

# Angiotensin II Receptor Blockage Prevents Diabetes-Induced Oxidative Damage in Rat Heart

(oxidative stress / renin-angiotensin system / type 1 diabetes / antioxidant enzymes / heart)

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**Abstract.** Current findings suggest a role for the angiotensin II (Ang II) signalling pathway in generation of reactive oxygen species and diabetes-induced cardiac complications. In this study we aimed to investigate the effect of angiotensin II type 1 (AT<sub>1</sub>) receptor blockage on some antioxidant enzymes such as glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), glutathione reductase (GR), glutathione-S-transferase (GST), glutathione peroxidase (GSH-Px), and catalase (CAT) in the heart of streptozotocin (STZ)-induced diabetic rats. The effect of AT<sub>1</sub> receptor blocker, candesartan-cilexetil (5 mg/kg/day for 4 weeks) was studied. Diabetes caused hyperglycaemia (4-fold of control) with significant increases in G6PD, 6PGD, GR, GSH-Px, CAT and no effect on GST in heart tissues as compared to normal control rats. Treatment of STZ-induced diabetic rats with candesartan-cilexetil had significant beneficial effects on these parameters without any side effect on control rats. These results suggest that Ang II can take part in induction of oxidative stress in diabetic rat heart and that blockage of its activity by AT<sub>1</sub> receptor blocker is potentially protective against diabetes-induced cellular damage.

## Introduction

Diabetes mellitus is a metabolic disorder that can manifest itself by cellular and functional abnormalities. Since heart is one of the most important target tissues of diabetes, the increased rate of mortality and morbidity of these patients has been attributed to diabetic cardiomyopathy (Fein and Sonnenblick, 1985; Flack et al., 1998). Furthermore, growing evidences showing impaired diastolic performance followed by depressed systolic function in diabetic patients support this hypothesis.

Increased production of reactive oxygen species (ROS) as well as nitrogen species is a well-known phenomenon in hyperglycaemic conditions. Although the aetiology of the hyperglycaemia-induced cellular damage has not been clarified yet, free radicals and oxidative stress are supposed to be the contributory factors underlying these abnormalities. The autooxidation of glucose, the formation of glycation end-products and the activation of NADPH oxidase have been suggested as possible sources of the augmented oxidative stress in diabetes. Thus, elevated free radical levels activate various subcellular signal transduction pathways including abnormal gene expression, which may cause myocardial cell death. Related with these suggestions, increased production of thiobarbituric acid-reactive substances (TBARS), which is an index of oxidative stress and lipid peroxidation, was reported in diabetic hearts (Kakkar et al., 1995; 1996; Babu et al., 2006; Tsutsui et al., 2007). The antioxidant enzymes (GSH-Px, GR, SOD, CAT) respond to the increased stress as a protective mechanism of the target tissue. In most of the studies, SOD, CAT, GSH-Px activities and/or levels were shown to be changed (Kakkar et al., 1995; 1996; Gumieniczek et al., 2002; Aksoy et al., 2003; Bukan et al., 2004; Okutan et al., 2005; Shirpoor et al., 2008) in diabetic hearts. However, there are still some controversies in these published results. While Kakkar et al. (Kakkar et al., 1995; 1996) have shown an increase in SOD, CAT and GSH-Px activities, unchanged GSH-Px (Okutan et al., 2005) and decreased

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Abbreviations: 6PGD – 6-phosphogluconate dehydrogenase, Ang II – angiotensin II, AT<sub>1</sub> – angiotensin II type 1, CAT – catalase, CDNB – chloro-dinitro benzene, G6PD – glucose-6-phosphate dehydrogenase, GR – glutathione reductase, GSH-Px – glutathione peroxidase, GSSG – oxidized glutathione, GST – glutathione-S-transferase, ROS – reactive oxygen species, STZ – streptozotocin, TBARS – thiobarbituric acid-reactive substances.

SOD (Bukan et al., 2004) activities were also reported. As well as their activities the levels of GSH, GSH-Px and SOD increase in diabetic rat hearts (Aksoy et al., 2003). The current findings of GR activity still remain to be conflicting in hyperglycaemic heart (Yadav et al., 1997; Strother et al., 2001; Gumieniczek et al., 2002; Kamuren et al., 2006). In addition, Yadav et al. (1997) reported an elevated G6PD level, which may result in an increase in NADPH production required for GR activity, and thus GSH production.

