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Intracellular Renin Protects Cardiomyocytes from Ischemic Injury in Diabetic Heart

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Abstract

Background: Local Renin-Angiotensin-Aldosterone system (RAS) is important in cardiac pathophysiology. We investigated the expression and distribution of intracellular renin after ischemia, and the effects of renin on mitochondrial function in diabetic heart.

Methods: In Goto-Kakizaki (DM) and Wistar (non-DM) rats, Langendorff-perfused hearts were subjected to ischemia by coronary artery ligation for 90 min. Infarct Size (IS) and expression of RAS components were examined. Mitochondrial membrane potential (ΔΨm), uncoupling protein-2 (UCP2), NAD/NADH ratio, and ATP were measured in renin-treated myocytes or isolated mitochondria.

Results: After ischemia, LV function (LVDP; 76 ± 4 vs. 52 ± 4 mmHg, LVEDP; 18 ± 2 vs. 29 ± 4 mmHg, $p<0.05$, DM; n=9, non-DM; n=9, respectively) was prevented and IS (44.2 ± 2.1 vs. 53.7 ± 2.9 %, $p<0.05$) was significantly small in DM hearts. These cardioprotective effects were abolished when DM hearts were treated with direct renin inhibitor (LVDP; 77 ± 3 vs. 55 ± 4 mmHg, LVEDP; 16 ± 1 vs. 27 ± 3 mmHg, IS; 40.8 ± 2.9 vs. 52.1 ± 3.4 %, $p<0.05$, DM; n=5, DM plus DRI; n=5, respectively). Renin expression in the ischemic area was increased in DM hearts. Electron microscopy showed predominant renin localization within mitochondria. In permeabilized myocytes or isolated mitochondria, renin hyperpolarized ΔΨm, increased NAD/NADH ratio and preserved ATP content. Ischemia-induced UCP2 expression was reduced in DM.

Conclusions: Intracellular renin, which mainly localizes within mitochondria, increased during ischemia and protected cardiomyocytes in diabetic hearts. This protective effect of renin is at least partially because of the reduction of UCP2 and the acceleration of electron transport chain, which resulted in the prevention of mitochondrial depolarization and ATP production.

Keywords: Renin; Diabetes mellitus; Ischemia; Mitochondria; Direct renin inhibitor

Abbreviation

RAS: Renin-Angiotensin-Aldosterone System; IS: Infarct Size; ΔΨm: Mitochondrial Membrane Potential; UCP: UnCoupling Protein; AngII: Angiotensin II; AGN: Angiotensinogen; DM: Diabetes Mellitus; DRI: Direct Renin Inhibitor; GK: Goto-Kakizaki; K-H: Krebs-Henseleit; LV: left ventricular; LAD: left artery descending; ARB: angiotensin receptor blocker; TTC: Triphenyl Tetrazolium Chloride; AAR: Area at risk; PVDF: Polyvinylidene difluoride; TBS-T: Tris-buffered saline with Tween; TMRE: Tetramethylrhodamine ethyl ester; JC-1: 5,5′,6,6′-tetrachloro- 1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide; LVDP: Left Ventricular Developed Pressure; LVEDP: Left Ventricular End Diastolic Pressure; RPP: Rate-Pressure Product; ETC: Electron Transport Chain; CTGF: Connective Tissue Growth Factor; TGF-β: Tissue Growth Factor-β; ROS: Reactive Oxygen Species; PPAR-γ: Peroxisome Proliferator-Activated Receptor-γ

Introduction

The local Renin-Angiotensin-aldosterone System (RAS) is regulated independently from the circulating RAS and is attributed to organ pathophysiology [1]. In local RAS, angiotensin II (AngII) in the interstitial space binds to AngII receptors present on adjacent cells and activates intracellular signaling pathways (paracrine effect); AngII can also acts inside the cell where RAS components are produced (intracrine effect) [2]. Among RAS components, renin initiates RAS by hydrolyzing angiotensinogen (AGN) to angiotensin I [3]. Renin is synthesized from a physiologically inactive precursor, prorenin, which has a prosegment that prevents the interaction between the enzymatically active site of prorenin and AGN. Prorenin can be either excreted immediately or converted into renin by proteolytic cleavage of the prosegment [4].

The existence of (pro) renin receptor that can bind to both renin and prorenin was reported. The binding to the (pro) renin receptor actives prorenin through the induction of conformational changes of the prosegment [5]. Receptor binding to prorenin/renin also triggers its own intracellular signaling pathways, independently from AngII generation [6].

An alternatively spliced renin transcript, which does not encode a secretory signal, was identified, and renin encoded by this transcript had a truncated prosegment, which compromises the secretory signal sequence and remains within the cytosol [7-9]. In the adrenal gland, the non-secreted intracellular renin exists predominantly within mitochondria [10]. The non-secretory renin was also found in cardiomyocytes [7,8]. In H9c2 cells, overexpression of the alternatively spliced renin transcript resulted in the compartmentation of non-

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secretory renin into mitochondria [11]. In a transgenic rat model expressing the alternative renin transcript, the cardiac renin activity was 5- fold higher than that of wild type [12].

In the heart, intracellular renin increases after myocardial infarction [13,14]. In diabetes mellitus (DM), there was a remarkable increase in circulating prorenin, whereas plasma renin level was low [15]. As for intracellular renin, there was an increase in intracellular renin, which elevated intracellular AngII levels and subsequently caused cardiac fibrosis and apoptosis in diabetic rat cardiac myocytes [16]. However, the dynamics of intracellular renin during ischemia/reperfusion in DM have not been examined.

Interestingly, recent clinical trials demonstrated that direct renin inhibitor (DRI) did not improve, but worsened prognosis of type-2 DM patients with cardiovascular disease when added to standard heart failure therapy [17,18]. This indicates that intracellular renin could play important and unexplored roles in DM heart pathophysiology. In addition, mitochondrial distribution of intracellular renin could be related to myocardial damage, because mitochondria are the key organelles for energy metabolism and cell death and survival.

The aims of this study are to examine renin expression and intracellular distribution during ischemia, and to investigate the effects of renin on mitochondrial function in diabetic hearts.

Materials and Methods

Animals

This investigation conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication, eighth edition, revised 2011), and was approved by the Hamamatsu University School of Medicine Animal Care and Use Committee. Fifteen-week-old Goto-Kakizaki (GK) rats and age-matched Wistar rats (male, 400-450g) were used.

