Full Length Research Paper

The effect of metformin and exercise on serum lipids, nitric oxide synthase and liver nitric oxide levels in streptozotocin-nicotinamide induced diabetic rats

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The objective of this study was to determine the effects of metformin treatment and exercise intervention on lipid metabolism, nitric oxide (NO), nitric oxide synthase (NOS) and glucose levels in streptozotocin-nicotinamide (STZ-NA) induced diabetic rats. Male wistar rats were used to conduct the study and they were divided into five groups of 8 rats in each. These groups are: Control group, diabetes group (STZ-NA), diabetes and metformin (DMet) group, diabetes and exercise (DE) group, and diabetes + exercise + metformin (DEMet) group. The STZ-NA induced diabetic rats were used as a model of type 2 diabetes. Metformin was orally administered to rats. For exercise intervention, rats were forced to run in a running wheel for 10 min each day (20 m/min/day). At the end of experimental period, blood glucose, glycated hemoglobin (HbA1c) levels, plasma lecithin:cholesterol acyltransferase (LCAT) levels, serum triglyceride (TG), cholesterol, high density lipoprotein (HDL), Apo A-I, NOS and liver NO levels were determined. Metformin caused beneficial changes in blood glucose, HbA1c levels, and HDL in type 2 diabetic rats. Both administration of metformin and exercise intervention independently caused beneficial changes in blood glucose. However, the surprising result found was that exercise intervention did not optimize the result of metformin treatment. The other finding of this study was that exercise intervention increased cholesterol and NOS level but exercise combined with metformin was not better than exercise alone. Thus, it appears from the present study that metformin and exercise combination has no effect.

Key words: Diabetes, exercise, lecithin:cholesterol acyltransferase (LCAT), metformin, nitric oxide synthase.

INTRODUCTION

Diabetes mellitus is one of the most widespread disease in both developed and developing countries. Type 2 diabetes is based mainly on decreased insulin responsiveness due to the down-regulation of hormone receptors on peripheral cells, leading to hyperglycemia (Hrabak et al., 2006). Treatment of hyperglycaemia in type 2 diabetes mellitus (T2DM) is necessary to relieve acute symptoms and to reduce the risk of chronic vascular complications (Bailey, 2011). Lifestyle modifications including diet and exercise are often insufficient for reducing glucose and weight, and most patients with type 2 diabetes will require pharmacotherapy to treat their hyperglycemia (Blevins, 2010).

Exercise is recommended for the treatment of type 2 diabetes to assist in weight management and glucose control and also it is shown that, it increases whole-body insulin sensitivity. Exercise has potential to improve the glucose lowering effect of oral antihyperglycemic therapy (Tang and Reed, 2001). Metformin belongs to biguanide family, used as an orally active antihyperglycemic drug in the treatment of human type 2 diabetes (Saravanan and Pari, 2006). Metformin controls hyperglycemia via ameliorating insulin sensitivity and decreasing insulin

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levels and it also regulates lipid profiles (Katakam et al., 2000). It is reported that metformin has antioxidant activity which is independent of its effect on insulin activity. Furthermore, Sartoretta et al. (2005) mentioned that metformin increases NO activity without increasing NO expression in n-STZ diabetic rats (Majithiya and Balaraman, 2006). NO is an important cellular regulator (Kingwell, 2000). The production of NO from L-arginine is synthesized by a family of enzymes called nitric oxide synthases (NOS) (Gao, 2010; Ozbayer et al., 2011). It is known that NO plays important roles on muscle contractility (smooth muscle, cardiac, and skeletal muscle), platelet aggregation, metabolism (glucose, lipid, and amino acid metabolism), neuronal activity, and immune responses (Gao, 2010). Morever, there is increasing evidence that NO is an important haemodynamic and metabolic regulator during the physical activity (Kingwell, 2000).

Studies also emphases that lower NO production causes hyperlipidemia (Zand et al., 2011; Ma et al., 2011; Goto et al. 1999). Hyperlipidemia is one of the metabolic complications of both clinical and experimental diabetes. Treatment of hyperlipidemia in diabetes involves improving of glycemic control, exercise and the use of lipid lowering diets and drugs (Saravanan and Pari, 2006).

Although metformin is widely used in the treatment of type 2 diabetes, its mechanism of action is unclear. There are few reports on short-term effects of the drug. Also the relationship between NO synyhesis and the metabolism of lipid and glucose is incomplete. The aim of this study was to evaluate the effects of short term metformin treatment and exercise intervention on lipid metabolism, lecithin:cholesterol acyltransferase (LCAT), NO, NOS and glucose levels in Streptozotocin-Nicotinamide induced type 2 diabetes.

MATERIALS AND METHODS

Animals

Three to four months old male Wistar rats (n=40) weighing 200 to 280 g were supplied from the Eskisehir Osmangazi University Experimental Research Center. Rats were housed in polycarbonate cages in a temperature (21±2°C) and humidity (45 to 55%) controlled room. They were fed with a standard rat chow (Oguzlar Yem, Eskisehir, Turkey) and allowed to drink water *ad libitum*. This study was approved by Eskisehir Osmangazi University İnstitutional Local Animal Care comittee. Animals were divided into five groups of 8 rats in each; (I) control, (II) diabetes (STZ+NA) group, (III) diabetes and exercise (DE) group, (IV) diabetes and metformin (DMet) group, and (V) diabetes + exercise + metformin (DEMet) group.

