



Cardioprotective effect of angiotensin-converting enzyme 2 and angiotensin II Type 2 receptor over-expression in ischemia reperfusion injury in diabetic rats

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ABSTRACT

Background: Major components of the renin-angiotensin system such as angiotensin II (Ang II), angiotensin converting enzyme (ACE), ACE2, Ang II Type 1 receptors (AT1R) and Ang II Type 2 receptors (AT2R) have been identified in human and rodent heart cells. Over-activation of this system has been reported to play a key role in diabetes. **Methods:** A total of 72 male albino rats (160-180 g) were divided into the following groups each containing 18 rats. Group 1: Control group, Group 2: Type 2 diabetic control rats, Group 3: Angiotensin receptor blocker (ARB)-protected diabetic rats and Group 4: ACE inhibitor (ACEI) protected diabetic rats. At the end of the experiment, half the number of rats in all groups was sacrificed, and the heart excised and perfused according to the Langendorff technique. Mechanical performance of the left ventricle (LV) of the heart was determined by the systolic pressure, the diastolic pressure, the heart rate and the peak rate of maximum LV pressure rise (dp/dt), which is considered as a good index of contractility. These mechanical performances were monitored during pre-ischemic; ischemic and post-ischemic reperfusion phases. From the other group of rats ($n = 9$), tissue was extracted from the heart for estimation of AT2R and ACE2 gene expression. **Results:** Treatment with ARBs and ACEIs significantly lowered blood glucose, insulin levels and homeostasis model assessment insulin resistance index compared to untreated diabetic rats. However, values did not return to control values. ARBs and ACEI improved myocardial performance and percentage recovery following ischemia reperfusion. The cardio protective effect was more pronounced in the ARBs Group. This positively correlated with increased AT2R and ACE2 expression in all studied Groups. However, there was no significant correlation between the level of AT2R and ACE2 expression in cardiac tissue. **Conclusion:** The use of ARBs and ACEIs in Type 2 diabetes mellitus significantly offered cardio-protection against ischemia-reperfusion injury either through improving the diabetic condition or by increasing the expression of AT2R and/or ACE2 in cardiac tissue.

KEY WORDS: Angiotensin converting enzyme 2, angiotensin II Type 2 receptors, cardiac ischemia reperfusion, diabetes, expression

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INTRODUCTION

Diabetes mellitus (DM) leads to high cardiovascular morbidity and mortality, as a result, of functional and morphological damage in diabetic hearts [1]. Myocardial dysfunction occurs frequently in diabetes, even in the absence of coronary or valvular heart disease, suggesting a primary diabetic cardiomyopathy [2].

The focus of this study is the participation of major hormonal system the renin-angiotensin system (RAS) in diabetes and diabetic heart dysfunction. Over-activation of this system has been reported to play a key role in diabetes [3]. Major

components of the RAS such as angiotensin converting enzyme (ACE), ACE2, angiotensin II (Ang II) Type 1 and Type 2 receptors (AT1R and AT2R) have been identified in human and rodent heart cells pointing to a local cardiac RAS [4,5].

AT2R is generally accepted to exert an opposing effect to AT1R in the cardiovascular system (CVS). Although Ang II is known to have direct effects on cardiomyocytes through the AT1R [6], the functional role of AT2R in the development of cardiomyopathy in diabetes is incompletely understood.

In the past few years, the classical concept of the RAS has experienced substantial conceptual changes. The importance

of the vasodeleterious axis of the RAS (ACE/Ang II/AT1R) in cardiovascular disease (CVD), as well as in diabetes and diabetic complications, is well established since ACE inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) are leading therapeutic strategies [7]. However, the impact of the vasoprotective axis of the RAS remains poorly understood.

Discovery of ACE2 has resulted in the establishment of a novel axis of the RAS involving ACE2/Ang (1-7)/Mas receptor. This vasoprotective axis counteracts the traditional proliferative, fibrotic, proinflammatory and hypertrophic effects of the ACE/Ang II/AT1R axis of the RAS. The concept that shifting the balance of the RAS toward the vasodilator axis by activation of ACE2 or its product, Ang (1-7) is beneficial has been supported by many studies in cardiac, pulmonary, and vascular fibrosis. Indeed, ACE2/Ang (1-7) activation is now considered to be a critical part of the beneficial actions of ACEIs and ARB drugs [8].

Whether AT2R and ACE2 interact in diabetes is unknown. However, since AT2R inhibits ACE activity [9], it is speculated that AT2R might increase ACE2/ACE ratio, which could be important in cardiac dysfunction in diabetes.

There are data suggesting that RAS blockade by ACEIs or ARBs may work as a cardio-protective mechanism through modulation of the expression of AT2R and/or the novel ACE2 [10]. However, the relation between AT2R and ACE2 expression in diabetic hearts needs to be clarified.

The aim of this study is to:

- Test and compare the possible protective effects of AT1R blockade by losartan and ACE inhibition by Lisinopril on the development of Type 2 diabetes in rats
- Test the possibility of improving the myocardial function and reducing the susceptibility to ischemia-reperfusion injury through the modulation of the gene expression of cardiac AT2R and ACE2
- Test the hypothesis of interaction between AT2R and ACE2 in diabetic heart dysfunction.

METHODS

Experimental Animals

A total of 72 male albino rats (160-180 g) was used in this study. The animals were housed in wire mesh cages at room temperature, with normal light-dark cycle. They had free access to water and to their respective diets. All of the experiments were performed in animal house of Cairo University in accordance with guidelines for the care and use of laboratory animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Rats were divided into the following groups, each containing 18 rats:

Group 1: Control group: Fed the commercial rat chew diet (12% calories as fat) for the whole time of the

experiment. Rats received intra-peritoneal (i.p.) saline injection on day 14.

