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Effects of Insuline on Oxidative Stress and Free Fatty Acid Level in Left Ventricular Muscles of Diabetic Rats

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Studies were carried out to examine and compare the effects of streptozotocin-diabetes on 3-nitrotyrosine (3-NT), Malondialdhyde (MDA) and lipid profiles related parameters in the heart from male rats. Effects of insulin (INS) treatment were also evaluated. The diabetic state severely compromised the 3-NT, MDA and lipid profiles defense mechanism in the left ventricular muscle tissue and the effects were more pronounced in the male rats. Wistar albino male rats were randomly divided into an untreated control group (C), a diabetic group (D) that was treated with a single intraperitoneal injection of streptozotocin (STZ) (45 mg kg^{-1}), D + INS group which were treated with INS one times a day by injection subcutaneous, respectively. Lipid profiles, HbA1c and blood glucose levels in the circulation and MDA and 3-NT levels in left ventricular muscle were measured. Treatment of diabetic rats with INS resulted in a time-dependent decrease in blood glucose. It is found that the lipid profile and HbA1c levels in D + INS group reached the untreated control group rat values at the end of the treatment period. It is found that the lipid profile and HbA1c levels in D + INS group reached the C rat values at the end of the treatment period. In group D, 3-NT and MDA levels were found to be increased when compared with C and D + INS groups. In the D + INS group, MDA levels were found to be decreased when compared with untreated control group. This study shows a direct correlation between hyperglycemia and the production of MDA and nitrotyrosine, a marker of oxidative stress, in diabetic rat left ventricular muscle.

Key Words: Diabetes mellitus, Malondialdehydem 3-Nitrotyrosine, Oxidative stress, Insuline.

INTRODUCTION

The sequels of chronic hyperglycemia in diabetes are manifested as micro- and macrovascular complications[1-3]. The macrovascular changes result in higher risk for myocardial infarction, congestive heart failure (CHF), coronary heart disease (CHD) and stroke, collectively grouped as cardiovascular diseases (CVDs)[1-5].

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There is growing evidence that oxidative stress the imbalance between free-radical production and antioxidant defense, is involved in the pathogenesis of cardiovascular diseases in diabetes[6]. It has been shown that hyperglycemia is associated with a simultaneous increase in the generation of superoxide anion (O^{2-}) and nitric oxide (NO)[7]. This increase is harmful because NO and O^{2-} react to produce peroxynitrite, a potent oxidant that lives for a long time[8]. The production of peroxynitrite can be indirectly inferred by the presence of 3-nitrotyrosine (3-NT) residues[9]. Increased 3-NT has been found in the plasma of diabetic patients[10] and there is evidence that an acute increase of glycemia induces an increase of 3-NT[11].

The aim of the present study was to evaluate whether hyperglycemia is accompanied by 3-NT generation and to explore the direct role of hyperglycemia on this phenomenon.

We measured tissue 3-NT levels in the left ventricular in insulin-treated diabetic rats and in healthy control subjects. Therefore we carried out experiments to determine and compare the 3-NT, MDA and lipid profiles parameter in left ventricular muscle tissue heart using STZ-diabetic male rats as a model system. Effects of treatment with insulin were also examined. The results of these experiments are described in the present communication.

EXPERIMENTAL

Animal handling and treatment protocol 24 healthy male Wistar albino male rats (250-310 g) were selected for the study. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health and approval of the ethics committee of our institution was obtained before the commencement of the study. The diabetic rat model used in present experiments was based on partial damage of pancreatic β-cells resulting from a single administration of STZ (45 mg kg^{-1}, STZ, Sigma Chemical Co., USA) intravenously (dissolved in 0.01 M sodium citrate, pH adjusted to 4.5). This model of experimental diabetes is associated with partial deficits in insulin secretion and consequential hyperglycaemia, without changes in peripheral insulin resistance[12]. STZ-injected animals were accepted as diabetic if blood glucose levels[13] were more than 200 mg dl using a glucometer (Aquo-Check, Roche) after a 1 week period and revealing at least three high blood glucose levels. We used three randomly constituted groups: (1) non-diabetic control animals (C): rats orally fed with standard rat nutrients and water. (2) Diabetic group (D), (3) INS-treated diabetic animals (D + INS): rats treated with 1 unit day^{-1} INS one times a day by subcutan. Animals were fed with standard rat nutrient and water without restriction throughout the experiment. INS-treated groups were given INS for 8 weeks and blood glucose levels as well as body weights were measured once weekly.