In the last decade, evidences indicating up-regulation of the cardiac renin-angiotensin system (RAS) in diabetic heart have increased (Sechi et al., 1994; Malhotra et al., 1997; Fiordaliso et al., 2000). Ang II is also suggested to increase the production of free oxygen radicals (Rajagopalan et al., 1996; Laursen et al., 1997; Berry et al., 2000; Griendling and Ushio-Fukai, 2000; Tsutsui et al., 2007) and to activate NAD(P)H-oxidase enzymes that may generate superoxide anions in various tissues including heart via AT<sub>1</sub> receptor stimulation (Rajagopalan et al., 1996; Griendling and Ushio-Fukai, 2000; Bendall et al., 2002; Lu et al., 2004; Rahman et al., 2004). Recently, Sugawara et al. (2005) showed that candesartan, an AT<sub>1</sub> receptor blocker, could increase the survival rate of the hippocampal neurons after global cerebral ischaemia via diminution of superoxide production. Fiordaliso et al. (2000) also observed an elevated myocyte death associated with up-regulation of RAS in diabetic heart. However, AT<sub>1</sub> receptor blockage suppressed this over-activation of RAS and decreased the loss of myocytes.

Thus, current findings suggest a role for the Ang II signalling pathway in generation of ROS and diabetes-induced cardiac complications. Recently, we have investigated the recovery effects of an Ang II receptor blocker, candesartan-cilexetil (5 mg/kg/day for 4 weeks) on the mechanical and electrophysiological properties of cardiomyocytes isolated from streptozotocin (STZ)-induced diabetic rats (Ozdemir et al., 2005). Despite that the recovery of parameters was significant by candesartan treatment, the underlying mechanism could not be clarified satisfactorily. The increased free radical generation because of up-regulated Ang II via diabetes could be one of the possible mechanisms. Related with this hypothesis, the antioxidant capacity of the tissues is important for determination of the oxidative status of diabetic heart and its relation to the Ang II signalling pathway. Therefore, in this study we investigated the role of AT<sub>1</sub> receptor blockage on antioxidant enzyme activities (G6PD, 6PGD, GR, GST, GSH-Px, and CAT) of the diabetic heart. We observed that candesartan-cilexetil could normalize the increased antioxidant enzyme activities of the heart in diabetic animals. Thus, we can conclude that Ang II may take part in generation of free radicals in diabetic hearts, and suppression of its activity by AT<sub>1</sub> receptor blocker may also decrease the oxidative stress-induced cellular damage.

## Material and Methods

### *Experimental groups*

Wistar rats of both sexes were used in this study (200–250 g body weight). Candesartan-cilexetil, an AT<sub>1</sub> receptor blocker was used as a homogenous suspension in distilled water. The rats were divided into four groups as: (a) control rats (Con); normal animals treated with vehicle, (b) candesartan-treated control rats (ConCan); normal rats treated intragastrically with candesartan-cilexetil (5 mg/kg body weight/day), (c) diabetic rats (DM); diabetic animals given vehicle, and (d) candesartan-cilexetil-treated diabetic rats (DMCan); diabetic rats treated with candesartan-cilexetil (5 mg/kg body weight/day). Diabetes was induced by a single intraperitoneal (ip) injection of streptozotocin (STZ, 50 mg/kg body weight and dissolved in 0.1 M citrate buffer, pH 4.5), while control rats received citrate buffer alone. A week following STZ injection, blood glucose levels were measured and rats with blood glucose at least threefold of the pre-injection levels were used as diabetics in our experiments. Diabetic rats were randomly separated into candesartan-cilexetil-treated and untreated groups. Treatment of all animals continued for four weeks. Candesartan-cilexetil and vehicle application to control and candesartan-cilexetil-treated control groups was done in a similar way. All rats had free access to standard rat chow and water. At the end of fifth week, the rats were anaesthetized with pentobarbital sodium (30 mg/kg body weight, ip) and hearts were excised rapidly for further experiments.

### *Tissue preparation for enzymatic assays*

All animals were sacrificed by decapitation. The hearts were removed and after washing with ice-cold sterile physiological saline solution, tissue samples were placed in liquid nitrogen, where they remained until the procedures for determining enzyme activities. Each sample was then homogenized with an ultra turax homogenizer with S18N-10G probe for approximately 3 min with 3 volumes of 50 mM potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 105,000 × g for 60 min at 4 °C in a Beckman ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA), and supernatants were used for the measurement of enzyme activities.