Group 2: Type 2 diabetic rats: Type 2 DM was induced by model introduced by Srinivasan *et al* (2005) [11].

Group 3: ARB-protected Type 2 diabetic rats: Losartan (AT1R blocker) 10 mg/kg/day [12] was added to drinking water daily from day 0 till the end of the experiment (21 days).

Group 4: ACEI-protected Type 2 diabetic rats: Lisinopril (ACEI), 10 mg/kg/day [12] were added to drinking water daily from day 0 till the end of the experiment (21 days).

Experimental Protocol

Induction of Type 2 DM model

Beginning on day 0, rats in Group 2, 3, and 4 were fed high-fat diet (58% calories as fat) for 2 weeks. On day 14, rats were injected with a single low dose of streptozotocin (STZ, 35 mg/kg i.p., in 0.01 M citrate buffer pH 4.3) to induce Type 2 DM [12]. Subsequent to treatment, rats had free access to food and water and were continued on their respective diets for the duration of study.

Characterization of Type 2 diabetes

Blood sampled from the retro-orbital plexus under diethylether anesthesia was used to measure plasma glucose and insulin concentration. Type 2 DM was confirmed by calculation of homeostasis model assessment insulin resistance index (HOMA-IR). Lower index indicates greater insulin sensitivity.

HOMA - IR =

$$\frac{\text{Fasting insulin } (\mu\text{IU/ml}) \times \text{Fasting glucose (mmol/L)}}{22.5}$$

At the end of the experiment (day 21), all experimental rats were sacrificed under anesthesia by i.p. injection of sodium thiopental (10 mg/100 g body weight). In half the number of rats of each group ($n = 9$), the hearts were rapidly excised and perfused according to the Langendorff technique [13]. In the rest of the rats ($n = 9$), tissue was extracted from the hearts for estimation of AT2R and ACE2 expression.

Langendorff technique for heart perfusion [13]

Hearts were immersed in ice cold modified Krebs-Henseleit solution containing heparin (5000 units) at ambient temperature (25°C). Hearts were then mounted on the aortic cannula and subsequently perfused according to the Langendorff technique with Krebs-Heinseleit medium at a hydrostatic pressure of 55 cm H₂O and bubbled with a mixture of 95% O₂ and 5% CO₂. The time between extraction of the hearts and their attachment to the Langendorff apparatus did not exceed 2 min.

The Krebs-Henseleit solution was prepared from the following concentration in (g): 6.926 NaCl, 2.1 NaHCO₃, 0.16 KH₂PO₄, 0.298 KCl, 0.294 MgSO₄.7H₂O, 0.264 CaCl₂ and glucose 1.982

in 1000 ml distilled water. A roller pump delivered the medium (flow = 15 ml/min) to an 8- μ m pore size, 47-mm diameter filter, a membrane oxygenator, a pre-heater, and the cannula. The temperature of the heart and of the perfusion medium was maintained at 37°C by an external water bath. A latex balloon filled with saline was introduced into the left ventricle (LV) and was connected to a pressure transducer to monitor performance. We measured the intra-ventricular balloon volume needed to increase end-diastolic pressure from 0 to 10-15 mmHg, afterward, the balloon volume was kept constant. After 30 min of stabilization, ischemia was induced by abolishing the coronary flow from 15 ml/min to 0 ml/min for 30 min. The hearts were then reperfused for 60 min with a coronary flow of 15 ml/min.

Measurements of myocardial function

Mechanical performance of the LV of the heart was determined by the systolic pressure, the diastolic pressure, the heart rate (HR) and the peak rate of maximum LV pressure rise (dp/dt), which is considered as a good index of contractility. These mechanical performances were monitored during pre-ischemic; ischemic and post-ischemic reperfusion phases by a balloon inserted into the LV and connected to a polygraph apparatus (San-ei Instrument, Ltd., Nee, Tokyo, Japan). The developed pressure was calculated (systolic pressure – Diastolic pressure).

Measurement of fasting blood insulin

Insulin concentrations were measured in previously frozen and thawed serum samples by enzyme immunoassay using the rat insulin ELISA kits.

Measurement of fasting blood glucose

Blood glucose was assayed by the method adopted by Trinder (1969) [14]. The test materials for this method were supplied as kits by “diamond diagnostics”.

Semi-quantitation of AT2R and ACE2 gene expression by reverse transcriptase polymerase chain reaction (RT-PCR)

At the end of the perfusion, hearts were removed from the perfusion apparatus, excess water was absorbed on tissue paper. About 30 mg of heart tissue was homogenized in RNA lysis buffer containing β -mercaptoethanol then the tissue homogenate was centrifuged at 200 $\times g$ for 20 min, then the clear lysate was subjected to the following.

RNA extraction

1. The clear lysate solution was transferred to a microcentrifuge tube by pipetting
2. 200 μ l 95% ethanol was added to the cleared lysate, mixed by pipetting 3-4 times, this mixture was transferred to spin column assembly, centrifuged at 240-250 $\times g$ for 1 min
3. The spin basket was taken from the spin column assembly, the fluid was discarded in the collection tube, and the spin basket was put into the collection tube
4. 600 μ l of RNA wash solution was added to the spin column assembly, and then centrifuged at 250 $\times g$ for 1 min

5. The collection tube was empty and placed in the rack, DNase mix was prepared by mixing 40 μ l yellow core buffer, 5 μ l 0.09 Mol $MgCl_2$ and 5 μ l of DNase solution was kept on ice for 1 min then about 50 μ l of freshly prepared DNase solution was added directly to the membrane inside the spin basket, and incubated for 20 min at 20°C, after the incubation, 200 μ l of DNase stop solution was added to the spin basket and centrifuged at 250 $\times g$ for 2 min
6. 600 μ l RNA wash solution was added and centrifuged at 250 $\times g$ for 1 min
7. The spin basket was transferred from the collection tube to the elution tube, and 100 μ l nuclease free water was added to the membrane, centrifuged at 240 $\times g$ for 1 min, the spin basket was removed and discarded; the elution tube containing the purified RNA capped and stored at –70°C.

