZ FVB mice can be used as a model of early DM1-associated nephropathy in an unaltered genetic background. Our mesangial matrix segmentation approach may be useful for laboratories that do not have access to bioinformatics resources. The novel finding in this study is that the mesangial expansion, at least in early DN in mice, is dependent on
CaMKII, and it can be prevented with chronic CaMKII inhibition, suggest-
ging that CaMKII inhibition has a promise as a therapeutic approach to prevent and/or treat the diabetic nephropathy.

Declarations

Author contribution statement

Che Ping Cheng: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Alexei Mikhailov: Conceived and designed the experiments; Per-
formed the experiments; Analyzed and interpreted the data; Wrote the paper.
Jen-Jar Lin: Conceived and designed the experiments; Performed the experiments.
Heng-Jie Cheng and Yixi Liu: Performed the experiments.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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References

[1] P. Fioretto, M. Mauer, Histopathology of diabetic nephropathy, Semin. Nephrol. 27 (2007) 195–207.
[2] R.M. Mason, N.A. Wahab, Extracellular matrix metabolism in diabetic nephropathy, J. Am. Soc. Nephrol. 14 (2003) 1358–1373.
[3] T. Sugiuira, E. Imaizumi, M. Morita, M. Hori, Calcium channel blockers inhibit proliferation and matrix production in rat mesangial cells: possible mechanism of suppression of AP-1 and CREB activities, Nephron 85 (2000) 71–80.
[4] C. Whitnside, S. Munk, K. Zhou, T. Miramyl, D.M. Templeton, Cleavage of intracellular calcium prevents mesangial cell proliferative responsiveness, J. Am. Soc. Nephrol. 9 (1998) 14–25.
[5] H. Soni, A. Adebiyi, Urotensin II-induced store-operated Ca(2+)/OS-channel contributes to glomerular mesangial cell proliferation and extracellular matrix protein production under high glucose conditions, Sci. Rep. 7 (2017), 18049.
[6] P.S. Banerjee, J. Ma, G.W. Hart, Diabetes-associated dysregulation of O-GlcNAcylation in rat cardiac mitochondria, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 6050–6055.
[7] F. Cuello, K. Lorenz, Inhibition of cardiac CaMKII to cure heart failure: step by step towards translation? Basic Res. Cardiol. 111 (2016) 66.
[8] O.O. Mesubi, A.G. Rokita, N. Abrol, Y. Wu, B. Chen, Q. Wang, J.M. Granger, A. Arora, S. Ojha, D. Vohora, Characterisation of streptozotocin induced diabetes mellitus in swiss albino mice, Global J. Pharmac. 3 (2009) 81–84.
[9] A. Moore, A. Shindikar, I. Fonisom-Nurse, F. Iu, P.E. Munasinghe, T.P. Ram, P. Saxena, S. Cofrey, W.R. Bunton, J.F. Galvin, M.J. Williams, C. Emami, P. Madeddu, R. Kates, Rapid onset of cardiomyopathy in STZ-induced female diabetic mice involves the downregulation of pro-survival Fim-1, Cardiovasc. Diabetol. 13 (2014) 68.
[10] J.R. Erickson, M.L. Joiner, X. Guan, W. Kutschke, J. Yang, C.V. Oddis, R.K. Bartlett, J.S. Lowe, S.E. O’Donnell, N. Aykin-Burns, M.C. Zimmerman, K. Zimmerman, A.J. Ham, R.M. Weiss, D.R. Spitz, M.A. Shea, R.J. Colbran, P.J. Mohler, M.E. Anderson, A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation, Cell 133 (2008) 462–474.
[11] M.E. Anderson, CaMKII and a failing strategy for growth in heart, J. Clin. Invest. 119 (2009) 1082–1085.
[12] P. Beaurevoir, M.L. Ozoux, G. Begis, V. Glaton, V. Briand, M.C. Philippo, C. Daveu, G. Vanaevers, S. Aymard, M. Mauget, L. Pollet, A. Filiatrault, S. Marchand, P. Gouriou, M. Caillou, B. Vasseur, P. Legrand, J. Vanrobaeys, L. Groban, C.P. Cheng, Critical role of the chymase/angiotensin-(1-12) axis in modulating cardiomyocyte contractility, Int. J. Cardiol. 264 (2019) 137–144.
[13] H.J. Cheng, Z.S. Zhang, K. Ohachi, T. Ueki, D.C. Sane, C.P. Cheng, Upregulation of functional beta(3)-adrenergic receptor in the failing canine myocardium, Circ. Res. 89 (2001) 599–606.
[14] C.P. Cheng, H.J. Cheng, C. Cunningham, Z.K. Shihab, D.C. Sane, T. Wannenburg, W.C. Little, Angiotensin II type 1 receptor blockade prevents alcoholic cardiomyopathy, Circulation 114 (2006) 226–236.
[15] Q. Shao, H.J. Cheng, M.F. Callahan, D.W. Kitzman, W.M. Li, C.P. Cheng, Overexpression myocardial inducible nitric oxide synthase exacerbates cardiac dysfunction and beta-adrenergic desensitization in experimental hypothyroidism, J. Int. Cardiol. 204 (2016) 229–241.
[16] P. Fioretto, M.W. Steffes, M. Mauer, Glomerular structure in nonproteinuric IDDM patients with various levels of albuminuria, Diabetes 43 (1994) 1358–1364.
[17] A. Arora, O. Duclos, D. Tamarelle, M.P. Pruniaux, A.J. Muslin, P. Janiak, Reversion of cardiac GlcNAcylation in rat cardiac mitochondria, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 3746–3751.
[18] A.M. Vunjak-Novakovic, G. Kalllich, S. Miller, S. Chen, E. Sadowski, D. Vranic, R. Katare, Rapid onset of cardiomyopathy in STZ-induced female diabetic mice involves the downregulation of pro-survival Fim-1, Cardiovasc. Diabetol. 13 (2014) 68.
[19] J.R. Erickson, M.L. Joiner, X. Guan, W. Kutschke, J. Yang, C.V. Oddis, R.K. Bartlett, J.S. Lowe, S.E. O’Donnell, N. Aykin-Burns, M.C. Zimmerman, K. Zimmerman, A.J. Ham, R.M. Weiss, D.R. Spitz, M.A. Shea, R.J. Colbran, P.J. Mohler, M.E. Anderson, A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation, Cell 133 (2008) 462–474.
[20] M.E. Anderson, CaMKII and a failing strategy for growth in heart, J. Clin. Invest. 119 (2009) 1082–1085.
[39] H. Saleh, E. Schlatter, D. Lang, H.G. Pasels, S. Heidenreich, Regulation of mesangial cell apoptosis and proliferation by intracellular Ca(2+) signals, Kidney Int. 58 (2000) 1876–1884.

