

Exacerbated cardiac injury induced by renal ischemia/reperfusion in Diabetes Mellitus-II

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Abstract

The present work was designed to investigate the role of Diabetes Mellitus Type-II (DM-II) on renal ischemia reperfusion (I/R)-associated pathophysiology in cardiac damage. DM-II in rats was induced by the administration of nicotinamide (230 mg/kg, *i.p.*) and streptozotocin (65 mg/kg, *i.v.*). *In vivo* renal I/R was performed in both DM-II and normal rats. Lipid peroxidation, xanthine oxidase activity, and nitric oxide levels were significantly increased in cardiac tissue after I/R in diabetic rats compared to I/R in normal rats. Levels of antioxidant enzymes were significantly reduced after I/R in diabetic rats compared to normal rats. Serum TNF- α levels and cardiac tissue myeloperoxidase activity were also significantly increased after I/R in DM-II rats. In conclusion, DM-II rats showed exaggerated cardiac damage by renal I/R injury.

Keywords: Diabetes Type-II, Ischemia, reperfusion, heart, inflammation, oxidative stress.

Introduction

Diabetes mellitus (DM) type-II is one of the leading causes of end stage renal disease (ESRD) (Maisonneuve et al. 2000). Diabetic patients are at a higher risk for ischemic conditions caused by decreased blood flow (Hokama et al. 2000). Thus, ischemia is described as a decrease in oxygen supply or an increase in oxygen demand. With increasing duration and severity of ischemia, greater cell damage can develop, with a predisposition to a spectrum of reperfusion-associated pathologies, collectively called reperfusion injury (Yellon and Baxter 2000). A recent study demonstrated a higher incidence of nephropathy in DM type-II compared to DM type-I patients (Yokoyama et al. 2000). Mechanisms behind the injury in diabetic nephropathy are not fully understood despite intense research. Diabetic patients may need renal transplantation later in life due to diabetic nephropathy; I/R injury is one of the dangerous complications of this procedure. Moreover, in DM rats, a comparatively short ischemia of 30 minutes, which in non-DM rats results in reversible acute renal failure, causes a progressive injury with end-stage renal failure (Melin et al. 1992).

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Reactive oxygen species (ROS) and nitric oxide (NO) play an important role in mediating cell damage during I/R injury (Basireddy et al. 2006). Inflammation contributes substantially to the pathogenesis of I/R with a central role for particular cells, adhesion molecules, and cytokines (Ysebaert et al. 2004). Neutrophils are the inflammatory cells that produce high levels of ROS during I/R injury. Myeloperoxidase (MPO) is found in neutrophils and is found to catalyze the formation of hypochlorous acid (HOCl), a toxic agent to cellular components, which initiates oxidative injury (Altunoluk et al. 2006). Renal I/R causes tissue injury via oxygen radicals and oxidative stress caused by an imbalance in the production of ROS and antioxidant capacity (Erdogan et al. 2006). Renal I/R injury may cause oxidative stress and increase lipid peroxidation in the tissue, something that is well documented in rat tissues (Emre et al. 2006).

Cardiac injury is one of the distant organ damage induced by kidney I/R (Kelly 2003). Acute renal failure and DM-II commonly associated with heart disease. Hence, the present investigation was designed to understand the role of DM-II on renal I/R-associated pathophysiology in cardiac damage using these diabetic rats.

Materials and Methods

Chemicals: Superoxide dismutase (SOD), crystalline beef liver catalase (CAT), 1,1,3,3-tetrahydroxypropane, glutathione (GSH), and Epinephrine Hydrochloride were purchased from Sigma Aldrich; USA. Tris buffer, Thiobarbituric acid, and Trichloroacetic acid were purchased from Himedia lab. Folin's phenol reagent was procured from S.D. Fine Chemicals Mumbai, India. All other chemicals used in the study were of laboratory grade.

Experimental groups and animals: Healthy adult Wistar rats (both sexes) weighing 200–250 g were used for this experiment whose protocol described herein was approved by the Institutional Animal Ethics Committee (IAEC) of Smt. R.B.P.M.C. Atkot. Permission was obtained from the committee for the purpose of control and supervision of experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The rats were assigned to three different groups ($n = 5$). Group-1: Normal sham-operated (underwent all surgical procedures without ischemia reperfusion in normal rats); Group-2: Normal rats; ischemia was produced for 30 min on day 25, followed by 24 h reperfusion (I/R control); Group-3: After induction of diabetes; I/R was produced on day 25 (DM + I/R control).