RT-PCR experiments

RT-PCR was done using the extracted RNA for detection of gene expression as follows: RNA was reverse transcribed using 12-15 μ l oligo (dT) 18 primer and was denaturated at 70°C for 2 min, the denaturated RNA was placed on ice for 5 min. 6.5 μ l of reverse transcription mixture was prepared containing: 50 mM Tris HCl pH 8.3, 50 mM KCl, 1-5 mM $MgCl_2$, 0.5 mM d NTPs, 1 unit/ml RNase inhibitor, 200 unit of moloney murine leukemic virus RT.

The mixture was added to the RNA and subjected to the following cycling condition needed for reverse transcription and synthesis of cDNA from RNA:

- 42°C for 1 h
- 95°C for 5 min to stop the reaction.

Then the PCR reaction was performed by adding PCR mix to a final volume of 100 μ l, the PCR mix contains: 10 m Mol/L Tris HCl pH 8.3, 50 m Mol KCl, 1.5 mMol $MgCl_2$, 0.001% gelatin, 250 μ Mol dNTPs mix, 2.5 unit Taq polymerase, 100 mMol of each primer

- Specific primer sequence for ACE2 (gene bank number NM 001012006):
5'CGCTGTCACCAGACAAGAA 3' (sense)
5'GCCATTATTTTCGTCCAATCC 3' (antisense)

The final PCR product was 139 bp in size.

- Specific primer sequence for AT2R (gene bank number NM 0124943):
5'GGA GCG AGC ACA GAA TTG AAA GC 3' (sense)
5'TGC CCA GAG AGG AAG GGT TGC C' (antisense)

The final PCR product was 445 bp in size.

- Specific primer sequence for b-actin (used as housekeeping gene) (gene bank accession number: NM 001014725.2)
5'-TTC-TACAATGAGCTGCGTGTGGC-3' (sense)
5'-AGAGGTCTTTACGGATGTCAACG-3'; (antisense)

The reaction mixture was then subjected to 40 cycles of:

- 95°C for 1 min
- 55°C for 1 min
- 72°C for 1 min.

After the last cycle, a final extension at 72°C for 1 min was done.

Agarose gel electrophoresis

All PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and visualized by ultra-violet transilluminator.

Gel documentation

The PCR products were semiquantitative using the gel documentation system (Bio Doc Analyze) supplied by Biometra, Germany using standard DNA with different concentration to make the curve from which concentration of PCR products was detected.

Statistical Analysis

The results were analyzed using SPSS computer software package, version 10.0 (Chicago, IL, USA). Data were presented as mean $\pm$ SD. Differences among the three groups were compared using one-way ANOVA. To study the relationship between the variables, Pearson's correlation coefficient was calculated. The results were considered statistically significant at $P < 0.05$.

RESULTS

As shown in Table 1, untreated diabetic rats (Group 2) showed significantly higher levels of fasting blood glucose, fasting insulin and HOMA-IR compared to non-diabetic control rats (Group 1). However, these levels decreased significantly in both protected groups; ARB (Group 3) and ACEI (Group 4) compared with untreated diabetic rats (Group 2) although the levels remained significantly higher than control rats (Group 1).

Moreover, there was no significant difference in these levels between the two protected groups (Group 3 and 4).

As shown in Table 2, the diabetic control group showed significantly higher levels of AT2R and ACE2 gene expression in cardiac tissue compared to non-diabetic control Group 1. Moreover, ACEIs protected rats (Group 4) showed significantly higher levels of ACE2 gene expression in cardiac tissue compared with diabetic control group, but there was no significant change in AT2R gene expression between ACEI protected rats (Group 4) and the unprotected diabetic control rats (Group 2).

Table 1: Levels of fasting blood glucose, fasting insulin and HOMA-IR test in the four studied groups

Calculated parameters	Group 1	Group 2	Group 3	Group 4	P
Glucose (mmol/L)	4.81 $\pm$ 0.51 ^a	12.14 $\pm$ 1.44 ^b	9.26 $\pm$ 1.26 ^c	9.50 $\pm$ 1.09 ^c	0.00
Insulin (uIU/ml)	10.58 $\pm$ 0.57 ^a	18.2 $\pm$ 2.76 ^b	15.47 $\pm$ 1.00 ^c	14.94 $\pm$ 2.26 ^c	0.00
HOMA	2.21 $\pm$ 0.21 ^a	9.79 $\pm$ 2.01 ^b	6.36 $\pm$ 1.18 ^c	6.22 $\pm$ 1.03 ^c	0.00

Groups bearing the same initials are not statistically significant from each other at $P < 0.05$. HOMA-IR: Homeostasis model assessment insulin resistance index

Most interestingly, ARB protected rats (Group 3), showed highly significant increase in the level of AT2R and ACE2 gene expression in cardiac tissue compared with control Group 1, unprotected diabetic group, and the other ACEI protected group.

As shown in Table 3, the unprotected diabetic rats (group 2) showed significantly lower levels of LV developed pressure (LVDP) (mmHg) and dp/dt (mmHg/s) compared to control Group 1 but there was no significant difference in the level of HR (b/min) between the 2 groups.

Regarding LVDP, both ARB and ACEIs protected rats (Group 3 and 4) showed significant improvement but still less than the control group. As regarding HR, the ARB protected diabetic rats Group 3 showed no significant change compared to the unprotected diabetic rats Group 2 and the control Group 1. However, the ACEIs protected diabetic rats (Group 4) showed significantly lower levels of HR compared with the control group and the unprotected diabetic rats (Group 2).