Isolated rat heart preparations and measurement of cardiac performance

After intraperitoneal administration of 50 mg/kg pentobarbital and cervical dislocation, the hearts were removed and retrogradely perfused in a Langendorff apparatus with a modified Krebs-Henseleit (K-H) buffer consisting of (in mmol/L) NaCl, 118.5; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 1.4; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; and glucose, 11.0 at 37°C. A water-filled latex balloon-tipped catheter was inserted into the left ventricle through the left atrium. The catheter was connected to a pressure transducer (Nihon Kohden, Japan) for continuous measurement of left ventricular (LV) pressure.

Assembly of the ex-vivo perfusion system

After 20 min stabilization, the left anterior descending (LAD) artery was occluded near its origin for 90 min. GK rats were randomly assigned into three experimental groups: ischemia, ischemia pretreated with a direct renin inhibitor (DRI; aliskiren, 1 μmol/L), and ischemia pretreated with an angiotensin receptor blocker (ARB; valsartan, 0.1 μmol/L). These drugs were added to the K-H buffer and perfused 20 min before ligation.

Measurement of myocardial infarction

After 90 min of ischemia, the heart was retrogradely perfused with 3 ml of 1% Evans blue (Sigma-Aldrich Chimie, France) to delineate the region of myocardial perfusion. The ligation of LAD was then released, and each heart was perfused with 1% of 2,3,5-triphenyl tetrazolium chloride (TTC) solution. The hearts were sliced across the long axis of the LV into 1.0 mm thick transverse sections. The sliced hearts were fixed for 24 h in 10% neutral buffered formalin solution (Wako Pure Chemical Ind., Ltd., Japan). The area at risk (AAR) and the infarct size (IS) in each slice were measured using planimetry (Excel software), and converted into percentages of the whole for each slice. The ratio of AAR to LV was calculated (AAR/LV). IS was expressed as the percentage of AAR (IS/AAR). In the first series of experiments where the difference of ischemic damage between DM and non-DM hearts were examined, 9 rats from DM and non-DM were used. In the second series of experiments, where the effects of direct renin inhibitor on ischemic damage in DM hearts were examined, total of 14 GK rats were divided into 3 groups (5 for DM, 5 for DM+DRI, 4 for DM+ARB).

Tissue preparation

After ischemia, the hearts were rapidly excised and placed in ice-cold phosphate-buffered saline. One transversal slide was used to perform immunohistochemistry and was embedded to obtain cryosections. The remaining ventricular slices were divided into ischemic area and non-ischemic area. Each tissue was used for Western blot assay and immunoelectron microscopy.

Immunohistochemistry

Heart transversal slices were frozen immediately in Optimal Cutting Temperature compound at -80°C. Frozen tissues were cut into 5 μm sections, and stained with hematoxylin-eosin (Supplemental Figure 1). The remaining sections fixed in 4% paraformaldehyde and incubated with antigen retrieval solution (Nichirei Bioscience, Japan) were heated in autoclave at 121°C for 20 min. The endogenous peroxidase was blocked with a 3% H_2O_2 solution in methanol. Slices were then stained with anti-renin (AnaSpec Inc., USA; 1:100) and anti-angiotensin II (Novus Biologicals, USA; 1:100) antibodies. After washing, the sections were incubated with Histofine Simple Stain MAX PO (R) (Nichirei Bioscience (code 414181), Japan). Non-immune rabbit serum (Sigma-Aldrich Chimie, France) was used as negative control for primary antibodies. The signals were visualized with a 3-amino-9-ethylcarbazole peroxidase substrate kit (Nichirei Bioscience, Japan).

Western blotting

Tissue lysates were prepared from homogenized samples with a ProteoExtract Cytosol/Mitochondria Fractionation kit (Merck Bioscience, Germany). Sample buffer containing 2-mercaptoethanol and Laemmli sample buffer was added to the lysate, and samples were boiled for 5 min at 100°C. Equal amounts (30 μg) of protein were separated on a 10% gradient mini gel and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking with 0.5% skim milk in Tris-buffered saline with Tween 20 (TBS-T), each membrane was incubated with primary anti-renin (AnaSpec, catalog number 54371; 1:250), anti-AngII (Novus Biologicals, catalog number NBP1-30027; 1:200), anti-uncoupling protein 2 (Santa-Cruz Biotechnology, catalog number sc-6525; 1:200), and anti-uncoupling protein 3 (Millipore, catalog number AB3046; 1:250) antibodies. For the incubation of first and secondary antibodies, Can Get Signal kit (TOYOBO, Japan) was used. Luminescence was used to visualize the bands. The membranes were subsequently incubated for 60 min in diluted appropriate secondary antibodies (1:2500).

Immunogold electron microscopy

For immunogold electron microscopy, the tissues were fixed in 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L cacodylic

acid and embedded in LR-white Resin. The samples were sectioned and collected on nickel grids. The sections were incubated with an anti-renin antibody (AnaSpec; 1:100) for 1 h at room temperature. After washing, immunogold labeling was performed by 1 h incubation with 10 nmol/L gold-labeled secondary antibody [Anti-Rabbit IgG (whole molecule)-gold antibody produced in goat; Sigma-Aldrich], and stained with uranyl acetate and lead citrate. Ultrathin sections were observed on transmission electron microscope (JEM-1220, JEOL, Japan). For the negative control, sections were incubated with non-immune rabbit serum (Sigma-Aldrich Chimie, France) and then subsequent incubation with gold-labeled secondary antibody was conducted.

Mitochondria preparation

Mitochondria were isolated by differential centrifugation. Briefly, heart tissue lysates were obtained from homogenized samples with the ProteoExtract Cytosol/Mitochondria Fractionation kit. After a centrifugation at 700 ×g for 10 min, the supernatant was washed twice by centrifugation at 10000 ×g for 30 min and purified by centrifugation for 4 h on OptiPrepTM density gradient (10-30%) (Cosmo Bio Co., LTD, Japan). On the other hand, the supernatant (from 10000 ×g) was centrifuged at 100000 ×g for 1 h. This supernatant was purified cytosolic fraction. This fraction was used western blotting.

The pellet was used for electron microscopic study. Electron microscopic observations showed very little contamination from broken mitochondria.