Induction of diabetes type-II and measurement of blood glucose level: DM-II was induced in rats by the administration of nicotinamide (NAD) (230 mg/kg, *i.p.*), 15 min prior to the single dose of streptozotocin (STZ) (65 mg/kg, *i.v.*) (Masiello et al. 1998). Control animals received an equal volume of saline. The STZ solution contained STZ in saline and a sodium citrate buffer, pH 4.0. Food, water consumption, weight gain, and blood glucose levels (by using standard diagnostic kits, Beacon Diagnostics Pvt. Limited) were recorded to monitor the degree of diabetes. Four weeks were allowed to elapse between the induction of diabetes and ischemic injury.

Renal I/R Injury: Diabetic and normal rats were anesthetized with ketamine (60 mg/kg *i.p.*) and diazepam (5 mg/kg *i.p.*). Body temperature was maintained throughout surgery at 37 ± 0.5 °C. The skin on the back was shaved and disinfected with povidone iodine solution. All rats underwent surgical exposure of the left and right renal pedicles via midline incision. To induce renal ischemia, both renal pedicles were occluded for 30 min with vascular clamps. After 30 min of occlusion, the clamps were removed and the kidneys observed before the 24 h reperfusion. At the end of each *in vivo* study, rats

were sacrificed and the kidneys quickly removed, placed in liquid nitrogen, and then stored at -70°C until assayed for oxidant and antioxidant parameters.

Blood pressure and cardiac injury markers: Blood pressure was measured non invasively at 5, 15, and 30 min during renal ischemia, and at 1, 6, 12, and 24 h during reperfusion by tail cuff method using LE 5002 storage pressure meter (LETICA scientific instruments, SPAIN) in all the above mentioned groups. At the end of experiment, animals were anesthetized under light ether anesthesia and blood samples were collected from the retro orbital plexuses of each rat. The serum was separated and lactate dehydrogenase (LDH), creatinine kinase (CPK) level was measured by spectrophotometer using biochemical diagnostic kits (Nicholas India Pvt. Ltd., India).

Lipid peroxidation and antioxidant enzymes: The heart was removed and kept in cold conditions (precooled in an inverted petridish on ice). Each heart was cross-chopped with a surgical scalpel into fine slices in chilled 0.25 M sucrose and quickly blotted on filter paper. The tissue was minced and homogenized in 10 mM Tris-HCl buffer, pH 7.4 (10 % w/v) with 25 strokes of a tight Teflon pestle of a glass homogenizer at a speed of 2500 rpm. The clear supernatant was used for assays of lipid peroxidation (MDA content) and endogenous antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and Glutathione peroxidase (GSHPx). MDA formation was estimated by the method of Slater and Sawyer (Slater and Sawyer 1971) reduced glutathione was determined by the method of Moron et al. (1979), and superoxide dismutase was determined by the method of Mishra and Fridovich (1972). Catalase was estimated by the method of Hugo Aebi as given by Colowick et al.; glutathione peroxidase was determined by the method of Paglia and Valentine (1967).

Xanthine oxidase activity: Tissue xanthine oxidase (XO) activity was measured spectrophotometrically by the increase in absorbance at 293 nm due to the formation of uric acid from xanthine (Prajda and Weber 1975). The phosphate buffer (pH 7.5) and xanthine were mixed with the supernatant sample and incubated for 30 min at 37°C . The reaction was stopped at 0 and 30 min by the addition of 100% trichloroacetic acid. Then, the mixture was centrifuged at 5000 g for 30 min and the activity was measured at 293 nm. One unit of activity was defined as 1 mmol of uric acid formed per minute at 37°C , pH 7.5.

Nitric oxide level: Nitrite (NO) was estimated by the method of Guevara et al. (1998). To 0.5 ml of tissue homogenate, 0.1 mL of sulphosalicylic acid was added and vortexed well for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. Protein-free supernatant was used for estimating nitrite levels. To 200 μl of the supernatant, 30 μl of 10 % NaOH was added, followed by 300 μl of Tris-HCl buffer and mixed well. To this, 530 μl of Griess reagent was added and incubated in the dark for 10–15 minutes and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained. The standard curve was prepared by using sodium nitrite solutions with concentrations in the range of 1–100 μM by diluting the nitrite standard solution.