Regarding dp/dt, both ARBs and ACEIs protected diabetic rats (Group 3 and 4) showed significantly higher values compared with the unprotected diabetic rats group that even reached values of the control group.

As shown in Table 4, the unprotected diabetic rats (Group 2) showed a significant decrease in the level of percentage recovery of the LV performance following ischemia-reperfusion (developed pressure (mmHg), contractility index dp/dt (mmHg/s) and HR (b/min) compared with control Group 1.

Regarding the percentage recovery of LVDP, both protected diabetic groups, ARB protected (Group 3) and ACEI protected (Group 4) showed significant improvement in the percentage recovery of LVDP compared with the untreated diabetic hearts with values reaching the control group.

Regarding the percentage recovery of HR, The ARB protected diabetic rats (Group 3) showed a significant increase in the level of percentage recovery of HR compared to the control Group 1, unprotected diabetic rats (Group 2) and to the ACEI protected groups (Group 4).

Regarding the percentage recovery of dp/dt, both protected diabetic groups (ARB-protected and ACEI-protected) showed significantly higher levels of percentage recovery of dp/dt following ischemia-reperfusion compared to unprotected diabetic rats (Group 2).

Table 2: Levels of AT2R and ACE2 gene expression in cardiac tissue in the four studied groups

Calculated parameters	Group 1	Group 2	Group 3	Group 4	P
AT2R mg/ml	0.18 $\pm$ 0.04 ^a	0.508 $\pm$ 0.06 ^b	1.69 $\pm$ 0.35 ^c	0.63 $\pm$ 0.03 ^{bd}	0.00
ACE2 mg/ml	0.108 $\pm$ 0.031 ^a	0.36 $\pm$ 0.085 ^b	1.26 $\pm$ 0.37 ^c	0.70 $\pm$ 0.11 ^d	0.00

Groups bearing the same initials are not statistically significant from each other at $P < 0.05$. AT2R: Angiotensin-II Type 2 receptors, ACE2: Angiotensin converting enzyme 2

Table 3: Left ventricular performance (developed pressure, contractility index (dp/dt) and HR) during the initial 30 min of perfusion in the four studied groups

Calculated parameters	Group 1	Group 2	Group 3	Group 4	P
LVDP mmHg	114.56±9.47 ^a	58.44±5.43 ^b	98.33±8.64 ^c	99.78±5.38 ^c	0.00
HR b/min	248.78±109.11 ^a	224.00±46.56 ^a	178.89±24.97 ^{ab}	134.00±36.67 ^b	0.00
dp/dt mmHg/s	116.44±7.126 ^a	65.78±4.969 ^b	106.78±4.944 ^{ac}	113.78±9.378 ^a	0.00

Groups bearing the same initials are not statistically significant from each other at $P < 0.05$. LVDP: Left ventricular developed pressure, HR: Heart rate

Table 4: The percentage recovery of the left ventricle performance following ischemia-reperfusion (developed pressure, contractility index dp/dt and HR) in the four studied groups

Calculated parameters	Group 1	Group 2	Group 3	Group 4	P
LVDP mmHg	78.144±1.93 ^{ab}	63.456±4.39 ^c	81.178±1.62 ^a	79.556±1.69 ^{ab}	0.00
HR b/min	77.100±1.90 ^a	65.98±7.14 ^b	86.74±4.02 ^c	80.52±3.49 ^a	0.00
Dp/dt mmHg/s	77.256±2.83 ^a	71.55±4.14 ^b	86.00±2.88 ^c	80.67±1.44 ^a	0.00

Groups bearing the same initials are not statistically significant from each other at $P < 0.05$. LVDP: Left ventricular developed pressure, HR: Heart rate

Interestingly, the improvement in percentage recovery of myocardial performance offered by AT1R blocker, reached levels higher than that of control non-diabetic rats (Group 1).

Correlations between Studied Parameters [Table 5]

When Pearson's correlation was performed in studied groups, significant positive correlation was found between the level of AT2R expression in cardiac tissue and the percentage recovery of the LV performance following ischemia-reperfusion "LVDP ($r = 0.412^{**}$), HR ($r = 0.546^{**}$) and contractility index dp/dt ($r = 0.610^{**}$) and also, there was significant positive correlation between the level of ACE2 expression in cardiac tissue and the percentage recovery of the LV performance following ischemia-reperfusion "LVDP ($r = 0.465^{**}$) and contractility index dp/dt ($r = 0.603^{**}$) and the percentage recovery the HR ($r = 0.624^{**}$). However, there was no significant correlation between the level of AT2R and ACE2 expression in cardiac tissue.

DISCUSSION

The identification of the local cardiac RAS, ACE2 as an angiotensin peptide processing enzyme, Mas as a receptor for Ang (1-7) and the possibility of Ang II signaling through AT1R and AT2R, have contributed to switch our understanding of the RAS from the classical limited proteolysis linear cascade to a cascade with multiple mediators, multiple receptors, and multi-functional enzymes.

Although most studies have addressed RAS over-activation as a consequence of diabetes, there is also research supporting a hypothesis implicating RAS over-activation as a cause of the disease and its cardiovascular complications.