Cardiomyocytes isolation and sarcolemmal membrane permeabilization

Myocytes were enzymatically isolated [19]. Briefly, the heart was excised and mounted on a Langendorff apparatus and perfused with solutions gassed with 95% O_2 - 5% CO_2 and maintained at 37°C and pH 7.4. After the initial perfusion, a Ca^{2+}-free solution containing enzyme (Collagenase S-l, Nitta Corporation, Japan) was perfused for 8-10 min. Immediately before the experiments, the cells were placed in a chamber and perfused with a Tyrode's solution consisting of (in mmol/L) NaCl, 143; KCl, 5.4; $MgCl_2$, 0.5; NaH_2PO_4, 0.25; $CaCl_2$, 1; glucose, 5.6; and HEPES, 5. For permeabilization of sarcolemmal membranes, cells were perfused with saponin (0.05 mg/mL) for 30 s in a calcium-free internal solution (in mmol/L) KCl, 50; K-aspartate, 80; Na-pyruvate, 2; HEPES, 20; $MgCl_2$-$6H_2O$, 3; Na2ATP, 2; and EGTA, 3. After the permeabilization, the concentration of free calcium ([Ca^{2+}]c) in the internal solution was changed to 177 nmol/L.

Measurement of mitochondrial membrane potential (ΔΨm)

Saponin-permeabilized myocytes were loaded with a continuous perfusion of the fluorescent indicator tetramethylrhodamine ethyl ester (TMRE; 10 nmol/L) for 20 min. Myocytes were then loaded with 100 ng/mL recombinant rat renin (AnaSpec) for 30 min. TMRE was excited at 543 nm, and emission signals were collected through a 560-nm long-pass filter.

ΔΨm of isolated mitochondria were measured with 5,5′,6,6′-tetrachloro-1,1′,3,3′ - tetraethylbenzimidazol - carbocyanine iodide (JC-1; Life Technologies Corporation). Isolated mitochondria were treated with 100 ng/mL recombinant rat renin for 30 min. After the treatment, mitochondria were incubated with 0.5 μmol/L JC-1 for 30 min. In the aliskiren or valsartan experiments, each drug was added 10 min before exposure to renin. The fluorescent intensity was measured with a microplate reader (Synergy HT; BioTek Instruments, Inc., USA). JC-1 was excited at 485/20 nm, and the emission signals were collected with 528/20 nm and 590/35 nm band-pass filters.

Measurement of NAD/NADH ratio

NAD/NADH ratio was measured using a fluorescent NAD/NADH detection kit (Cell Technology Inc., USA). Isolated mitochondria were treated with renin for 30 min. After transferring the mitochondria into a 1.5 mL Eppendorf tube, either 200 mL of NAD or NADH extraction buffer and 200 mL of NAD/NADH lysis buffer were added to the respective tubes. Homogenates were heated at 60°C for 15 min. After cooling, reaction buffer and opposite extraction buffer were added. The tubes were vortexed and spined at 5000-8000 ×g for 5 min. The supernatants were measured using a fluorescent microplate reader with excitation at 530 nm and emission at 590 nm.

Measurement of ATP concentration

Isolated mitochondria were treated with renin for 60 min. Mitochondrial ATP production rate was determined with a luciferase assay (Toyo Ink Co., Ltd., Japan) according to the manufacturer's instructions.

Statistics

Data are presented as means ± SEM, and the number of cells or experiments is denoted by n. Statistical analyses were performed using two-way ANOVA of repeated measurements, followed by the Bonferroni test. A p value of <0.05 was accepted as statistically significant.

Results

Table 1 summarizes body weight, heart weight, and laboratory data for age-matched Wistar (non-DM) and GK (DM) rats. Preischemic cardiac function of spontaneously beating hearts from non-DM and DM rats is shown in Table 2. Intrinsic heart rate was lower in DM. There were no differences in LV developed pressure (LVDP) and LV end diastolic pressure (LVEDP) between DM and non-DM. Consequently, rate-pressure product (RPP) was significantly lower in DM.

DM preserved cardiac function after regional ischemia

Figure 1(a) demonstrates LV pressure during ischemia in non-DM and DM hearts. Hearts were subjected to regional ischemia by LAD artery ligation for 90 min. At the end of ischemia, LVDP was higher and LVEDP was lower in DM compared with non-DM (76 ± 4 vs. 52 ± 4 mmHg, 18 ± 2 vs. 29 ± 4 mmHg, $p<0.05$, respectively) (Figures 1(b) and 1(c)). Figure 1(d) shows cross-sectional images obtained from non-DM and DM hearts. There was no significant difference in AAR between DM and non-DM (48.0 ± 3.2 vs. 51.9 ± 2.5 %, Figure 1(e)). However, IS was significantly smaller in DM compared with that in non-DM (44.2 ± 2.1 vs. 53.7 ± 2.9 %, $p<0.05$, Figure 1(f)). These results indicate that DM hearts are more tolerant to ischemic stress than non-DM hearts. When nuclear condensation (by Hoechst 33342 staining) and the expression of apoptosis-related proteins (Bax, Bcl-2 and caspase 3) were examined, there were no significant changes between two groups (data not shown).

Renin expression in the ischemic area was increased in DM

Immunohistochemistry was performed in heart sections obtained from non-DM and DM hearts using anti-renin (Figure 2(a)) or anti-AngII (Figure 2(b)) antibodies. Renin increased both in DM and non-DM hearts, and the increase in renin was greater in ischemic areas than in non-ischemic areas. The difference of renin expression between ischemic and non-ischemic areas was small in non-DM heart. No difference in the level of AngII was observed between ischemic and non-ischemic areas in both DM and non-DM hearts.

	Age (days)	Glucose (mg/dl)	Insulin (ng/ml)	Body weight (g)	Heart weight (g)	HW/BW (x1000)
non-DM	100±1	75.5±2.7	2.6±0.3	426.3±14.1	2.17±0.02	5.06±0.17
DM	101±1	197.0±14.3**	0.9±0.3**	412.9±8.6	2.24±0.02	5.51±0.11*

HW heart weight, BW body weight. Data are shown as means ± SEM, n=8 animals per group.*p<0.05 compared with non-DM.**p<0.01 compared with non-DM.

Table 1: Characteristics and laboratory data from non-DM and DM rats at 15 wk of age.

