CHARACTERIZATION OF A RAT MODEL OF INSULIN DEFICIENCY INDUCED VASCULAR COMPLICATION

Nora Desoky,Hany M. El-Bassossy*& Ahmed Fahmy
Department of Pharmacology, Faculty of Pharmacy, Zagazig University, Egypt
*Department of Pharmacology, Faculty of Pharmacy, King Abdulaziz University, Kingdom of Saudi Arabia**

ABSTRACT

Vascular dysfunction is one of the important diabetic complications. Here we fully characterized a rat model of streptozotocin (STZ) induced vascular complication.

Diabetes was induced by single intraperitoneally injection of STZ (50 $mg.kg^{-1}$, ip).Blood pressure (BP) and isolated aorta responses phenylephrine (PE), KCl, acetylcholine (ACh), sodium nitroprusside (SNP) were recorded after 6, 8 and 10 weeks of STZ injection.In addition, serum levels of glucose, tumor necrosis factor α (TNFα), lipids, advancedglycation end products (AGEs), and arginase activity were determined. Furthermore, aortic reactive oxygen species(ROS) generation, hemeoxygenase-1 expression and collagen deposition were examined.

Streptozotocin injection resulted in a significant hyperglycemia after 3 days of injection which was stable for 10 weeks. Diabetes was associated with a significant increasein BP after 6 weeks which was stable at 8 weeks. Aorta isolated from diabetic animals showed exaggerated contractility to PE and KCland impaired relaxation to ACh compared to control after 6 weeks which were clearer at 8 weeks of STZ injection. In addition, diabetic animals showed significant increases in serum levels of lipids, AGEs, TNFα and arginase enzyme activity after 8 weeks of STZ compared to control. Furthermore, aortae isolated from diabetic animals were characterized by increased ROS generation and collagen deposition.

In conclusion, injecting rats with STZ at dose 50 $mg.kg^{-1}$ produces a model of diabetic vascular complication after 8 weeks that are characterized by hypertension, disturbed vascular reactivity, elevated serum lipids, inflammatory cytokines and enzymes and enhanced aortic ROS generation and collagen deposition.

Key words: Diabetes; aorta; hypertension; vascular reactivity, rat

Abbreviations: ANOVA, analysis of variance; NO, nitric oxide; PE, phenylephrine; ROS, reactive oxygen species; SNP, sodium nitroprusside;Ach, acetyl choline; BP, blood pressure.

INTRODUCTION

Diabetes mellitus is a complex, multisystem disease that representsthe most common metabolic disorder. Many of the complications of diabetes are vascular in origin,and this puts diabeticsat an increased risk of cardiovascular diseases [1].Human diabetics have a markedly increased morbidity and mortality due to cardiovascular disease[2;3].

Severalfactors have been reported to explain the vascular dysfunction and acceleratedatherosclerosis in diabetes, including hyperglycemia itself, production of advanced glycation end products (AGEs), hyperlipidemia and hypertension. Increased oxidative stress is particularly of greatimportance as a pathogenic factor of diabetes-associatedvascular disease[4;5].Changes in vascular responsiveness to vasoconstrictors and vasodilatorsare mainly responsible for development of some vascularcomplications of diabetics[6]. Most of thesecomplications are due to augmentedgeneration of reactive oxygen species (ROS) which lead to endotheliumdysfunction [7]

In spite of the significant developments in antidiabetic therapy, diabetic complications continue to be seriously deleterious. Many of the clinical complications of diabetes may be ascribed to alterations in vascular structure and function, with subsequent end-organ damage and death [8]. Therefore, preventing vascular complications in diabetes can save diabetic patient from other complications.

New drugs are continually being tested and new strategies developed to prevent and treat diabetes. In thesestudies, different experimental animal models of diabeteshave been used. One of the most important diabetes models is streptozotocin (STZ)-induced diabetes in the rat[9]. However, little is known about the onset of developing vascular complications and their characteristics. The aim of the present work was to fully characterize the rat streptozotocin model in order to provide supporting information for researchers who wish to work on diabetic vascular complications.

2. Materials and methods

2.1 Animals

Adult male Wistar rats weighing 140–160 g (Zagazig University, Zagazig, Egypt) were housed in clear polypropylene cages (4rats per cage) and kept on a light–dark cycle of equal duration, under constant environmental conditions. Rats were fed commercially available rat normal pellet diet and water *ad libitum*. Experimental design and animal handling were according to the guidelines of the Ethical Committee of the Faculty of Pharmacy, Zagazig University, for Animal Use.