*a) G6PD Assay:* Enzyme activities were determined spectrophotometrically using an LKB Ultraspec Plus (4054 UV/visible) spectrophotometer, by monitoring the NADPH production at 340 nm and at 37 °C (Betke et al., 1967). The 500 µl assay mixture contained 50 µl of 2 mmol/l NADP<sup>+</sup> and 50 µl of 6 mmol/l G6P, 100 µl of 100 mmol/l Tris/HCl buffer, pH 8.0 (containing 10 mM MgCl<sub>2</sub>), 280 µl distilled water and 20 µl supernatant. Assays were carried out in duplicate and the activities were followed for 40 s. The reaction was linear during this period.

One unit (U) of activity is the amount of enzyme required to reduce one µmol of NADP<sup>+</sup> per min under

the assay conditions. Specific activity was defined as units per mg of protein.

- b) *6PGD Assay*: Activities were measured by substituting 50  $\mu$ l 0.6 mM 6-phosphogluconate as substrate in the assay mixture given above for glucose-6-phosphate dehydrogenase measurement (Pearse and Rosemeyer, 1975).
- c) *GR Assay*: The activity of this enzyme was measured according to the modified Staal method (Acan and Tezcan, 1989). The 500  $\mu$ l incubation mixture contained 250  $\mu$ l of 0.2 mol/l sodium phosphate buffer, pH 7.4; 50  $\mu$ l of 10 mmol/l GSSG; 50  $\mu$ l of 2 mmol/l NADPH, 10  $\mu$ l supernatant and 140  $\mu$ l distilled water. A decrease in the absorbance of NADPH at 340 nm was monitored spectrophotometrically at 37 °C. A unit of activity (U) was defined as the amount of enzyme that catalyses the oxidation of 1 micromol of NADPH in 1 min under these conditions.
- d) *GST Assay*: Enzyme activity was assayed by measuring the conjugation of reduced glutathione with 1-chloro-2,4-dinitrobenzene, as described by Habig and et al. (1974). The 500  $\mu$ l incubation mixture contained 250  $\mu$ l of 0.2 mol/l sodium phosphate buffer (pH 6.5), 25  $\mu$ l of 20 mmol/l GSH, 25  $\mu$ l of 20 mmol/l CDNB, 5  $\mu$ l supernatant and 195  $\mu$ l distilled water.
- e) *GSH-Px Assay*: 105,000 g supernatants were used for determination of the glutathione peroxidase activity level (Beutler, 1971), 5  $\mu$ l of each sample was incubated for 10 min at 37 °C in a 495  $\mu$ l incubation mixture containing 50  $\mu$ l of 100 mM potassium phosphate buffer, pH 7.0, 5  $\mu$ l of 100 mM GSH, 10  $\mu$ l of 200 mM EDTA, 5  $\mu$ l of 400 mM sodium azide, 50  $\mu$ l of 2 mM NADPH, 320  $\mu$ l of distilled water and 50  $\mu$ l glutathione reductase (10 U/ml). After the incubation period, the reaction was initiated by addition 5  $\mu$ l of 10 mM H<sub>2</sub>O<sub>2</sub>. The decrease in OD of the system was measured for 30 s at 340 nm. A similar mixture excluding GSH was used as a blank. The definition of the unit was the same as that of GR.
- f) *CAT Assay*: Catalase activity was determined according to Aebi (1984). The reaction was started by addition of H<sub>2</sub>O<sub>2</sub> and initial absorbances were approximately A = 500. The decrease in absorbance was followed for 15 s at 240 nm. The 500  $\mu$ l incubation mixture contained 338  $\mu$ l of 50 mmol/l potassium phosphate buffer, pH 7.0; 160  $\mu$ l of 3% (w/w) H<sub>2</sub>O<sub>2</sub> (30-fold diluted); 2  $\mu$ l supernatant (500-fold diluted).

Protein concentrations were determined by the method of Bradford using BSA as standard (Bradford, 1976).

### Chemicals

Glucose-6-phosphate, NADP<sup>+</sup>, NADPH, nitrate reductase, flavin adenin dinucleotide, 6-phosphogluconate, and glutathione (oxidized form); Tris [Tris (hydroxymethyl) aminomethane] were obtained from Sigma Chemical Co., St. Louis, MO, glutathione (reduced form) from E. Merck, Darmstadt, Germany. The other chemicals were analytical grade and obtained from Sigma Chemical Co.

### Statistical Analysis

The values were represented as mean  $\pm$  SEM. Data were analysed by Student's *t*-test and P values lower than 0.05 were accepted as significant.