Isolation of left ventricular muscles: Wistar albino rats were anesthetized with ether. Hearts were rapidly removed and the left ventricular muscles were dissected. The muscle was mounted in a Petri cup (about 2 mL volume) and perfused continuously

(6-8 mL min^{-1}) with oxygenated (95 % O_2 and 5 % CO_2) Krebs buffer (constituents in mmol L^{-1}: 113 NaCl, 4.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 1.9 $CaCl_2 \cdot 2H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 11.5 glucose, pH 7.4) solution at a constant flow rate.

Biochemical analysis

Measurements of HbA1c and lipid parameters: Blood plasma HbA1c was determined immunoturbidimetrically. Triacylglycerol (TAG), total cholesterol (TC) and high-density lipoprotein-cholesterol (HDL-C) were analyzed by glycerophosphate oxidase, peroxidase/4-aminophenazone (GPO/PAP), cholesterol oxidase, peroxidase/4-aminophenazone (CHOD/PAP) and direct COHD/PAP enzymic colorimetric methods, respectively. The content of very low-density lipoprotein-cholesterol (VLDL-C) and low-density lipoprotein-cholesterol (LDL-C) was calculated according to the equation described by Friedewald *et al.*[14] All these parameters were determined by a Cobas Integra 800 biochemical analyzer (Roche Diagnostics, GmbH, Mannheim, Germany). Measurement of malondialdehyde. A tissue specimen of 50 mg was homogenized in 0.15 mol L^{-1} KCl. After the homogenate had been centrifuged at 1600 g, the MDA levels in tissue homogenate supernatant were determined by the thiobarbituric acid (TBA) reaction according to Yagi *et al.*[14]. The principle of the method is based on measuring the absorbance of the pink colour produced by the interaction of TBA with MDA at 530 nm. Values were expressed as nmol mL^{-1}.

Measurement of 3-nitroyrosine (3-NT) and tyrosine: 3-Nitroyrosine (3-NT) and tyrosine were procured from Sigma Chemical (St. Louis, USA). H_2O_2, sodium acetate, citrate, NaOH, HCl, H_3PO_4, KH_2PO_4 and K_2HPO_4 were purchased from Merck Chemical (Deisenhofen, Germany). All organic solvents were HPLC grade. The tissues were homogenized in ice-cold phosphate-buffered saline (pH 7.4). Equivalent amounts of each sample were hydrolyzed in 6 N HCl at 100 ºC for 18-24 h and then samples were analyzed on an Agilent 1100 series HPLC apparatus (Germany). The analytical column was a 5 mm pore size Spherisorb ODS-2 C18 reverse-phase column (4.6 × 250 mm; HICHROM, Waters Spherisorb, UK). The guard column was a C_{18} cartridge (HICHROM, Waters Spherisorb, UK). The mobile phase was 50 mmol L^{-1} sodium acetate/50 mmol L^{-1} citrate/8 % (v/v) methanol, pH 3.1. HPLC analysis was performed under isocratic conditions at a flow rate of 1 mL min^{-1} and UV detector set at 274 nm. 3-NT and tyrosine peaks were determined according to their retention times and the peaks were confirmed by spiking with added exogenous 3-NT[15] and tyrosine (10 µmol L^{-1}). 3-NT levels were expressed as 3-NT/total tyrosine.