Myeloperoxidase activity: MPO activity was measured in tissues by a procedure similar to that documented by Hillegas et al. Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0) and centrifuged at 41,400 g (10 min). Pellets were suspended in 50 mM Phosphate buffer containing 0.5 % hexadecyltrimethylammonium bromide (HETAB). After three freeze-thaw cycles with sonication between the cycles, the samples were centrifuged at 41,000 g for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of the reaction mixture containing 50 mM Phosphate buffer, *o*-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in the absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g of tissue.

TNF- α quantification by ELISA: Serum levels of TNF- α were determined by using an enzyme-linked immunosorbent assay (ELISA) (Endogen, mouse TNF- α kit, Pierce Biotech Int., Rockford, Illinois, USA) according to the manufacturer's instructions.

Statistical analysis: All the values are expressed as mean \pm SEM. Statistical significance was tested between more than two groups using one-way ANOVA followed by the Bonferroni multiple comparisons test using a computer-based fitting program (Prism, Graphpad 5.). Differences were considered to be statistically significant when $P < 0.05$.

Results

Blood pressure and cardiac injury markers: Non-diabetic and diabetic rats demonstrated a reduction in mean blood pressure but that didn't statistically significant (Table 1). Heart rate was found to be elevated in both Non-diabetic and diabetic rats after 30 min of ischemia compared to normal rats ($P < 0.05$). Non-diabetic and diabetic rats demonstrated a significant ($P < 0.01$) increase in CPK and LDH levels after I/R. In the diabetic I/R group CPK and LDH levels significantly ($P < 0.05$) increased compare to non-diabetic I/R rats (Table 2).

Table 1. Mean blood pressure; and Heart Rate during the experiments

Group	Baseline	Time for ischemia (min)			Time for reperfusion (h)			
		5	15	30	1	6	12	24
Mean blood pressure (mmHg)								
NC	98 \pm 2.4	99 \pm 3.2	96 \pm 2.1	97 \pm 1.2	98 \pm 2.3	99 \pm 2.7	99 \pm 4.3	97 \pm 1.8
I/R	101 \pm 3.5	95 \pm 2.3	88 \pm 2.5	82 \pm 1.4	79 \pm 2.1	75 \pm 3.1	70 \pm 1.3	64 \pm 1.3
DM+I/R	110 \pm 2.5	98 \pm 2.3	87 \pm 2.5	72 \pm 3.4	69 \pm 1.1	65 \pm 1.1	60 \pm 2.3	59 \pm 3.3
Heart Rate (beats/min)								
NC	358 \pm 9.2	355 \pm 5.6	365 \pm 5.6	354 \pm 4.5	360 \pm 1.7	361 \pm 6.7	356 \pm 4.7	358 \pm 1.3
I/R	352 \pm 6.2	340 \pm 7.3	310 \pm 4.5	264 \pm 4.2 ^s	258 \pm 6.5 ^s	245 \pm 5.6 ^s	240 \pm 3.2 ^s	232 \pm 4.8 ^s
DM+I/R	348 \pm 5.2	330 \pm 6.2	301 \pm 3.8	252 \pm 5.4 ^s	241 \pm 7.1 ^s	231 \pm 6.2 ^s	228 \pm 3.9 ^s	202 \pm 3.4 ^s

Values are mean \pm SEM ($n = 5$), analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests. ^s $P < 0.05$ vs normal control

Table 2. Lactate dehydrogenase; and CPK after renal I/R in normal, and diabetic rats

Group	LDH (U/L)	CPK (U/L)
NC	1201 \pm 94.9	18.65 \pm 1.00
I/R	2581 \pm 174.8 ^{ss}	38.48 \pm 2.33 ^{ss}
DM+I/R	3487 \pm 175.9 ^{*,sss}	49.43 \pm 2.72 ^{*,sss}

Values are mean \pm SEM ($n = 5$), analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests. * $P < 0.05$ vs I/R; ^{ss} $P < 0.01$, ^{sss} $P < 0.001$ vs normal control.