[40] J.R. Erickson, B.J. He, L.M. Grumbach, M.E. Anderson, CaMKII in the cardiovascular system: sensing redox states, Physiol. Rev. 91 (2011) 889–915.

[41] M.V. Singh, M.E. Anderson, Is CaMKII a link between inflammation and hypertrophy in heart? J. Mol. Med. 89 (2011) 537–545.

[42] A.T. Roe, M. Fritsk, W.E. Louch, Targeting cardiomyocyte Ca2+ homeostasis in heart failure, Curr. Pharmaceut. Des. 21 (2015) 431–448.

[43] A.P. Braun, H. Schulman, The multifunctional calcium/calmodulin-dependent protein kinase: from form to function, Annu. Rev. Physiol. 57 (1995) 417–445.

[44] W.B. Reeves, T.E. Andreoli, Transforming growth factor beta contributes to progressive diabetic nephropathy, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 7667–7669.

[45] Y. Zhang, J. Feng, Q. Wang, S. Zhao, S. Yang, L. Tian, P. Meng, J. Li, H. Li, Hyperglycaemia stress-induced renal injury is caused by extensive mitochondrial fragmentation, attenuated MAPK1 signalling, and activated JNK-CaMKII-fos1 biological Axis, Cell. Physiol. Biochem. 51 (2018) 1778–1798.

[46] N.S. Dhallal, P.K. Ganguly, S.K. Bhullar, P.S. Tappia, Role of catecholamines in the pathogenesis of diabetic cardiomyopathy (1), Can. J. Physiol. Pharmacol. 97 (2019) 815–819.

[47] M. Schecter, C. Melzer Cohen, I. Yanuv, A. Rozensz, G. Chodick, J. Bodegard, L.A. Leiter, S. Verma, H.J. Lambers Heerspink, A. Karazik, O. Mosenzon, Epidemiology of the diabetes-cardio-renal spectrum: a cross-sectional report of 1.4 million adults, Cardiovasc. Diabetol. 21 (2022) 104.

[48] J. Chen, T. Fleming, S. Katz, M. Dewenter, K. Hofmann, A. Saadatmand, M. Kronlage, M.P. Werner, B. Pokranda, F. Schreiter, J. Lin, D. Katz, J. Morgenstern, A. Elwakiel, P. Sinn, H.J. Grone, H.P. Hammes, P.P. Nawroth, B. Iermann, C. Sticht, B. Brugger, H.A. Katus, M. Hagenmüller, J. Backs, CaMK kinase II-delta is required for diabetic hyperglycaemia and retinopathy but not nephropathy, Diabetes 70 (2021) 616–626.

[49] D. Nasal, D. Gratz, T.J. Hund, Challenges and opportunities for therapeutic targeting of calmodulin kinase II in heart, Front. Pharmacol. 11 (2020) 35.

[50] M.E. Anderson, A.P. Braun, Y. Wu, T. Lu, W. Wu, H. Schulman, R.J. Sung, KN-93, an inhibitor of multifunctional Ca++/calmodulin-dependent protein kinase, decreases early afterdepolarizations in rabbit heart, J. Pharmacol. Exp. Therapeut. 287 (1998) 996–1006.

[51] M. Warren, K.J. Sciuto, T.G. Taylor, V. Garg, N.S. Torres, J. Shibayama, K.W. Spitzer, A.V. Zaitsev, Blockade of CaMKII depresses conduction preferentially in the right ventricular outflow tract and promotes ischemic ventricular fibrillation in the rabbit heart, Am. J. Physiol. Heart Circ. Physiol. 312 (2017) H752–H767.

[52] R. Toedebusch, A. Belenchia, L. Pulakat, Diabetic cardiomyopathy: impact of biological sex on disease development and molecular signatures, Front. Physiol. 9 (2018) 453.

[53] M. Yu, M. Karon, R. Young, Associations between sex and incident chronic kidney disease in a prospective diabetic cohort, Nephrology 20 (2015) 451–458.