## Results

After five weeks following STZ injection, the rats showed a significant increase in plasma glucose levels, as expected. Diabetes also caused a marked reduction in body weight compared to control animals. Treatment with candesartan-cilexetil did not modify any of these parameters (Table 1).

Diabetes caused significant increases in the G6PD and 6PGD activities of diabetic hearts, whereas candesartan-cilexetil treatment reversed these effects of diabetes to the normal levels. Nevertheless, neither the G6PD nor the 6PGD activities were affected by candesartan-cilexetil administration in the control group (Fig. 1A and B).

The activity of CAT was found to be elevated significantly in the diabetic hearts at the end of the experimental period. However, treatment of diabetic animals with candesartan-cilexetil for five weeks decreased the high activity to about the control level (Fig. 1C). Treatment of control rats with candesartan-cilexetil for the same period did not cause any significant change in this parameter.

Although the GR and the GSH-Px activities increased significantly in the diabetic group at the end of 5-week diabetes, the GST activity did not change in this period. However, 5-week candesartan-cilexetil treatment of the diabetic rats caused significant decreases in GR and

Table 1. General characteristics of the animals

GROUPS	Body weight (g)		Blood glucose (mg/dl)	
	Start	Final	Start	Final
Con (N = 10)	218.0 $\pm$ 2.2	238.8 $\pm$ 13.9	105.3 $\pm$ 1.6	105.7 $\pm$ 3.0
DM (N = 8)	220.3 $\pm$ 7.3	215.6 $\pm$ 17.3	104.9 $\pm$ 1.8	408.9 $\pm$ 10.8*
DMCan (N = 9)	220.2 $\pm$ 8.1	207.4 $\pm$ 14.6	107.7 $\pm$ 2.1	400.8 $\pm$ 18.4*
ConCan (N = 10)	207.6 $\pm$ 6.8	241.2 $\pm$ 10.1	108.5 $\pm$ 2.0	110.4 $\pm$ 1.9

Values are mean  $\pm$  SEM. Control (Con), diabetic (DM), candesartan-cilexetil-treated (5 mg/kg/day) diabetic (DMCan), and candesartan-cilexetil-treated control (ConCan) animals. \*P < 0.05 is the significance level compared to the control group and N represents the number of animals. Start and Final represent the initial and final situation of the animals during the experimental protocol.

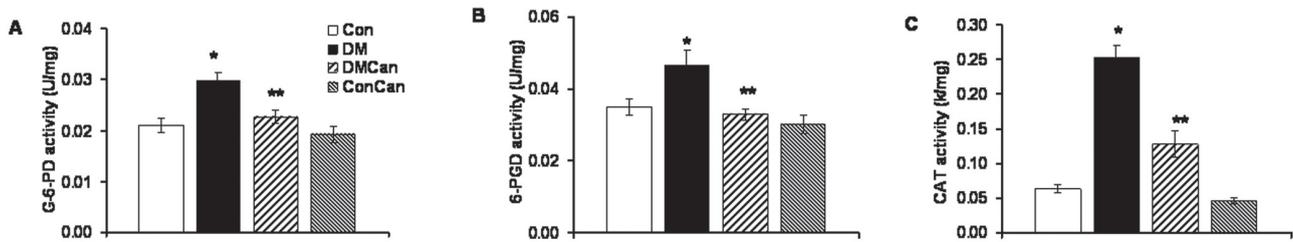


Fig. 1. Effect of candesartan-cilexetil treatment on altered antioxidant enzymes of the diabetic heart. \*  $P < 0.05$  with respect to control; † $P < 0.05$  with respect to diabetes

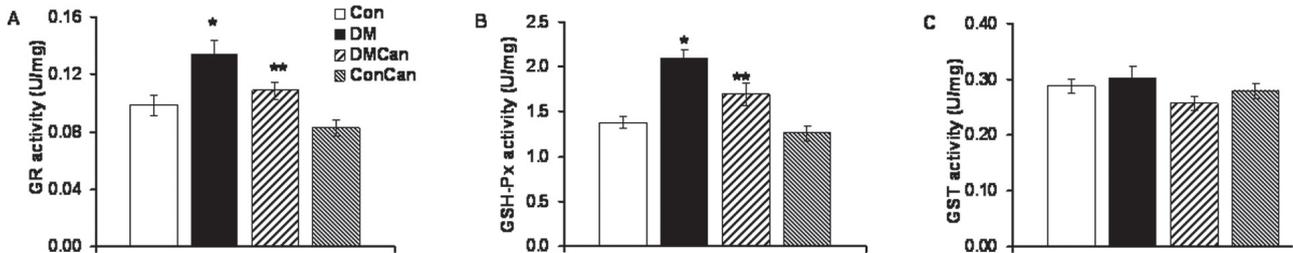


Fig. 2. Beneficial effects of candesartan-cilexetil treatment on glutathione enzymes of diabetic rat heart. \*  $P < 0.05$  with respect to control; † $P < 0.05$  with respect to diabetes