	Heart rate (beats/min)	LVDP (mmHg)	LVEDP (mmHg)	RPP (mmHgxbeats/min)	Peak LV +dP/dt (mmHg/sec)	Peak LV -dP/dt (mmHg/sec)
non-DM	300.1±10.2	112.4±6.8	5.4±0.5	34330±2055	2366±102.3	-1864±100.9
DM	253.1±6.2**	104.3±3.4	6.5±0.5	26160±1084**	2242±150.0	-1666±102.8

LVDP left ventricular developed pressure, LVEDP left ventricular end diastolic pressure, RPP: rate-pressure product. Data are shown as means ± SEM, n=8 animals per group.**p<0.01 compared with non-DM.

Table 2: Preischemic cardiac parameter on Langendorff system.

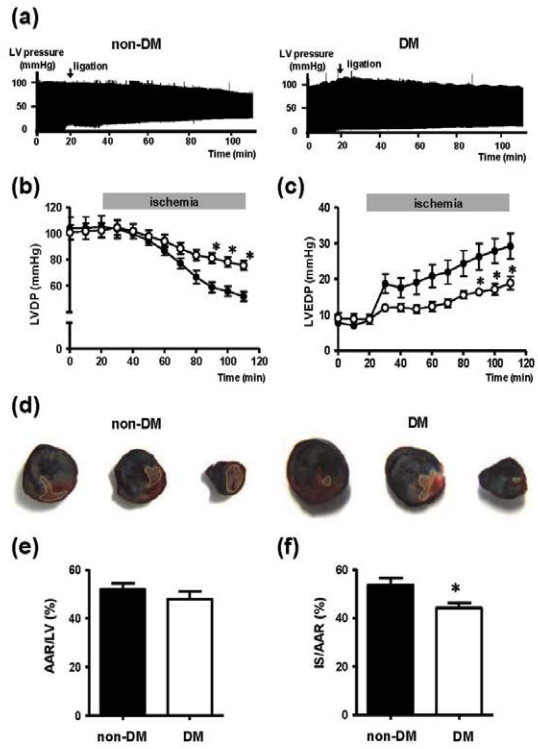

Figure 1: Ischemic injury is less severe in diabetic hearts. Representative recordings of LV pressure in non-DM and DM hearts **(a).** Time courses of the changes in LVDP **(b)**, and LVEDP **(c)** during ischemia in non-DM (closed circles, n=9) and DM hearts (open circles, n=9). Representative cross-sectional images from non-DM and DM hearts after ischemia **(d)**. The infarct region was delineated with white line. Summarized data of area at risk (AAR, **(e)**) and infarct size (IS, **(f)**). non-DM, n=9; DM, n=9. Values are means ± SEM; *p<0.05 compared with non-DM.

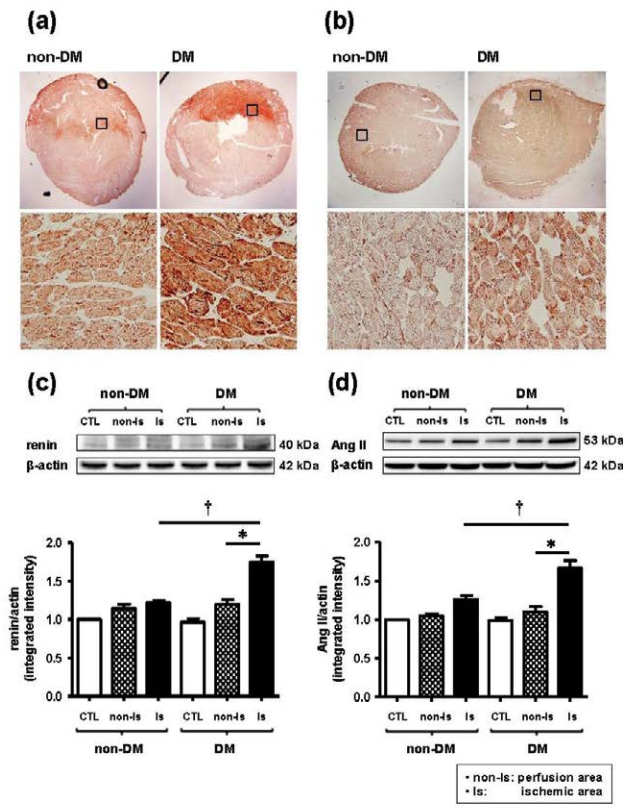

Figure 2: Expression of renin and angiotensin II (AngII) during ischemia. Representative immunohistochemistry images of renin **(a)** and AngII **(b)** after ischemia from non-DM and DM hearts, with low magnification (upper panels, ×1.25) and high magnification (lower panels, ×40). Western blot analysis of renin **(c)** and AngII **(d).** The bar graph shows the summarized data of control (CTL), non-ischemic area (non-Is) and ischemic area (Is) in non-DM and DM hearts (n=7 for renin, and n=4 for AngII in each bar). Data are expressed as integrated intensity normalized to non-DM control. Data represent means ± SEM. *p<0.05 vs. DM non-Is; †p<0.05 vs. non-DM Is.

In Western blotting analysis, renin expression in ischemic area was significantly increased in DM compared with non-DM hearts (1.7 ± 0.1- vs. 1.2 ± 0.1- fold of non-ischemic whole heart, p<0.05, Figure 2(c)). When renin expression was compared between ischemic and non-ischemic areas in DM hearts, there was a significant increase in renin expression in ischemic area (1.7 ± 0.1- vs. 1.2 ± 0.1- fold of non-ischemic whole heart, respectively, p<0.05, Figure 2(c)). As for AngII expression, in contrast to immunohistochemistry, AngII in ischemic area was significantly increased compared with that in non-ischemic area in DM hearts (1.7 ± 0.1- vs. 1.3 ± 0.1- fold of non-ischemic whole heart, p<0.05, Figure 2(d)).

Renin was expressed within mitochondria and cytosol in DM hearts

In Immunoelectron microscopy, after 90 min of ischemia, renin immunoreactivity was observed in both mitochondria (i) and cytosol (ii), and renin was predominantly distributed within mitochondria

(Figure 3(a)) in DM. We did not detect renin immunoreactivity either in the cytosol or in the mitochondria in non-DM hearts (data not shown).