Statistical analysis: Statistical analysis was performed by using SPSS 11.5.1 software (Lead Technologies, Inc., USA). All data represent mean-standard error of the mean (SEM) of n observations. For all experiments, statistical analysis was performed by one-way ANOVA followed by post-hoc analysis with the Bonferroni test to detect differences between control and experimental groups. Comparison within the same group was done by a paired Student's t-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Effects of insulin on blood glucose and body weight tolerance in diabetic rats: Treatment of diabetic rats with insulin resulted in a time-dependent decrease in blood glucose. The reduction in blood glucose becomes significant by week 3 of treatment compared to the diabetic groups ($p < 0.05$) (Fig. 1). At the end of the study period, the diabetic group had lower body weights than the control group ($p < 0.05$). Treatment of diabetic rats with insulin for 8 weeks showed a significant increase (18.5 %) in the body weight compared to the untreated diabetic group ($p < 0.05$) (Fig. 2).

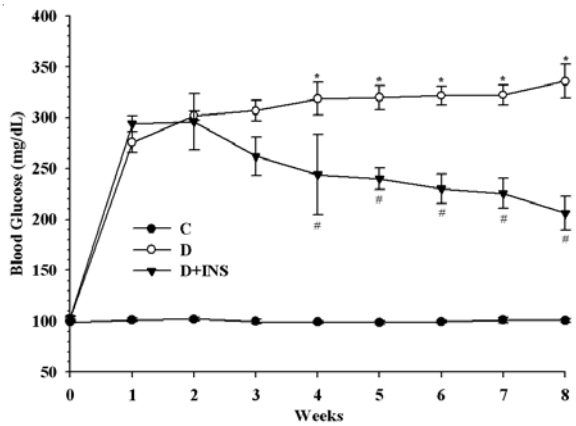

Fig. 1. Time-course effect of insulin on blood glucose. Rats were treated with insulin for 8 weeks. Blood glucose was determined every other week. Data are presented as mean ± SD. *$p < 0.05$ in D + INS groups compared with D. #$p < 0.05$ in D compared with C. the same group in the same week. $p < 0.05$; paired Student's t-test

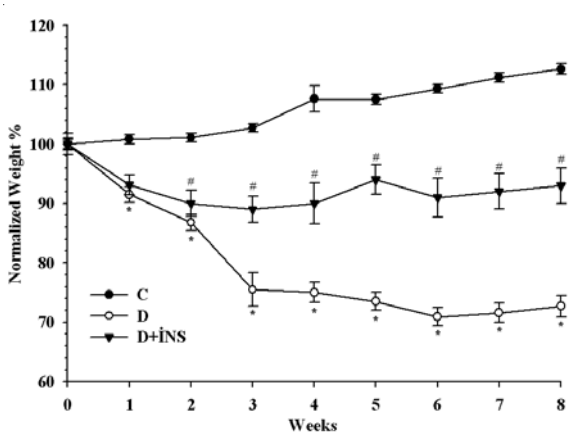

Fig. 2. Effect of insulin on body weight. Rats were treated with insulin for 8 weeks. Body weight was determined every other week. Data are presented as mean ± SD. *$p < 0.05$ in D groups compared with D + INS. #$p < 0.05$ in D compared with C. the same group in the same week; paired Student's t-test

Effects of insulin on HbA1c and lipid profiles tolerance in diabetic rats: Lipid profiles and HbA1C levels of study groups are shown in Fig. 3. Insulin had significant effects on 1 HbA1C and lipid profiles in diabetic rats ($p < 0.05$) Fig. 4. HbA1C and TC, TAG and VLDL levels were significantly increased in diabetic group compared with other groups ($p < 0.05$). LDL-C levels were not significantly different between groups (Fig. 4).

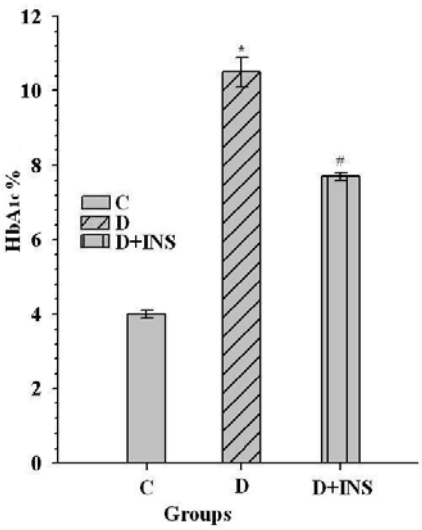

Fig. 3. Plasma HbA1c of experimental and control groups. C indicates control; D, diabetic; D + INS, diabetic + insulin. Data are expressed as mean ± SEM. *p < 0.05 in D compared with C,. #p < 0.05 in D compared with D+INS, C