In an attempt to clarify the role played by the RAS in the development of diabetes, the present study confirmed the protective effect of inhibition of the RAS with either ACEIs or ARBs on the development of Type 2 DM following HFD + STZ injection. As shown in Table 1, rats in Group 3 (ARB protected group) and Group 4 (ACEI protected group), showed

Table 5: Correlation between the level of AT2R and ACE2 expression in cardiac tissue with the percentage recovery of the left ventricle performance following ischemia-reperfusion (developed pressure, contractility index dp/dt and HR)

	AT2	ACE2
LVDP % recovery		
Pearson correlation	0.412 ^{**}	0.465 ^{**}
Significant (2-tailed)	0.001	0.000
N	63	63
HR % recovery		
Pearson correlation	0.546 ^{**}	0.624 ^{**}
Significant (2-tailed)	0.000	0.000
N	63	63
Dp/dt % recovery		
Pearson correlation	0.610 ^{**}	0.603 ^{**}
Significant (2-tailed)	0.000	0.000
N	63	63

^{**}Correlation is significant at the 0.05 level (2-tailed).

AT2R: Angiotensin-II Type 2 receptors, ACE2: Angiotensin converting enzyme 2, LVDP: Left ventricular developed pressure, HR: Heart rate

significantly lower values of glucose, insulin and HOMA-IR compared to diabetic rats (Group 2). However, values did not return to control values.

Our results are consistent with several clinical trials showing that RAS agents modify the natural course of diabetes itself. In this context, it was recently found that the incidence of Type 2 DM in patients with hypertension or congestive heart failure is significantly reduced by the inhibition of the RAS with either ACEIs or AT1R blockers [15,16].

In spite of firm clinical data, the mechanisms underlying protective effect of RAS blockade appear to be complex and have yet to be solved.

Initial reports demonstrated that inhibition of AT1R prevents decline of glucose transporter-4 (GLUT-4) in diabetic rat heart, and further studies reported that chronic administration of ACEIs or AT1R antagonists to insulin-resistant rodents can increase protein expression of GLUT-4 in skeletal muscle

and myocardium. ACEIs increase GLUT-4 concentration/translocation and activate hexokinase, one of the major enzymes of glucose pathway. These changes are probably secondary to activation of the PI3-kinase signaling pathway by enhancing tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and improvement of PI3-kinase-IRS-1 [17,18].

Recently, attention has been focused on the role of ARBs in the preservation of pancreatic β -cell function resulting in significant reductions in reactive O₂ species, protein kinase C (PKC) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activities in pancreatic cell islets leading to enhanced β -cell survival [16].

It has been postulated that the beneficial effects observed with ARBs and ACEIs are mediated through the newly discovered member of RAS; Ang (1-7), binding to the G-protein-coupled Mas receptor [19], known to inhibit Ang II responses [20]. Mas knockout (Mas^{-/-}) mice exhibit many common features of metabolic syndrome [21]. Mas^{-/-} mice also exhibit impaired insulin sensitivity and glucose tolerance, reduced adipose glucose uptake and impaired GLUT4 expression. This study clearly demonstrates that loss of the Mas receptor leads to metabolic dysfunction and implicates the ACE2/Ang (1-7)/Mas receptor axis in the development of insulin resistance.

Chronic diabetes alters the structure and function of the human heart, and individuals with DM usually develop a specific cardiac dysfunction known as diabetic cardiomyopathy. Several mechanisms involved in the development of cardiomyopathy have been postulated, including alterations in Ca⁺⁺ signaling pathways and glucose metabolism, enhanced oxidative stress and stimulation of local cardiac RAS [22].

In the present study, we examined the effect of induction of diabetes on cardiac performance of non-diabetic and diabetic rats before and after ischemia reperfusion injury. Table 3 shows that in the unprotected diabetic heart (Group 2 rats) there was a significant decrease in the LV performance and contractility index compared to non-diabetic hearts. On the other hand, Table 4 shows that the percentage recovery in diabetic rats was significantly lower than non-diabetic hearts.

Several mechanisms have been proposed to explain how all of the pathologies involved in the progression of diabetic cardiac defects might result from hyperglycemia. It was recently reported that these defects may result partially from altered local Ca⁺⁺ signaling due to a dysfunction of cardiac protein kinase A mediated ryanodine receptor Ca⁺⁺ release channel. Increased PKC isoform expression and increased polyol pathway flux are two main hypotheses presented to describe how hyperglycemia might cause all of the diabetic complications [23].

Furthermore, it has been demonstrated that hyperglycemia activates the local cardiac RAS and enhances Ang II activity in diabetes [24]. Ang II may have direct electrophysiological effects and could act as an endogenous arrhythmogenic agent [25], with mechanisms involving enhanced free radical formation,

as well as release of sarcoplasmic Ca⁺⁺ in cardiac myocytes via activation of a phosphatidylinositol response [26].

To get further insight on the potential role of RAS activation in diabetic heart dysfunction, the present study also examined the possible protective effects of RAS blockade by ARBs and ACEIs on cardiac performance in diabetic hearts before and after ischemia-reperfusion injury.

As shown in Table 3, RAS blockade by ARBs and ACEIs improved LV performance significantly (but not to control levels), while the contractility index improved to control values in both groups of rats pre-treated with ARBs and ACEIs. Moreover, Table 4 shows significantly higher values in percentage recovery of contractility index in ARBs-protected group compared to non-diabetic and unprotected diabetic hearts.

The present results are consistent with other experimental and clinical studies which demonstrated that myocardial ischemia induces activation of various components of the RAS in the acute phase of myocardial infarction (MI) and the post-infarction remodeling process [27]. Pharmacological inhibition of the RAS by administration of renin inhibitors, ACEIs, and ARBs has shown beneficial effects on the pathological processes of MI in both experimental animal studies and clinical trials [28].

However, the potential mechanisms responsible for the cardioprotective effect of RAS inhibition remain unclear.

One proposed mechanism is that ATR density and mRNA expression are elevated in the diabetic heart [29]. Blockade of the RAS in STZ-treated rats attenuated cardiac dysfunction partially through restoration of sarcoplasmic Ca⁺⁺ handling. In a parallel study, blockade of the RAS reversed diabetes-induced Ca⁺⁺ loading of the SR and depletion of ryanodine receptors [30].