2.2 Study protocol

Animals were randomly divided into four experimental groups (6 animals each); control, diabetic (6 weeks),diabetic (8 weeks), diabetic (10 weeks). Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 50 $mg.kg^{-1}$).

At the end of the study, blood pressure was measured and rats were anesthetized with diethyl ether. Blood was collected from the retro-orbital plexus and centrifuged (3000×g, 4°C, 20 min) to separate serum that was analysed for glucose, insulin, tumour necrosis factor α (TNFα), cholesterol, low density lipoprotein cholesterol (LDL-C), advanced glycation end products (AGEs) and arginase activity. Then, through opening the abdomen, descending thoracic aorta was carefully excised and placed in a Petri dish filled with cold Krebs–Henseleit buffer containing (in mM): NaCl 118.1, KCl 4.69, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.7, $MgSO_4$ 0.5 and $CaCl_2$ 2.5. The aorta was cleaned of excess connective tissue and fat and cut into rings of approximately 3 mm in length. For each animal, one aortic ring was suspended in an organ bath for studying vascular reactivity while the second was snap frozen until measurement of reactive oxygen species (ROS) generation. The third rapidly stored in 10 % neutral formalin solution for examining aortic collagen deposition andhemeoxygenase-1 expression.

2.3 Serum analysis

Serum glucose was determined using blood glucose meter system (one touch brand, U.S.A), according to the method[10].

Serum arginase activity was measured as previously described [11] using arginase assay kit® (Biodiagnostic, Cairo, Egypt). The method depends upon the colorimetric determination of the arginase enzyme product, urea. The serum was incubated for 1 hour withreaction mixture containing the activator (manganesesulphate) and substrate (arginine) in carbonate buffer (pH 9.5).The produced urea was condensed with diacetylmonoxime in an acid medium in the presence of ferric chloride (oxidant) and carbazide (accelerator). Then the absorbance was measured at 525nm.

Serum AGEs was determined as previously described[12;13]. In brief, serum was diluted 1:15 in saline and the fluorescence intensity at (λex = 370 or 440 nm) was determined by LS45 fluorescence spectrophotometer (PerkinElmer®, Cairo, Egypt).

Serum TNF-α level was determined by enzyme-linked immunosorbent assay (ELISA) using Quantikine® kit (R&D systems, Cairo,Egypt) that contained Escherichia coli-expressed recombinant rat TNF-α and antibodies raised against the recombinant factor.

Total cholesterol was determined enzymatically with the cholesterol oxidase/p-aminophenazone method using Boehringer Mannheim® colorimetric kit (Mannheim, Germany). Serum was mixed in a tube containing the precipitation reagent (phosphotungstic acid and magnesium chloride) and incubated for 15 min. After centrifugation then supernatant was mixed with reagent to measure high density cholesterol (HDL-C) content, and the clear supernatant was used for determination of its cholesterol content using method determined enzymatically with the cholesterol oxidase/p-aminophenazone method using Boehringer Mannheim® colorimetric kit (Mannheim, Germany). Finally, LDLcholesterol was calculated according to the following Friedewald equation: total cholesterol−(HDL cholesterol+1/5 TGs) [14].

2.4Blood pressure measurement

Blood pressure (BP) was measured indirectly in a conscious and slightly restrained rat by the tail cuff method as previously described [15;16]. For these measurements,rats were conditioned to the restraint and the warming chamber for10–20 min/day for at least 3 days before measurements. BP measurementswere performed from 7:00 to 12:00 AM by the same investigator.After 5–10 min of stabilization in a worming chamber (35 °C), a typical run involved 10 repetitions of the automated inflation–deflation cycle. The mean of 6 readings within a 5–10 mmHg range wastaken as the blood pressure.

2.5Vascular reactivity

Vascular reactivity was measured using the isolated artery technique as previously described [17;18]. Thoracic aorta rings were suspended under 8 mN resting tension in individual 30 ml organ chambers containing Krebs–Henseleit buffer at 37°C and aerated with 95% oxygen, 5% carbon dioxide. Ring tension was determined by use of an isometric force transducer (Biegestab K30, HugosachsElektronik, March, Germany). Force displacement was recorded with a PowerLab Data Interface Module connected to a PC running Chart software (v4.2, ADI Instruments, Chalgrove, Oxon, UK). Rings were equilibrated for 60 min during which time, the bath solution was changed every 30 min. Before beginning the experiment, vessel viability was assessed by exposing arteries to KCl (80 mM). This was repeated until stable responses