To confirm the presence of renin in mitochondria, renin expression was examined in the mitochondrial fraction after 90 min of ischemia in DM. Figure 3(b) shows that there was a band for renin both in cytosolic and mitochondrial fractions. The purity of mitochondrial fraction was identified using of prohibitin (as the mitochondrial marker), and DM1A (as the cytosolic marker). These results indicated that renin was preferentially localized in mitochondria after ischemia in DM hearts.

DRI decreased renin expression and deteriorated cardiac function in DM hearts after ischemia

We next investigated the effect of inhibition of renin or AngII on ischemic tolerance in DM. Langendorff-perfused hearts were pretreated with DRI (aliskiren, 1 μmol/L) or ARB (valsartan, 0.1 μmol/L) for 20 min before LAD artery ligation. Figure 4(a) demonstrates the representative recordings of LV pressure during ischemia in DM, DM plus aliskiren, and DM plus valsartan. LVDP was significantly reduced (55 ± 4 vs. 77 ± 3 mmHg, $p<0.05$, Figure 4(b)), and LVEDP was significantly elevated (27 ± 3 vs 16 ± 1 mmHg, $p<0.05$, Figure 4(c)) in DM perfused with aliskiren compared with DM without aliskiren treatment. Unlike the aliskiren treatment, pretreatment with valsartan did not alter cardiac function after ischemia in DM hearts (Figures 4(b) and 4(c)). Aliskiren worsened IS in DM (40.8 ± 2.9 % vs. 52.1 ± 3.4 %, $p<0.05$, Figures 4(d)-4(f)).

Immunohistochemistry of DM hearts demonstrated that pretreatment of aliskiren suppressed renin (Figure 5(a)) and AngII expressions (Figure 5(b)) in ischemic areas. Similarly, western blotting data showed that aliskiren treatment significantly decreased renin expression (1.5 ± 0.1- vs. 1.9 ± 0.1-fold, $p<0.05$, Figure 5(c)). Pretreatment of aliskiren slightly, but not significantly, decreased AngII expression. (Figure 5(d)). These results indicated that DRI, but not ARB deteriorates the ischemic tolerance of DM hearts.

Renin hyperpolarized ΔΨm

To examine the effect of renin on mitochondrial function, ΔΨm of isolated mitochondria obtained from DM hearts were measured with the voltage sensitive fluorescent JC-1. After a 60 min exposure to renin, there was an increase in JC-1 ratio, which indicated hyperpolarization of ΔΨm. When isolated mitochondria were pretreated with aliskiren (from 1 to 10 μmol/L), renin-induced ΔΨm hyperpolarization was concentration dependently inhibited (Figure 6(a)). In contrast, pretreatment of isolated mitochondria with valsartan (from 0.1 to 1 μmol/L) did not alter renin-induced hyperpolarization of ΔΨm (Figure 6(b)).

Next, the effect of renin on ΔΨm was examined in permeabilized non-DM adult rat cardiomyocytes. To measure ΔΨm, cells were loaded with TMRE (Figure 6(c)). Figure 6(d) shows time courses of the changes in ΔΨm during the perfusion of renin. After 60 min of perfusion, most of the cells remained rod shape in both control and renin perfused group. Renin increased TMRE intensity (117.4 ± 2.7 % vs 99.9 ± 4.7 % of baseline, $p<0.05$), indicating hyperpolarization of ΔΨm. The increase in TMRE intensity by renin was inhibited in the presence of aliskiren (105.2 ± 4.0 % vs 117.4 ± 2.7 %, $p<0.05$). These results suggested that renin affects ΔΨm independently from AngII.

Renin accelerates electron transport chain (ETC) and suppresses uncoupling protein-2 (UCP2) expression

We examined ATP contents in renin-treated isolated mitochondria

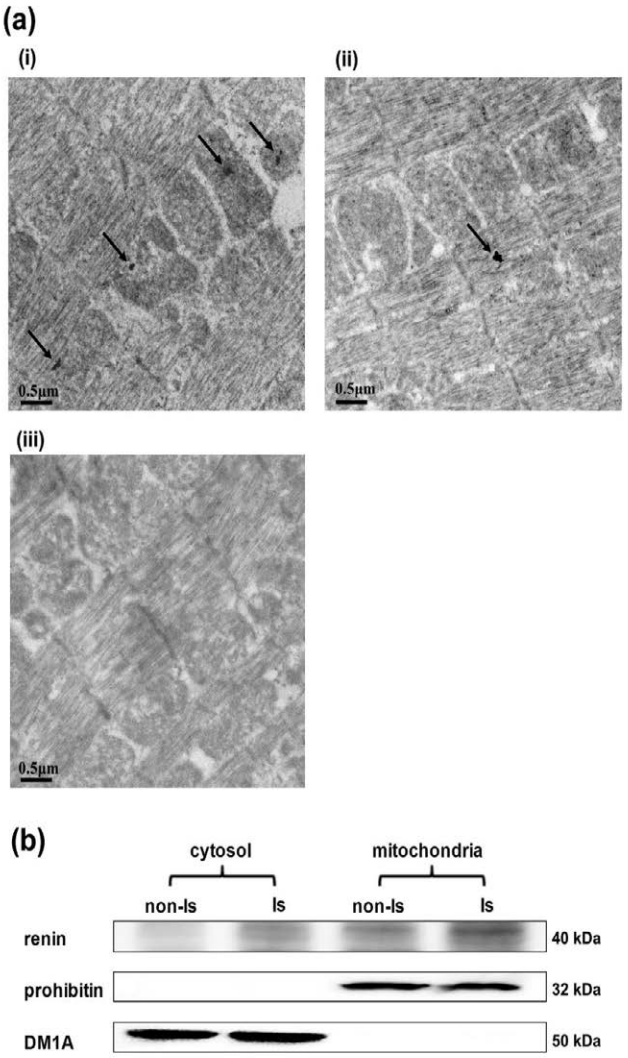

Figure 3: Subcellular renin distribution after ischemia in diabetic heart. Immuno-electron microscopic localization of renin **(a).** **(i)** Arrows indicate renin distribution in mitochondria and **(ii)** cytosol. **(iii)** For the negative control, non-immune rabbit serum was used instead of anti-renin antibody. **(b)**Western blotting of renin in mitochondrial and cytosolic fragments after ischemia in diabetic hearts (prohibitin and DMIA were used as the mitochondrial and cytosolic markers, respectively).

obtained from DM and non-DM hearts. Renin increased ATP concentration, and pretreatment with aliskiren (10 μmol/L) inhibited the effect of renin on ATP concentration both in DM and non-DM (Figure 7(a)).