Most of the cardiovascular effects of Ang II are thought to be mediated through the AT1R. The AT1R has been shown to be coupled to several post-receptor signaling pathways, including NADPH oxidase, generating O₂ free radicals, which have detrimental effects on the CVS [31].

Selective blockade of AT1R with ARBs results in an elevation of the levels of circulating Ang II, which can then stimulate the unopposed AT2R [32]. Thus, it is hypothesized that the beneficial effects of ARBs may be mediated, at least in part, through AT2R activation. However, the exact role of AT2R is still an open question.

The level of AT2R in the adult is relatively low and limited to certain organs; however, the AT2R is re-expressed and/or upregulated under pathophysiological conditions such as in patients with ischemic heart disease, heart failure, dilated cardiomyopathy and stroke [33].

Furthermore, blocking the formation and action of Ang II by ACEIs and ARBs may have beneficial therapeutic outcome

in the treatment of CVD through accelerated processing of ACE2-Ang(1-7)-Mas axis. It has been recently demonstrated that overexpression of Ang (1-7) can attenuate ischemic induced cardiac pathophysiology [34].

To test the various hypotheses on the mechanism of action of RAS inhibitors in diabetic heart protection, the levels of AT2R expression and ACE2 expression in cardiac tissue were evaluated in our present study. Interestingly, the unprotected diabetic control rats showed significantly higher levels of AT2R and ACE2 gene expression in cardiac tissue (compared to those of non-diabetic rats).

These results are consistent with several observations and experimental evidence showing elevated AT2R and ACE2 expression at the initial stages of several pathologies including DM, which decline with progression of the disease. This might indicate a protective role for both AT2R and ACE2 [35].

Interestingly, ARB protected rats, showed significantly higher level of AT2R and ACE2 gene expression in cardiac tissue than that of control Group 1, unprotected diabetic group, and the other ACEI protected group.

ARBs are known to antagonize the effect of Ang II by blocking AT1R and also by upregulating the ACE2 expression, thereby enhancing Ang 1-7 actions. Our results confirm that both AT1R blockade and overexpression of ACE2 resulted in marked recovery of myocardial dysfunction after ischemia-reperfusion injury.

Jugdutt and Menon (2004) [36] reported that ARBs significantly limited the increase in left atrial pressure, promptly limited the deterioration of LV dp/dt_{max} , dp/dt_{min} , ejection fraction and diastolic function, limited infarct expansion and thinning, and limited infarct size in the dog model of ischemia-reperfusion injury. In this study, both ARBs increased AT2R protein in the post-ischemic-reperfused zone, with no change in AT1R protein.

Le Corvoisier *et al.* (2010) [37] observed that the treatment with either ARBs or ACEIs significantly increased AT2R density in the LV of high-salt diet mice.

Dong *et al.* (2012) [38] reported that the overexpression of AT2R exhibit, improved baseline LV systolic function, as well as preservation of systolic function after MI.

Qi *et al.* (2012) [39] demonstrated that moderate cardiac-selective overexpression of AT2R protects cardiac function and attenuates cardiac remodeling post MI. These beneficial effects involve restoration of the RAS balance and prevention of the upregulation of fibrotic factors (Collagen I and Collagen III).

Taken together, these results suggest that AT2R is upregulated in the tissues following injury or insult to somehow reduce and/or repair the injured tissue. It is conceivable that the endogenous upregulation of AT2R may not be adequate to exert any significant beneficial effects, or may only be transient in nature and thus not able to restore normal function. There are

several reports suggesting that AT2R protects cardiac functions, or mediates part of the protective effects of AT1R antagonists [40,41].

However, there are opposing studies that do not support a cardioprotective effect for AT2R overexpression. Chronic overexpression of AT2R has been reported to depress myocardial contractility in transgenic mice. Other investigators have reported that transgenic overexpression of AT2R in cardiomyocytes *in vivo* results in enhanced hypertrophy and dilated cardiomyopathy [42].

Cardiac overexpression of ACE2 or Ang (1-7) mediated by Lenti-viral vector preserved cardiac functions and attenuated LV wall thinning following MI [43]. Whereas, chronic Ang (1-7) treatment not only attenuated the development of heart failure in the MI model, but also prevented cardiac hypertrophy and fibrosis in rats [44].

Although it is now generally accepted that ACE2 plays a role in cardiac remodeling, the exact means by which ACE2 activity affords cardioprotection are unclear. Potential mechanisms include increased Ang II degradation and increased formation of Ang (1-7) which activates the G protein-coupled Mas receptor. Mas receptor activation by Ang (1-7) opposes many of the AT1R-mediated actions (vasoconstriction, hypertrophy, fibrosis), thereby improving cardiac function and remodeling and attenuating heart failure. Mas receptors are also localized to cardiac myocytes and activate NO production [45].

There are several literature reports that suggest the concept that AT2R exerts its protective effects on the heart post MI through interacting with ACE2-Ang (1-7)-Mas axis [46]. It has also been demonstrated that ACE2 activity or Ang (1-7) forming activity directly correlated with AT2R density [47]. In addition, ARB (irbesartan) or ACEIs (ramipril) treatment significantly increased AT2R density in the LV of high-salt diet mice, and the ACEIs induced change was more pronounced than that of the ARB.

It has also been suggested that Ang (1-7) may act through both AT2R and Mas receptors, as both AT2R antagonist, and Mas receptor antagonist abrogated the Ang (1-7)-evoked vasoprotection and atheroprotection and the reciprocal changes in eNOS and superoxide in a model of atherosclerosis [48]. Thus, it is conceivable that Ang (1-7) may be a ligand for the AT2R.