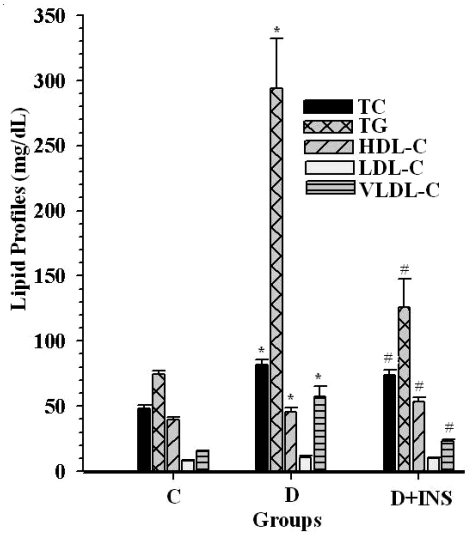

Fig. 4. Plasma lipid metabolic profiles of experimental and control groups. C indicates control; D, diabetic; D + INS, diabetic + insulin. Data are expressed as mean ± SEM. *p < 0.05 in D compared with C, #p < 0.05 in D compared with D + INS, C, TAG, triacylglycerol; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; VLDL-C, very low-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol

Effects of insulin on malondialdehyde levels in diabetic rats: Treatment of diabetic rats with INS for 8 weeks brought about a significant decrease in MDA levels compared with the D and C groups ($p < 0.05$). MDA levels in D + INS group were significantly decreased compared with the D group ($p < 0.001$). In the diabetic group, MDA levels were found to be increased compared with the C group and the differences between these groups were significant ($p < 0.015$) (Table-1).

Effects of rosiglitazone on 3-nitroyrosine levels in diabetic rats: In the diabetic group, 3-NT levels were significantly increased compared to control rats with RSG ($p < 0.001$). Treatment of diabetic rats with insulin for 8 weeks caused a significant decrease in 3-NT levels compared to the diabetic group ($p < 0.001$) (Table-2).

TABLE-1
MALONDIALDEHYDE

Groups	MDA (namol/mL)
C (N = 8)	23.0 ± 1.1
D (N = 8)	30.0 ± 0.9#
D + INS (N = 8)	19.2 ± 2.1*

Effects of INS on the MDA levels of rat left ventricular papillary muscle. C = control rats, D = diabetic rat, D + INS insulin-treated diabetic rats. *$p < 0.05$ in D + INS group compared with D. #$p < 0.05$ in D compared with D + INS, C

TABLE-2
MEASUREMENT OF 3-NITROYROSINE

Groups	3- NT (10 µmol/L)
C (N = 8)	0.08 ± 0.07
D (N = 8)	0.10 ± 0.02#
D + INS (N = 8)	0.07 ± 0.04*

Effects of INS on the 3-nitrotyrosine/total tyrosine levels of rat left ventricular. C = control rats, D = diabetic rat, D + INS: Insulin-treated diabetic rats. *$p < 0.05$ in D + INS group compared with D. #$p < 0.05$ in D compared with D + INS, C

In the present study, following induction of diabetes by injection of streptozotocin, treatment with insulin prevented the increase in blood glucose levels, as well as the reduction in body weight, apparently by preventing the development of metabolic changes due to diabetes. However, treatment of the diabetic rats with insulin prevent hyperglycemia or the weight loss in this group. Oxidative damage was observed in the experimentally diabetic rat left ventricular tissues, as assessed by the increase in 3-NT, malondialdehyde and lipid prolifies levels. The impaired responses were partly restored by insulin but were restored to the diabetes levels following treatment with insulin.