To investigate the mechanism of renin-induced hyperpolarization of ΔΨm, we assessed NAD/NADH ratio in mitochondrial fractions obtained from DM and non-DM hearts. An increase in NAD/NADH represents acceleration of ETC, which results in hyperpolarization of ΔΨm. Renin tended to increase NAD/NADH ratio in isolated mitochondria from non-DM hearts, and significantly increased NAD/NADH ratio in isolated mitochondria from DM hearts (Figure 7(b)), suggesting the acceleration of ETC activity.

Finally, we examined the UCP2 expression in DM and non-DM hearts after 90 min ischemia. In perfused hearts, after 90 min of

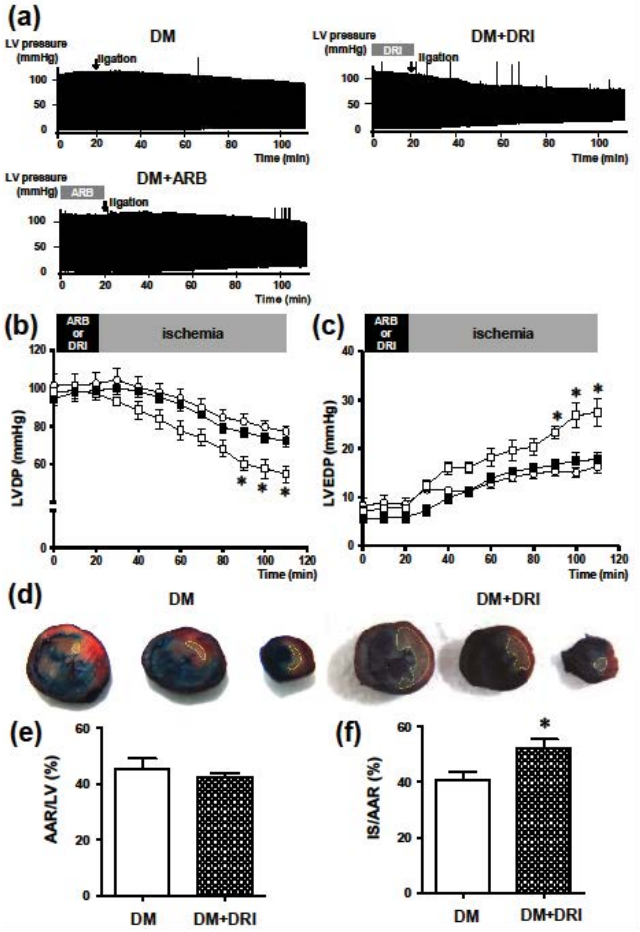

Figure 4: DRI but not ARB abolished ischemic tolerance in DM. Representative recordings of LV pressure during ischemia from DM, DM plus DRI, and DM plus ARB hearts **(a)**. DRI or ARB was added 20 min before coronary ligation. Time courses of the changes in LVDP **(b)** and LVEDP **(c)** during ischemia in DM (open circles, n=5), DM plus DRI (open squares, n=5), and DM plus ARB (closed squares, n=4). Representative cross-sectional images after ischemia obtained from DM and DM plus DRI hearts **(d)**. The infarct region was delineated with white line. Summarized data of AAR **(e)** and infarct size (IS) **(f)**. DM, n=5; DM plus DRI, n=5.Values are means ± SEM. *p<0.05 compared with DM hearts.

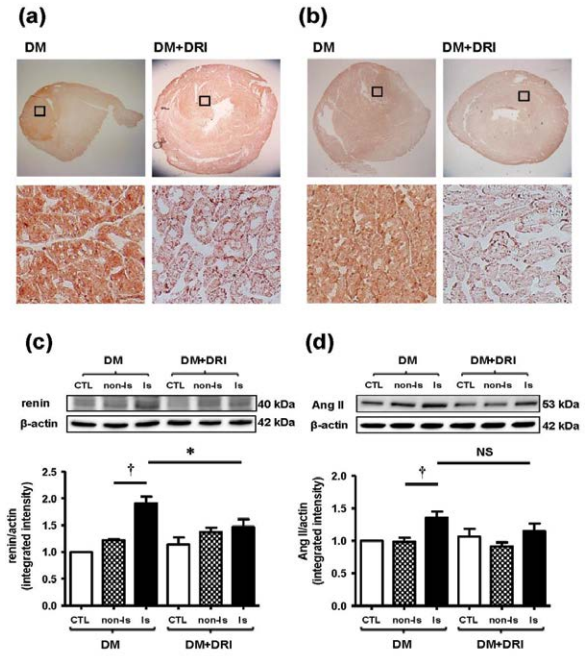

Figure 5: DRI inhibited renin expression after ischemia in DM hearts. Representative immunohistochemistry images of renin **(a)** and angiotensin II (AngII) **(b)** after ischemia from DM and DM plus DRI hearts. Western blot analysis of renin **(c)** and AngII **(d)**. Bar graph shows the summarized data of control (CTL), non-ischemic area (non-Is) and ischemic area (Is) in DM (n=5 for renin, and n=5 for AngII in each bar) and DM plus DRI (n=5 for renin, and n=5 for AngII in each bar) hearts. Data are expressed as integrated intensity normalized by non-DM control. Data represent means ± SEM. *p<0.05 vs. DM Is; †p<0.05 vs. DM non-Is.

ischemia, UCP2 expression increased in non-DM hearts (1.1 ± 0.1- vs. 1.4 ± 0.1-fold, p<0.05), but there was no increase in UCP2 expression in DM hearts. This inhibition of UCP2 up-regulation was abrogated when DM hearts were pretreated with aliskiren (Figure 7(c)), indicating that intracellular renin prevented the increase in UCP2 expression in DM hearts. These results suggest that renin-induced hyperpolarization of ΔΨm could be at least partially because of the acceleration of ETC and the reduction of UCP2 expression.

Discussion

We demonstrated that intracellular renin expression increases during ischemia in diabetic hearts, and that increased renin protects cardiomyocytes from ischemic injury. This protective effect of renin is at least partially because of the prevention of mitochondrial depolarization and ATP production. Our results indicated that in contrast to previously reported adverse effects of tissue RAS, intracellular renin plays an important role to protect myocytes from ischemic stress in diabetic heart.