The cross talk between ACE2 and AT2R could not be confirmed in our study as no correlation was found between gene expression of the AT2R and ACE2 in cardiac tissue of all studied groups. Further experimentation would be required to test this hypothesis.

Based on the above, therapeutic approach that will amplify the AT2R in addition to the ACE2-Ang (1-7) axis could provide further protection against the development of diabetic CVD. It turns out that the merits of currently used drugs – ARBs, ACEIs – lay beyond their direct effects on suppression of the

ACE-Ang-AT1R axis as they also increase cardiac AT2R, ACE2 and Ang (1-7) significantly, and shed light on the usually dark side of RAS.

REFERENCES

- Douglas G, Bendall JK, Crabtree MJ, Tatham AL, Carter EE, Hale AB, et al. Endothelial-specific Nox2 overexpression increases vascular superoxide and macrophage recruitment in ApoE^{-/-} mice. *Cardiovasc Res* 2012;94:20-9.
- Tziakas DN, Chalikias GK, Kaski JC. Epidemiology of the diabetic heart. *Coron Artery Dis* 2005;16 Suppl 1:S3-10.
- Peti-Peterdi J, Kang JJ, Toma I. Activation of the renal renin-angiotensin system in diabetes – new concepts. *Nephrol Dial Transplant* 2008;23:3047-9.
- Danser AH, Saris JJ, Schuijt MP, van Kats JP. Is there a local renin-angiotensin system in the heart? *Cardiovasc Res* 1999;44:252-65.
- Carey RM, Siragy HM. Newly recognized components of the renin-angiotensin system: Potential roles in cardiovascular and renal regulation. *Endocr Rev* 2003;24:261-71.
- Kaschina E, Grzesiak A, Li J, Foryst-Ludwig A, Timm M, Rompe F, et al. Angiotensin II type 2 receptor stimulation: A novel option of therapeutic interference with the renin-angiotensin system in myocardial infarction? *Circulation* 2008;118:2523-32.
- Ma TK, Kam KK, Yan BP, Lam YY. Renin-angiotensin-aldosterone system blockade for cardiovascular diseases: Current status. *Br J Pharmacol* 2010;160:1273-92.
- Verma A, Shan Z, Lei B, Yuan L, Liu X, Nakagawa T, et al. ACE2 and Ang-(1-7) confer protection against development of diabetic retinopathy. *Mol Ther* 2012;20:28-36.
- Hunley TE, Tamura M, Stoneking BJ, Nishimura H, Ichiki T, Inagami T, et al. The angiotensin type II receptor tonically inhibits angiotensin-converting enzyme in AT2 null mutant mice. *Kidney Int* 2000;57:570-7.
- Okumura M, Iwai M, Ide A, Mogi M, Ito M, Horiuchi M. Sex difference in vascular injury and the vasoprotective effect of valsartan are related to differential AT2 receptor expression. *Hypertension* 2005;46:577-83.
- Srinivasan K, Viswanad B, Asrat L, Kaul CL, Ramarao P. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: A model for type 2 diabetes and pharmacological screening. *Pharmacol Res* 2005;52:313-20.
- Ferrario CM, Jessup J, Chappell MC, Averill DB, Brosnihan KB, Tallant EA, et al. Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. *Circulation* 2005;111:2605-10.
- Skrzypiec-Spring M, Grotthus B, Szelag A, Schulz R. Isolated heart perfusion according to Langendorff - still viable in the new millennium. *J Pharmacol Toxicol Methods* 2007;55:113-26.
- Trinder P. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. *J Clin Pathol* 1969;22:158-61.
- Elliott WJ, Meyer PM. Incident diabetes in clinical trials of antihypertensive drugs: A network meta-analysis. *Lancet* 2007;369:201-7.
- Lardizabal JA, Deedwania PC. The role of renin-angiotensin agents in altering the natural history of type 2 diabetes mellitus. *Curr Cardiol Rep* 2010;12:464-71.
- Henriksen EJ, Jacob S, Kinnick TR, Teachey MK, Krekler M. Selective angiotensin II receptor antagonism reduces insulin resistance in obese Zucker rats. *Hypertension* 2001;38:884-90.
- Csibi A, Communi D, Müller N, Bottari SP. Angiotensin II inhibits insulin-stimulated GLUT4 translocation and Akt activation through tyrosine nitration-dependent mechanisms. *PLoS One* 2010;5:e10070.
- Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, et al. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A* 2003;100:8258-63.
- Sampaio WO, Souza dos Santos RA, Faria-Silva R, da Mata Machado LT, Schiffrin EL, Touyz RM. Angiotensin-(1-7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension* 2007;49:185-92.
- Santos SH, Fernandes LR, Mario EG, Ferreira AV, Pôrto LC, Alvarez-Leite JI, et al. Mas deficiency in FVB/N mice produces marked changes in lipid and glycemic metabolism. *Diabetes* 2008;57:340-7.
- Yaras N, Bilginoglu A, Vassort G, Turan B. Restoration of diabetes-induced abnormal local Ca²⁺ release in cardiomyocytes by angiotensin II receptor blockade. *Am J Physiol Heart Circ Physiol* 2007;292:H912-20.
- Ozdemir S, Ugur M, Gürdal H, Turan B. Treatment with AT(1) receptor blocker restores diabetes-induced alterations in intracellular Ca(2+) transients and contractile function of rat myocardium. *Arch Biochem Biophys* 2005;435:166-74.
- Giacchetti G, Sechi LA, Rilli S, Carey RM. The renin-angiotensin-aldosterone system, glucose metabolism and diabetes. *Trends Endocrinol Metab* 2005;16:120-6.
- Goette A, Lendeckel U. Electrophysiological effects of angiotensin II. Part I: Signal transduction and basic electrophysiological mechanisms. *Eurpace* 2008;10:238-41.
- Sonoyama K, Igawa O, Miake J, Yamamoto Y, Sugihara S, Sasaki N, et al. Effects of angiotensin II on the action potential durations of atrial myocytes in hypertensive rats. *Hypertens Res* 2005;28:173-9.
- Dai W, Kloner RA. Potential role of renin-angiotensin system blockade for preventing myocardial ischemia/reperfusion injury and remodeling after myocardial infarction. *Postgrad Med* 2011;123:49-55.
- D'Amore A, Black MJ, Thomas WG. The angiotensin II type 2 receptor causes constitutive growth of cardiomyocytes and does not antagonize angiotensin II type 1 receptor-mediated hypertrophy. *Hypertension* 2005;46:1347-54.
- Fang ZY, Prins JB, Marwick TH. Diabetic cardiomyopathy: Evidence, mechanisms, and therapeutic implications. *Endocr Rev* 2004;25:543-67.
- Liu SF, Malik AB. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L622-45.
- Privratsky JR, Wold LE, Sowers JR, Quinn MT, Ren J. AT1 blockade prevents glucose-induced cardiac dysfunction in ventricular myocytes: Role of the AT1 receptor and NADPH oxidase. *Hypertension* 2003;42:206-12.
- Matsubara H. Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. *Circ Res* 1998;83:1182-91.
- Zhu YZ, Chimon GN, Zhu YC, Lu Q, Li B, Hu HZ, et al. Expression of angiotensin II AT2 receptor in the acute phase of stroke in rats. *Neuroreport* 2000;11:1191-4.
- Qi Y, Shenoy V, Wong F, Li H, Afzal A, Mocco J, et al. Lentivirus-mediated overexpression of angiotensin-(1-7) attenuated ischaemia-induced cardiac pathophysiology. *Exp Physiol* 2011;96:863-74.
- Bogacka I, Xie H, Bray GA, Smith SR. The effect of pioglitazone on peroxisome proliferator-activated receptor-gamma target genes related to lipid storage *in vivo*. *Diabetes Care* 2004;27:1660-7.
- Jugdutt BI, Menon V. Upregulation of angiotensin II type 2 receptor and limitation of myocardial stunning by angiotensin II type 1 receptor blockers during reperfused myocardial infarction in the rat. *J Cardiovasc Pharmacol Ther* 2003;8:217-26.
- Le Corvoisier P, Adamy C, Sambin L, Crozatier B, Berdeaux A, Michel JB, et al. The cardiac renin-angiotensin system is responsible for high-salt diet-induced left ventricular hypertrophy in mice. *Eur J Heart Fail* 2010;12:1171-8.
- Dong B, Yu QT, Dai HY, Gao YY, Zhou ZL, Zhang L, et al. Angiotensin-converting enzyme-2 overexpression improves left ventricular remodeling and function in a rat model of diabetic cardiomyopathy. *J Am Coll Cardiol* 2012;59:739-47.
- Qi Y, Li H, Shenoy V, Li Q, Wong F, Zhang L, et al. Moderate cardiac-selective overexpression of angiotensin II type 2 receptor protects cardiac functions from ischaemic injury. *Exp Physiol* 2012;97:89-101.
- Oishi Y, Ozono R, Yano Y, Teranishi Y, Akishita M, Horiuchi M, et al. Cardioprotective role of AT2 receptor in postinfarction left ventricular remodeling. *Hypertension* 2003;41:814-8.
- Oishi Y, Ozono R, Yoshizumi M, Akishita M, Horiuchi M, Oshima T. AT2 receptor mediates the cardioprotective effects of AT1 receptor antagonist in post-myocardial infarction remodeling. *Life Sci* 2006;80:82-8.
- Nakayama M, Yan X, Price RL, Borg TK, Ito K, Sanbe A, et al. Chronic ventricular myocyte-specific overexpression of angiotensin II type 2 receptor results in intrinsic myocyte contractile dysfunction. *Am J Physiol Heart Circ Physiol* 2005;288:H317-27.
- Grobe JL, Mecca AP, Lingis M, Shenoy V, Bolton TA, Machado JM,