were achieved (usually two exposures). For studying the vasoconstrictor responsiveness of aorta, cumulative concentrations of phenylephrine (PE, 10^{-9} to 10^{-5} M) or KCl (10 to 100 mM) were added to the organ bath and the response was recorded. For studying the vasodilator responsiveness of aorta, rings were first precontracted with submaximal concentrations of PE ($3x10^{-7}$ in case of diabetic group and 10^{-6}other groups). The submaximal concentration of PE was chosen to give similar precontraction in all studied groups. The cumulative concentrations of acetylcholine (ACh, 10^{-9} to 10^{-5} M) or sodium nitroprusside (SNP, 10^{-9} to 10^{-5} M) were then added to the organ bath and the response was recorded.

2.6Haem oxygenase-1(HO-1) immunohistochemistry

Immunohistochemistry of HO-1 protein in rat paraffin embedded aorta sections was assessed using the method described previously [19]with some modification. The method uses primary antibody to detect the HO-1 in sections followed by fluorophore conjugated antibody.Slides were heated at 55ºc for 30 min. The sections were deparaffinizedin two changes of xylene (5min each) then in two changes of 100% ETOH(5min each).The sections were rehydrated through rinsing once with graded alcohols 95%ETOH and 70%ETOH for 2 min each then rinsed for 5 min with distilled H_2O. Endogenous peroxidase activity was saturate by incubating slides in 3%H2O2 solution (100ml 30% H_2O_2+ 900ml Methanol) for 30 min then rinsedwith 3 changesof distilled H_2O(5 min each). Sections were then incubated in antigen retrieval solution (1gm sodium citrate in 500ml dH_2O,pH=6) in water bath at 90ºc for 30 min to unmask the antigenic epitope then the solution was allowed to cool slowly to room temperature for 20 minutes(it is important to let the temperature ramp down slowly to allow the protein molecules to fold properly). Then sections were rinsed in 3 changes of1xPBS (5 min each), 10xPBS was prepared (80gmNaCL + 1.94gmKCL + 2.45gm KH_2PO_4 + 14.2gmNa_2HPO_4 complete to 1L and adjust pH at 7.2). Non specific binding was blocked by incubating the sections with 50 µl blocking solution (0.5ml rabbit serum +9.5ml 1xPBS + 100µl Triton) for 30 min at room temperature.The sections were then incubated with 50 µl of the rabbit anti-heme oxygenase-1 primary antibody (dilution 1:200 in blocking solution) overnight in cold room. Then sections were rinsed in 3 changesof 1xPBS(5 min each) then incubated with 50 µl of the Alexafluor conjugated gout anti-rabbit secondary antibody (dilution 1:10,000 in blocking solution) for 2hr in dark then rinsed in 3 changesof 1xPBS(5min each) then the coverslip was mounted with 20µl of fluorescence mounting media and left in dark for 20 hr before examination by LEICA DM500 fluorescence microscope with excitation at ʎ=478 and emission at ʎ=495 nm. Images were acquired with identical acquisition parameters, with minimum gain to avoid interference by tissue autofluorescence. The aorta from each rat was divided between all treatment groups. Sections treated with the secondary antibody alone did not show specific staining.

2.7 Collagen deposition examination

The aorta was rapidly dissected out and tissue sections(5 mm) fixed by immersion at room temperature in 10%neutral formalin solution. For collagen synthesis examination, paraffin-embedded tissue sections of aorta were stainedwith collagen-masson'strichrome stain. Sections were examinedunder light microscope.

2.7Intracellular of reactive oxygen species (ROS) generation

The intracellular levels of ROS were determinedby the fluorescent probedichlorofluoresceindiacetate (DCF-DA) according to the method described by [20]with slight modification. Frozen aorta were homogenized in 20 parts (w/v) of 0.32 mol/l sucrose solution in ice and the centrifuged at 2000 rpm at 4 °Cfor 15 min. Then, 100 µl of the supernatant was added to 1900 µl18:1 40 mmol/l Tris (pH 7.4):HEPESbuffer (10 mmol/l HEPES, 120 mmol/l NaCl, 2.5mmol/l KCl, 1.2 mmol/l NaH_2PO_4, 0.1 mmol/l $MgCl_2$,5 mmol/l $NaHCO_3$, 6 mmol/l glucose, and 1 mmol/l $CaCl_2$) containing 5 µM DCF-DA plus 0.1% Pluronic F-127. The fluorescence was measured (λex=485, λem=515nm) before and after 2 h incubation at 37°C. The difference in fluorescence per hour was used as indicative for ROS formation.