Intracellular renin in cardiomyocytes

In the local RAS, synthesized AngII in the interstitial space acts in an autocrine or paracrine manner and plays more important roles in tissue pathology than in the circulating RAS. Recent evidences revealed that the action of intracellular AngII (iAngII) is initiated inside the cell, and this type of RAS is described as an intracrine or intracellular RAS [2].

Renin has been shown to exist and contribute to the syntheses of iAngII within cardiomyocytes [16]. In the heart, intracellular renin can be uptaken from the circulation and/or locally synthesized [20]. Under normal conditions, internalization of plasma prorenin/renin into the cytosol may be dominant [21,22]. Because we used Langendorff-perfused hearts, where serum renin was washed out within several minutes, the uptake from the circulation can be excluded [23]. Studies with transgenic animal models evidenced that the heart is a site of extrarenal renin production [12]. Cardiac renin is a prosegment truncated prorenin encoded by an alternative renin transcript, which lacks a secretory signal and remains inside the cell [7-9]. In the heart, renin expression is stimulated under pathophysiological conditions [13,24-26]. In diabetic animals and patients, plasma renin level was decreased [15]. However, there was an increase in intracellular renin in streptozotocin-induced diabetic rat hearts or in rat neonatal ventricular myocytes when exposed to high glucose [16,26]. These imply that intracellular renin may have distinct roles from circulating renin/prorenin in the pathophysiology of diabetic hearts.

We demonstrated that distribution of intracellular renin was

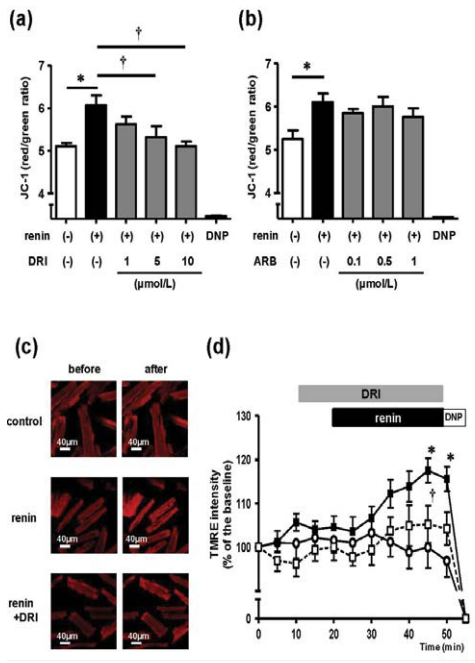

Figure 6: Renin hyperpolarized ΔΨm. Effects of renin on ΔΨm in isolated mitochondria from DM hearts. Summarized JC-1 data after 60 min application of renin in the presence or absence of DRI ((**a**); control; renin; renin plus DRI (1 μmol/L); renin plus DRI (5 μmol/L); renin plus DRI (10 μmol/L), n=14 for each group) or ARB ((b); control; renin; renin plus ARB (0.1 μmol/L); renin plus ARB (0.5 μmol/L); renin plus ARB (1 μmol/L), n=8 for each group). DNP was referred as maximum depolarization. Values are means ± SEM. *- p<0.05 vs. Control; †- p<0.05 vs. DRI-5 μmol/L or 10 μmol/L. Representative confocal tetramethylrhodamine ethyl ester (TMRE) images before and after 30 min exposure to renin in the presence (open squares, n=16) or absence (closed squares, n=22) of DRI **(c)**. The time course of the changes in TMRE intensity during the perfusion of renin in permeabilized non-DM myocytes. DRI was perfused as indicated in the bar. Open circle represents perfusion of internal solution without renin (control, n=20) **(d).** Values are means ± SEM. *p<0.05 vs. Control; †p<0.05 vs. renin.

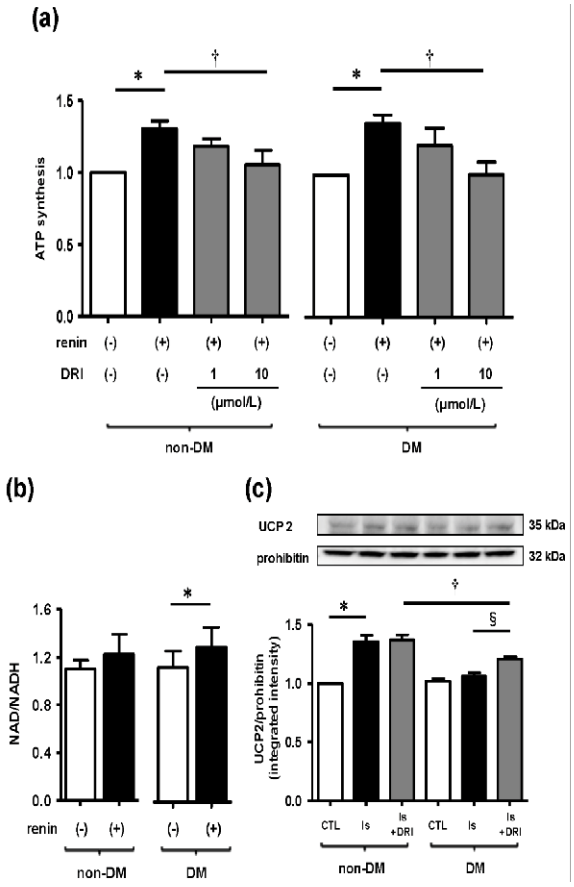

Figure 7: Effects of renin on ATP production, NAD/NADH ratio, and UCP2 expression. ATP concentration in isolated mitochondria from non-DM and DM after 60 min exposure to renin **(a)**. control; renin; renin plus DRI (1 μmol/L); renin plus DRI (10 μmol/L), n=5 for each group. Values are means ± SEM. *p<0.05 vs. Control; †p<0.05 vs. DRI. NAD/NADH ratio after 60 min application of renin in non-DM and DM hearts **(b).** control, n=7; renin, n=7. Western blot analysis of UCP2 after 90 min of ischemia in non-DM and DM hearts **(c)**. CTL; Is; Is plus DRI, n=9 for each group. Values are means ± SEM. *p<0.05 vs. non-DM control; †p<0.05 vs. non-DM Is plus DRI (10 μmol/L); §p<0.05 vs. DM Is.

predominant within mitochondria, suggesting a necessity to consider subcellular RAS. Consistent with our results, overexpression of cardiac alternative renin transcript lacking signal for secretion resulted in an increase of a non-secretory renin mainly within mitochondria in H9c2 cells [11]. Although the mechanisms causing the increase in mitochondrial renin content have not been fully resolved, it is reported that prosegment truncated prorenin can be imported into mitochondria [7-9].