Lipid peroxidation and antioxidant enzymes in cardiac tissue

The cardiac tissue MDA content was elevated in the normal and diabetic groups after induction of I/R injury, compared to the normal control group ($P < 0.05$, $P < 0.01$ respectively). However, the DM + I/R group had higher cardiac MDA levels compared to the I/R group ($P < 0.05$). I/R in diabetic rats resulted in a significant decrease in cardiac tissue GSH ($P < 0.05$) when compared to I/R in normal rats. Diabetic animals that underwent renal I/R had no effect on cardiac GSHPx or SOD levels when compared with I/R animals. The CAT activity of the DM + I/R group was decreased in comparison with the I/R group ($P < 0.05$), whereas XO

activity was increased in the DM + I/R group in comparison with the I/R group ($P < 0.05$) (Table 3).

Myeloperoxidase activity, serum TNF- α and NO level

Myeloperoxidase activity, an accepted indicator of neutrophil infiltration, was significantly higher in the cardiac tissue of the DM+I/R group than in the I/R group ($P < 0.05$). Serum TNF- α levels were significantly higher in DM + I/R rats than in the I/R control rats ($P < 0.05$). The levels of NO were increased in the DM+I/R group in comparison with the I/R group ($P < 0.05$) (Table 4).

Table 3. Lipid peroxidation; reduced glutathione; glutathione peroxidase; superoxide dismutase; catalase; and xanthine oxidase in cardiac tissue after renal I/R in normal, and diabetic rats.

Groups	NC	I/R	DM + I/R
MDA (Nmolc/gm of tissue)	71.50 \pm 3.15	95.33 \pm 3.17 ^{\$}	125.0 \pm 6.19 ^{*,^{ss}}
GSH (μ molc/gm of tissue)	2.86 \pm 0.45	1.7 \pm 0.06	0.44 \pm 0.08
GSHPx (Unit/mg of protein)	0.34 \pm 0.06	0.19 \pm 0.03	0.09 \pm 0.03
SOD (Unit/gm of tissue)	20.83 \pm 3.1	8.63 \pm 0.98 ^{\$}	6.33 \pm 1.25 ^{*,^{ss}}
CAT (μ molc H ₂ O ₂ consumed/min/gm of tissue)	213.3 \pm 11.45	132.5 \pm 6.29 ^{\$}	70.83 \pm 5.83 ^{*,^{ss}}
XO (Unit/gm of protein)	0.71 \pm 0.03	1.24 \pm 0.05 ^{\$}	1.48 \pm 0.08 ^{*,^{ss}}

Values are mean \pm SEM ($n = 5$), analyzed by one-way ANOVA followed by Bonfferoni's multiple comparison tests. * $P < 0.05$ vs I/R; ^{\$} $P < 0.05$, ^{ss} $P < 0.01$ vs normal control.

Table 4. Myeloperoxidase; tissue nitric oxide; and TNF- α in cardiac tissue after renal I/R in normal and diabetic rats.

Groups	NC	I/R	DM+I/R
BGC (mmol/L)	4.9 \pm 0.92	4.7 \pm 0.84	34.48 \pm 1.51 ^{***, ^{sss}}
MPO (Unit/gm of tissue)	0.32 \pm 0.06	4.21 \pm 0.60 ^{\$}	6.28 \pm 0.28 ^{*,^{\$}}
NO (μ molc/gm of tissue)	0.66 \pm 0.05	1.19 \pm 0.06 ^{\$}	1.84 \pm 0.07 ^{*,^{\$}}
TNF- α (pg/ml)	60.63 \pm 9.30	120.8 \pm 7.34 ^{\$}	221.3 \pm 12.58 ^{*,^{ss}}

Values are mean \pm SEM ($n = 5$), analyzed by one-way ANOVA followed by Bonfferoni's multiple comparison tests. * $P < 0.05$, *** $P < 0.001$ vs I/R; ^{\$} $P < 0.05$, ^{ss} $P < 0.01$, ^{sss} $P < 0.001$ vs normal control.

Discussion

In the present study, we used 30 minutes of ischemia as the importance of the ischemic duration is well established-it has been previously shown that a longer period of ischemia causes more severe injury (Melin et al. 1992). The degradation of ATP to hypoxanthine and xanthine via inosine is enhanced with the duration of ischemia (Gerlach et al. 1963). The restoration of ATP levels during reperfusion is slower after prolonged ischemia (Stromski et al. 1986). Temperature is a critical factor in ischemic injury—hyperthermia, especially during the ischemic phase, leads to more severe renal I/R injury. Raising the temperature from 37 to 39.5 °C during ischemia leads to a 100 % increase in blood urea nitrogen (BUN) in a model using 30 minutes of ischemia and uninephrectomy (Zager 1990). In order to keep the temperature constant, we used a servo-controlled heating pad that kept the temperature in the rat at 37.5°C. An important question in this study is how DM-II could cause the increased sensitivity cardiac injury induced by renal I/R, which has been observed in DM animals. Several possible explanations exist: i) the increased sensitivity to cardiac damage could be due to hyperglycemia

per se, ii) shortage of insulin could also be involved, iii) secondary effects of hyperglycemia such as the formation of advanced glycosylated end products, increased oxidative stress, hemodynamic alterations, and formation of NO could also be involved.