2.9Drugs and chemicals

The following drugs and chemicals were used: STZ, ACh, PE, SNP (Sigma-Aldrich, Munich, Germany); DCF-DA, pluronic F-127 (molecular probes, Cairo, Egypt). All the used chemicals were dissolved in double distilled water.

2.10Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed by either student t test or analysis of variance (ANOVA) followed by Newman-Keuls' post hoc test as appropriate using

computer based fitting program and used for comparison (Prism 5, Graphpad, CA, USA).

3. RESULTS

3.1 Serum parameters

Intra-peritoneal administration of STZ in a dose of 50 $mg.kg^{-1}$led to a significant hyperglycemia. Theelevated blood glucose levelreached maximum 3 days after STZ injection ($p<0.001$), declined a little bit (but still statistically different from control) 1 weeksafter STZ ($p<0.001$). The developed hyperglycemiawere stable in the weeks 6 ($p<0.05$), 8 ($p<0.01$) and 10 ($p<0.05$) after STZ injection (Fig. 1).

The STZ-induced hyperglycemia was associated with significant elevations in serum levels of total cholesterol ($p<0.05$), LDL-cholesterol($p<0.05$), TNFα ($p<0.001$), AGEs ($p<0.01$) and arginase activity ($p<0.001$, tables 1) after 8 weeks of STZ injection.

3.2 Blood pressure

Diabetic animals were characterized by significantly higher systolic and diastolic BP 6 weeks after STZ injection compared with control (both at $p<0.001$, Fig 2a). The elevations in systolic and diastolic BP reached steady higher levels at 8 weeks after STZ injection compared with control (both at $p<0.001$, Fig 2b). However, the developed diabetes did not significantly affect the pulse (Fig 2c).

3.3 Vascular reactivity

Cumulative addition of PE(10^{-9} to 10^{-5} M) or KCl (10^{-2} to 10^{-1} M) to the organ bath resulted in concentration dependent contraction of aorta in all the groups (Fig. 3). Diabetes induced by STZ resulted in larger increases in isolated aorta responsiveness to PEcompared with control. The isolated diabetic aorta hyperresponsiveness to PE started from the week 6 till the week 10 after STZ injection, reflected by a significant increase in apparent E_{max}6 ($p<0.001$), 8 ($p<0.05$) and 10 ($p<0.001$, Fig 3a) weeks after STZ injection.Diabetes led also to a similar increase in the aorta responsiveness to KCl as shown by the significant increases in apparent E_{max}6, 8 and 10 (all at $p<0.001$, Fig 3b) weeks after STZ injection.

Cumulative addition of ACh (10^{-9} to 10^{-5} M) to the organ bath resulted in concentration-related decreases in the tension of aortic artery rings precontracted with PE (Fig. 4a). Diabetes was associated with a significant decrease in isolated aorta responsiveness to ACh 8 weeks after STZ injection, reflected by a significant decrease in apparent E_{max} ($p<0.05$)compared with the control (Figure4a). On the other hand, diabetes did not show any significant effect on isolated aorta responsiveness to SNP 6, 8 or 10 weeks after STZ injection (Fig.4b).

3.4 HO-1 expression, glycogen deposition and histopathology

Aorta isolated from diabetic animals showed a clear increase in glycogen deposition 8 weeks after STZ injection compared with control (Fig 5a). However, isolated diabetic aortae did not show significant change in HO-1 immunofluorescence 8 weeks after STZ injection (Fig 5b).

3.5 Intracellular reactive oxygen species (ROS) generation

Aorta isolated from diabetic rats 8 weeks after STZ injection were characterized by a significant increase in ROS generation compared with control ($p<0.05$, table 1).

4. DISCUSSION

The purpose of this study is to fully characterize the vascular complications associated with the STZ diabetic modeland illustrate the mechanism of these vascular complications. We have shown that rats develop clear vascular complications 8 weeks after STZ injection. The developed vascular complications were characterized by hypertension, disturbed vascular reactivity, altered vascular structure and dyslipidaemia. In addition, the vascular complications were associated with low grade inflammation and oxidant stress.