- et al.* Prevention of angiotensin II-induced cardiac remodeling by angiotensin-(1-7). *Am J Physiol Heart Circ Physiol* 2007;292:H736-42.
44. Wang LJ, He JG, Ma H, Cai YM, Liao XX, Zeng WT, *et al.* Chronic administration of angiotensin-(1-7) attenuates pressure-overload left ventricular hypertrophy and fibrosis in rats. *Di Yi Jun Yi Da Xue Xue Bao* 2005;25:481-7.
 45. Stewart JA Jr, Lazartigues E, Lucchesi PA. The angiotensin converting enzyme 2/Ang-(1-7) axis in the heart: A role for MAS communication? *Circ Res* 2008;103:1197-9.
 46. Chang SY, Chen YW, Chenier I, Tran Sle M, Zhang SL. Angiotensin II type II receptor deficiency accelerates the development of nephropathy in type I diabetes via oxidative stress and ACE2. *Exp Diabetes Res* 2011;2011:521076.
 47. Zisman LS, Keller RS, Weaver B, Lin Q, Speth R, Bristow MR, *et al.* Increased angiotensin-(1-7)-forming activity in failing human heart ventricles: Evidence for upregulation of the angiotensin-converting enzyme Homologue ACE2. *Circulation* 2003;108:1707-12.
 48. Tesanovic S, Vinh A, Gaspari TA, Casley D, Widdop RE. Vasoprotective and atheroprotective effects of angiotensin (1-7) in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2010;30:1606-13.

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