In our study, animals subjected to renal I/R demonstrated an increase in the cardiac tissue MDA levels and an attenuated antioxidant enzyme pool. Renal I/R-induced oxidative stress in DM-II was associated with impaired cardiac function, leading to a marked increase in serum LDH and CK. Oxidative stress and inflammatory response might play a pathophysiological role in renal I/R injury in DM-II, given the knowledge that oxidative stress is implicated both in the complications of DM-II and renal I/R. Elevated oxidative stress has been demonstrated in cerebral (Aragno et al. 2000) and intestinal (Salas et al. 1999) I/R in diabetic rats. The combined oxidative stress from two sources may thus increase the total level of ROS. Infiltration of inflammatory cells is one of the main features of renal I/R injury in DM-II rats. The infiltrate mainly consists of cells identified as macrophages/monocytes and T-lymphocytes. The inflammatory response is increased acutely after I/R of the intestines in diabetic animals (Panese et al. 1996). After a brief ischemia of the intestine, ROS levels are also increased and the increase is more pronounced in diabetes (Salas et al. 1999). Elevated inflammatory response and generation of oxidative stress in DM-II might be responsible for exaggerated cardiac injury.

Cardiac MPO activity increased after renal I/R, consistent with leukocyte infiltration and activation. Active neutrophils show high MPO activity in the tissue as an inflammatory response (Sakr et al. 1992). The present work demonstrated that the high cardiac MPO activity after induction of I/R in DM-II rats, is very important because it clearly shows high leukocyte infiltration in cardiac tissue. Neutrophils play a major role in oxidant injury via mechanisms such as the action of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or the MPO system. Hypochlorous acid produced largely from stimulated neutrophils by MPO activity, causes oxidation of other molecules such as proteins, amino acids, carbohydrates, nucleic acids, and lipids, expanding cardiac tissue damage (Kelly KJ 2003). The nitric oxide system may be involved in the increased sensitivity to I/R in DM-II. There is evidence for increased NO-production in the STZ-DM kidney (Goor et al. 1996); the reaction of NO with O_2^- results in peroxynitrite formation, a potent and aggressive cellular oxidant, and causes the formation of 3-nitro-L-tyrosine. Nitrite/nitrate levels, as the end products of nitric oxide conversion, were found to be increased in blood plasma and aortic tissue in diabetic animals in comparison with nondiabetic animals (Sudnikovich et al. 2007), something that was confirmed by elevated NO levels in our study.

Several mechanisms might be responsible for the exaggerated cardiac injury seen in DM-II, for instance, the restoration of hyperglycemia (Table 4); previous work supports the importance of blood glucose concentration (BGC) in I/R injury. In our study, we found severe cardiac injury when I/R was performed in DM-II rats in whom BGC was higher than in normal rats. Hyperglycemia, the elevated BGC, during I/R could be deleterious for the heart. Numerous studies have investigated the influence of hyperglycemia and diabetes in cerebral ischemia. Diabetes is associated with a worse outcome after stroke in humans, and elevated blood glucose

predisposes for a more severe cerebral injury even in non-DM patients (Pulsinelli et al. 1983). Taken together, these studies suggested a role for reperfusion in the harmful effect of hyperglycemia in ischemic injury.

In our study, increases in MDA, XO activity, and decreases in SOD, CAT, GSH, and GSHPx in DM + I/R rats demonstrated the induction in nuclear oxidative stress. In addition, the outer membrane of mitochondria becomes permeabilized in response to ROS, resulting in the translocation of Bax from cytosol to the mitochondria and the release of cytochrome C. The release of cytochrome C into the cytosol leads to the formation of the apoptosome, which stimulates the activation of procaspase-9 and procaspase-3. Active caspase-3 activates caspase-activated DNAase, leading to DNA fragmentation (Timmers et al. 2009, Chen et al. 2008).

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