In the present study, diabetes was associated with a significant increase in both systolic and diastolic BP after 6 weeks which was stable at 8 weeks.The elevated systolic (afterload) BP in diabetic animals could be due to cardiac complication and/or aortic stiffness while the elevated diastolic (preload) BP is attributed to the impaired vascular reactivity observed in diabetic animals. Aortic stiffness has been reported to be responsible for augmented pressure in late systole[21]. Previous literatures reported elevated systolic blood pressure in diabetes [22]. This elevation in systolic BP could be due to the increase inpulse and the dyslipidemia associated with diabetes. Dyslipidemia has an importanteffect on pulse in diabetes. It has been reported that increase in triglycerides andcholesterol levels were associated with increase in heart rate and are major coronaryrisk factors[23]. Furthermore, clinical trials have proved that alterationof these atherosclerotic risk factors clearly decreases cardiovascular risk [24-26]. In this study, we found significant elevations in serum levels of total cholesterol and LDL-C in diabetic animals 8 weeks after STZ injection.

We focused on the impairment in vascular reactivity because of its importance in development of hypertension [27]. The present study has shown that aorta isolated from diabetic animals showed exaggerated contractility to PE and KCl and impaired relaxation to ACh compared to control after 6 weeks which were clearer at 8 weeks of STZ injection. Our result is in consistence with previous studies which have reported that diabetes is associated with enhanced vasoconstriction to different vasoconstrictors[28;29]. Diabetes-evoked vasoconstriction may be maintained by two independent mechanisms: an increased release of endothelium-derived vasoconstrictor prostanoids and an enhanced Ca^{2+} influx and/or sensitivity of the vascular smooth muscle cells [30]On the other hand, the impairment in endothelial dependent relaxation in diabetes is thought to be mainly mediated by inhibition of NO generation [31].

The roles of low grade inflammation in the development of vascular complications in the present diabetic model were investigated by monitoring serum levels of TNF-α (one of the important inflammatory cytokine), arginase enzyme (an important enzyme in vascular inflammation), aortic ROS generation and HO-1 (homeostatic enzyme) expression. In the present study, we found significant increases in serum levels of TNF-α, arginase enzyme, and aortic ROS generation in diabetic animals 8 weeks after STZ injection. Plasma levels of TNF-α were found to increase in patients with both type 1 and type 2 diabetes and are significantly correlated to elevated fasting glucose levels, it has also been suggested to represent an important link between metabolic deregulations and inflammation in diabetes [32].Elevated ROS is linked to the formation of AGEs during prolonged hyperglycaemia in diabetes as both hyperglycaemia and oxidative stress in diabetes promote AGEs accumulation[33].AGEs and their intermediates have been found to contribute to vascular complications associated with diabetes and increased oxidative stress [34].In the present work we have reported a significant increase in serum level of AGEs in diabetic animals.Superoxide, one of the ROS produced mainly by NADPH oxidase and increased in diabetes, directly decreases NO bioavailability by reacting with NO, forming peroxynitrite at a rate up to four times faster than superoxide metabolism by superoxide dismutase [35]. During diabetes, impaired vascular function is closely associated with oxidative stress and vascular inflammation [36], both of which have been associated with increases in arginase activity and expression [37]. Arginase, which L-arginine to urea and ornithine, competes directly with NOS for L-arginine. Hence increases in arginase activity can decrease tissue and cellular arginine levels, reducing its availability to eNOS[38]. This may lead to decreased NO production and increased production of superoxide by eNOS[39]. We examined HO-1 expression as one of the most important homeostatic mechanisms. Expression of HO-1 prevents the development of atherosclerotic lesions[40], lowers blood pressure[41]and prevents vascular dysfunction[42]in a variety of experimental conditions. In the present study, we did not notice any major change in HO-1 expression in aorta isolated from diabetic animals compared with control.

The structural changes in the vasculature were also investigated in the present study. We found a clear increase in collagen deposition in aorta isolated from diabetic animals compared with control. Collagen is the major biomechanical constituent of the vessel wall;pathological modifications in fibrous collagen might alter the wall stiffness and consequently derange vascular function(Kesava, 2004). Collagen in the blood vessel wall ,with time and under the effect of AGEs, undergoes significant nonenzymic glycosylation (glycation), which may have a considerable bearing on the atherosclerotic process, especially the accelerated atherogenesis associated with diabetes [43-45].

5. CONCLUSION

In conclusion, single dose of 50 $mg.kg^{-1}$ of STZ produces a model of diabetic vascular complication after 8 weeks that are characterized by hypertension, disturbed vascular reactivity, increased serum lipids, inflammatory cytokines and enzymes, ROS generation and aortic collagen deposition.

6. ACKNOWLEDGMENT

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