Several animal studies using a model of diabetes have examined the effect of treatment insulin (INS) on abnormal lipid parameters. Significant decreases in triacylglycerol (TAG); total cholesterol (TC) and very low-density lipoprotein-cholesterol (VLDL-C) were noted in all studies[13]. In a study, insulin gave rise a decrease of lipid profiles and MDA of diabetic rats treated with insulin[13]. In present study, the administration of INS, decreased TC, TAG and VLDL-C, TC levels in the STZ diabetic rat. HDL-C and LDL-C, levels were not statistically significant between the D and D+INS groups.

Patients with Type 2 diabetes have modified levels of various markers of oxidative stress, indicating an overproduction of free radicals, which have a key role in the development of diabetic vascular complications[12,13]. When focusing on diabetic vascular disease, it is the fine balance between the levels of superoxide (O^{2-}), peroxynitrite ($ONOO^{-}$) and NO that is the key in determining the extent of vascular damage. This property may reflect 'preventative' action since these agents do not show direct antioxidant scavenging activity on free radicals, but block several mechanisms

that in hyperglycaemic or hyperlipidaemic conditions lead to the generation of oxidative stress.

However in this study, we demonstrate that differentiation of rat left ventricular tissue is associated with increased production of reactive oxygen species (ROS). Soluble uric acid stimulated an increase in NADPH oxidase activity and ROS production in mature adipocytes but not in preadipocytes. The stimulation of NADPH oxidase-dependent ROS by uric acid resulted in activation of MAP kinases p38 and ERK1/2, a decrease in nitric oxide bioavailability and an increase in protein nitrosylation and lipid oxidation. Collectively, present results suggest that hyperuricemia induces redox-dependent signaling and oxidative stress in adipocytes. Since oxidative stress in the adipose tissue has recently been recognized as a major cause of insulin resistance and cardiovascular disease, hyperuricemia-induced alterations in oxidative homeostasis in the adipose tissue might play an important role in these derangements.

This study, shows that the hypertriglyceridemia state is accompanied by a significant increase of 3-NT in diabetic rat left ventricular. The demonstration that lower glycemic levels, in the presence of the same level of triglycerides after insulin NPH administration, is associated with less production of 3-NT suggests a specific and direct role of hyperglycemia in favouring 3-NT formation. The possibility that hyperglycemia may lead to NT formation is supported by studies showing the presence of 3-NT in the kidney, heart and plasma of diabetic patients[10,16] and in aortic tissue from diabetic cynomolgus monkeys[17]. The direct role of high blood glucose levels has been substantiated by showing that acute hyperglycemia induces 3-NT overproduction, even in the plasma of healthy subjects and in working hearts from rats[11,18].

Therefore, it is not surprising that the hyperglycemia phase, which is accompanied in diabetic rat left ventricular by a marked increase of glycemia, will show an acute increase in 3-NT. It is also interesting to underline that 3-NT increase after in diabetic subjects, whose diabetic triglyceride and blood glucose levels showed only a slight and significant increase.

Peroxynitrite is a potent oxidant and nitrating agent that leads to a host of potentially harmful events, including VLDL peroxidation[19], depletion of antioxidant defenses[20] and inactivation of enzymes[21]. In addition, it can be directly cytotoxic for endothelial cells[22]. All these events may convincingly be involved in the pathogenesis of atherosclerosis and CVD. This hypothesis is strongly supported by the recent finding that the increased apoptosis of myocytes, endothelial cells and fibroblasts in heart biopsies from diabetic patients and in hearts from streptozotocin-induced diabetic rats[23] is selectively associated with the levels of 3-NT found in those cells. Furthermore, the demonstration that 3-NT can induce endothelial dysfunction by itself[24] and that it is present in atherosclerotic lesions in humans and diabetic cynomolgus monkeys[17] is additional evidence that peroxynitrite production may be strongly involved in atherogenesis. In conclusion, the present study demonstrates

a strong correlation between hyperglycemia and 3-NT production in with 8 weeks insulin-treated diabetic rats. Because 3-NT is a marker of the production of peroxynitrite, which in turn is a putative determinant of oxidative stress and cardiovascular disease, present data reinforce the hypothesis that the post diabetes state, particularly in diabetic subjects because of their marked hyperglycemia, may have an important role in the pathogenesis of cardiovascular disease through the production of oxidative stress.

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