Renin contributes to ischemic tolerance in diabetic heart

Whether diabetic heart is resistant to ischemia has been a matter of debate [27-30]. Ischemic tolerance appears to be dependent on duration, severity and type of diabetes, and the severity of the ischemic stress. The mechanisms of ischemic tolerance in diabetic heart have been attributed to metabolic alteration, changes in ionic current, less severe Ca^{2+} overload caused by reduced Na^{+}/H^{+} exchanger activity, and intracellular signal transduction pathway [31,32].

In this study, intrinsic heart rate was lower in DM compared to no-DM. This is in line with previous report, which showed that HR was significantly lower in GK rats compared with Wistar control animals at 2, 7 and 15 months [33]. The prolongation of diastolic interval has been reported to increase myocardial angiogenesis via the stimulation of angiogenic growth factors and receptors [34]. Therefore, the possibility that lower HR-related factors, such as lower RPP and increased angiogenesis, could affect the degree of ischemic injury cannot be excluded. However, there were several studies demonstrating that hearts from GK rats were more resistant to ischemic damage [29,35]. In addition, unlike the HR of preischemia, there was no difference in HR after ischmeia between GK rats and Wistar control in this study (data not shown).

To our knowledge, this is the first report to demonstrate that intracellular renin could be involved in the mechanisms of ischemic tolerance in diabetic heart. In this study, although AngII expression was increased after ischemia, ARB did not abrogate the ischemic tolerance but DRI did, indicating that the effect of renin on ischemic tolerance is independent on subsequently produced iAngII. In our results, DRI reduced the expression of intracellular renin after ischemia in DM heart. Aliskiren has been reported to reduce the gene expression of cardiovascular diseases-related proteins including connective tissue growth factor (CTGF) and tissue growth factor-β (TGF-β) [36,37]. Aliskiren also reduced the gene expression of (pro)renin receptor in the kidney of in vivo diabetic model of mouse-renin expressing rat

(TG(mRen-2)). However, in the same diabetic rat model, in contrast to our results, aliskiren increased renal renin gene expression [37]. Thus, although we did not investigate renin gene expression, there seems to be a discrepancy for the effect of aliskiren on renin gene expression. The discrepancy of the effects of aliskiren on renin expression may be due to the different experimental conditions, such as in-vivo or ex-vivo protocol, different organs (kidney or heart), and transgenic model for renin or not. Further studies are required to elucidate the effect of aliskiren of renin gene expression.

Renin hyperpolarized ΔΨm in permeabilized myocytes and in isolated mitochondria, and increased ATP contents in isolated mitochondria obtained from DM hearts. In contrast, perfusion of AngII did not alter ΔΨm in permeabilized myocytes (Supplemental Figure 2). These results imply the direct effects of renin on mitochondria, and suggest that renin protects myocardium from ischemic damage by preserving mitochondrial function. Interestingly, overexpression of cytosolic renin prevented necrosis but increased apoptosis in H9c2 cell [11].

In this study, the activity of ETC assessed by NAD/NADH ratio increased after renin exposure in isolated mitochondria from DM hearts, suggesting that the activation of ETC by renin accelerates mitochondrial respiration and hyperpolarizes ΔΨm. How renin directory induces these changes in mitochondria remains to be elucidated. Recently Abadir et al. reported that AngII receptor type 1 and 2 are present in mitochondria. These AngII receptors play significant roles in the regulation of metabolism, transcription, and gene expression [38]. The (pro)renin receptor [5], and mannose 6-phoshate receptor [39] have been known to be present in cardiac sarcolemma and to interact with renin. However, the existence of these receptors in mitochondrial membrane has not been demonstrated.

In addition, an alternative possibility that renin affects mitochondrial function in concert with cytosolic proteins should be considered. In the heart, UCP2 and UCP3 increase during ischemia/reperfusion. Although cardioprotective effects of UCPs by reducing reactive oxygen species (ROS) in ischemia/reperfusion have been reported, UCPs afford deleterious effects for cardiac function by impairing energy metabolism [40,41]. In our study, expression of UCP2 after ischemia was less in DM than in non-DM hearts. The expression of UCP3 was not altered both in DM and non-DM hearts after ischemia (data not shown). Cardiac UCP2 is regulated by multiple factors including peroxisome proliferator-activated receptor-γ (PPAR-γ) [42]. PPAR-γ stimulates UCP2 expression and its activity is elevated in diabetic hearts [43]. Because renin and PPAR-γ functionally counteract each other in cardiomyocytes [44], increased intracellular renin could reduce the activity of PPAR-γ, resulting in the inhibition of UCP2 expression [42].

Clinical implication

A recent clinical study, which tested whether the addition of DRI to standard heart failure therapy would improve clinical outcomes in patients with acute heart failure, revealed that although overall results were not affected, only a subgroup of patients with DM had poor prognosis [17]. Another clinical trial, which tested the effects of DRI in patients with type-2 DM and chronic kidney or cardiovascular disease, was terminated prematurely due to the not favorable risk/benefit ratio of DRI [18]. Microcirculation-related ischemic events could happen more frequently in these patients than in non-DM patients. Elimination of the cardioprotective effect of intracellular renin by DRI within small ischemic areas may explain why DRI worsened the outcomes of DM patients.

Conclusion

We conclude that intracellular renin expressed within cardiomyocytes has a beneficial effect to prevent mitochondrial dysfunction and the reduction of ATP contents, and that these events contribute to the ischemic tolerance in diabetic heart. The cardioprotective effect of renin is independent on subsequent iAngII production. Because mitochondrial distribution of intracellular renin may alter mitochondrial function, subcellular RAS may play pivotal roles for the pathogenesis of ischemic injury in diabetic heart. Further investigations are required to examine the role of intracellular renin not only during ischemia, but also after ischemia/reperfusion or longer time courses using in vivo myocardial infarction models.

Competing Interests

The authors declare that they have no competing interests.

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