GSH-Px activities. Moreover, there was no additional change in GST activity after the treatment of the diabetic or control animals with candesartan-cilexetil (Fig. 2A, B, and C).

## Discussion

The present study demonstrated that  $AT_1$  receptor blocker candesartan restored the activities of the antioxidant enzymes due to protection of the heart against diabetes-induced oxidative stress. Diabetes can alter the activities of antioxidant enzymes in the heart (Strother et al., 2001; Gumieniczek, 2005; Kamuren et al., 2006; Shirpoor et al., 2008), which is a well-known implication of increased ROS generation. Increased Ang II levels in diabetic heart is a common finding (Sechi et al., 1994; Malhotra et al., 1997; Fiordaliso et al., 2000). An important result of elevated Ang II is promoted activation of NADPH-oxidase, which is a major source of ROS (Berry et al., 2000; Griendling and Ushio-Fukai, 2000; Das et al., 2004; Lu et al., 2004). Ang II-dependent stimulation of NADPH-oxidases may lead to an increase in superoxide ( $O_2^{\bullet-}$ ) generation, which is converted to hydrogen peroxide ( $H_2O_2$ ) by SOD. However,  $H_2O_2$  would remain a reactive free radical that may also cause cellular damage, unless CAT and GSH-Px convert it to  $H_2O$  and annihilate its harmful potency. Therefore,  $AT_1$  receptor blockage would inhibit the Ang II-dependent oxidation pathway and thus decrease production of ROS with a resultant decrease of antioxidant enzymes activities.

G6PD and 6PGD activities were found to be increased in diabetic heart and these are most probably due to increased NADPH production, a substrate of NADPH-oxidases. The activities of antioxidant enzymes GR,

GSH-Px and CAT also increased in diabetic hearts, and  $AT_1$  receptor blocker candesartan-cilexetil normalized their activities significantly. Our results suggest that  $AT_1$  blockade may decrease the Ang II-induced free radical production in diabetic heart. Thus, as the ROS generation mechanism is suppressed and the free radical levels decreased, the activity of the antioxidant enzymes returns to its basal levels. Accordingly,  $AT_1$  receptor blockade reversed the increased enzyme activities in diabetic rat hearts. In addition, the elevated G6PD and 6PGD activities decreased by candesartan-cilexetil administration in diabetic animals. Therefore, reduction of the activity of these enzymes that promote NADPH generation, which is a substrate of NADPH-oxidases, may suppress the Ang II-induced free radical production.

The over-activation of Ang II signalling by diabetic condition seems to be critical for triggering ROS production in the heart through activation of NAD(P)H oxidase. We measured the oxidant level of the heart by monitoring lipid peroxidation in our previous study (Ulusu and Turan, 2005) and showed that it increased significantly in diabetic animals, which was confirmed by the data published by other researchers (Gumieniczek, 2005; Babu et al., 2006; Tsutsui et al., 2007; Shirpoor et al., 2008). Therefore, our data related to GSH-Px, GR, and CAT in the heart tissues can demonstrate that the antioxidant defence mechanism altered due to increased ROS and  $AT_1$  receptor blockage can significantly protect heart against oxidative damage. From this point, it is tempting to suggest that increased enzyme activities were a compensatory response to oxidative stress due to an elevation of hydrogen peroxide and lipid peroxidation. Recent findings (Tsutsui et al., 2007) that showed significant reduction of TBARS levels in diabetic heart after candesartan treatment support this proposal and

thus our hypothesis, which attributes a protective role to AT<sub>1</sub> receptor blockage against oxidative stress induced by diabetes.

In summary, Ang II may take part in ROS production in diabetic rat heart, and suppression of its activity by AT<sub>1</sub> receptor blocker decreases its potency and protects the tissue against diabetes-induced ROS generation and cellular damage. This subject needs further studies to clarify the underlying mechanisms.

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