Experimental design

At the beginning of the experimental period, tail blood samples were taken for blood glucose determination by using glucometer (Accutrend, Boehringer Mannheim, Germany). Rats in each group

except control group were given intraperitoneally (i:p) 290 mg/kg of nicotinamide dissolved in saline. After 15 min, they were given intraperitoneally 60 mg/kg STZ dissolved in citrate buffer (pH 4.5) (Maisello et al., 1998).

After administration of STZ and NA, fasting blood glucose levels were determined every week from tail veins of all animals during the next 5-week period using glucometer (Accutrend, Boehringer Mannheim, Germany). Fasting blood glucose levels were measured. The animals that showed stable hyperglycemia (range, 115 to 120 mg/dl) were used for experiments as diabetics. After acclimatization period of 5 weeks following the induction of diabetes, the experimental period lasted for six days.

(I) Control group: Any medication or treatment was given to control group.
(II) Diabetes (STZ+NA) group: The diabetes group did not receive any medication.
(III) Diabetes and exercise (DE) group: The DE group did not receive any medication. Instead, they were forced to run in a running wheel at a speed of 20 m/min for 10 min each day.
(IV) Diabetes and metformin (DMet) group: The DMet group received metformin orally at 50 mg/kg for the first day and adding of 50 mg/kg metformin for each day and finally for the last day of the experimental period the rats received 300 mg/kg metformin orally (Katakam et al., 2000).
(V) Diabetes + exercise + metformin (DEMet) group: DEMet group recieved metformin orally at 50 mg/kg for the first day and adding of 50 mg/kg metformin for each day and finally for last day of the experimental period the rats received 300 mg/kg metformin orally (Katakam et al., 2000) similar to DMet group. Additionally, they were forced to run in a running wheel at a speed of 20 m/min for 10 min each day (Copp et al., 2010).

At the end of experimental period, the tail blood samples were taken for blood glucose determination for the second time. Intracardiac whole blood samples were collected, under ether anesthesia, into two different tubes. First tubes were centrifugated at 3500 g for ten minutes to separate the serum and used for the determination of lipid profiles. The second tubes including EDTA, used firstly for the determination of HbA1c and then used for lecithin cholesterol acyltransferase (LCAT) assay. HbA1c was measured by using Roche-Hitachi kit via Hitachi 911 Analyzer.

After the collection of blood samples, rats were sacrified. Under aseptic conditions, a midline incison was made and their livers were removed.

Liver nitrite

NO as a free radical is relatively unstable in oxygenated solutions where it rapidly and spontaneously reacts with molecular oxygen to yield a variety of nitrogen oxides. It was demonstrated that the only stable products formed by spontaneous decomposition of NO in oxygenated solutions are nitrites and nitrates, thus they were measured as indicators for NO production (El-Mahmoudy et al., 2005). Liver tissues were homogenized in Tris-HCl buffer (5 mM containing 2 mM EDTA, pH 7.4). The supernatants were prepared by centrifugation at 2000 g for 5 min (Futter et al., 2001). Nitrate in supernatants was assayed by a modification of cadmium (Cd)-reduction method; the produced was nitrite determined by diazotization of sulfanilamide and coupling to naphthylethylenediamine. After samples were deproteinized by Somogyi reagent, the nitrate was reduced by Cu-coated Cd in glycine buffer at pH 9.7 (2.5 to 3 g of Cd granules for a 4-ml reaction mixture). Nitrite was then measured spectrophotometrically by adding of the 2.5 ml of deproteinized sample, 1 ml of H_2O, 1 ml of sulfanilamide solution and 1 ml of N-naphthylethylenediamine solution. Absorbances were determined at 545 nm after 20 to 60

min of incubation. The concentration of nitrite levels in the serum was determined by using standard curve, which was constructed by using sodium nitrite as the standard (Cortas and Wakid, 1990). The amount of protein was evaluated by using biurethe method (Layne et al., 1957).

NOS assay

NOS level in the serum was measured by using nitric oxide assay kit (BIOXYTECH®, Oxis International, Inc, Portland, OR, USA, Cat. No: 22113) via Alpha Prime Multiplate ELISA test Analyzer (SFR1/AES Laboratoire, France).

Lecithin: Cholesterol acyltranferase assay

LCAT activity in plasma was measured by the colorimetric method described by Nagasaki and Akanuma (1977). Synthetic dipalmitoyl lecithin solution was added to the incubation medium and then the change in free cholesterol content following the incubation period was analyzed by a combined enzymatic method by using cholesterol oxidase and peroxidase.

Serum lipids

Lipid profiles (serum triglyceride, TG, cholesterol, high density lipoprotein, HDL, Apo A-I) were measured by using Roche-Hitachi kit via Roche Diagnostic Modular Analyzer.

Statistical analysis

Data were expressed as mean ± standard error. Statistical Package for the Social Sciences (SPSS) standard version 13.0 for Windows was used to evaluate the data. Statistical analyses were performed by one-way analysis of variance (ANOVA) test. Groups were compared by using Fisher LSD test.

RESULT

The glucose levels in tail venoz samples taken at the beginning and at the end of the study were similar to that of the control rats (p>0.05). Blood glucose levels were increased significantly in STZ-NA group compared to the controls (p<0.001). On the other hand, decreases in all of the intervention groups (DMet, DE, DEMet) according to the STZ-NA group (p<0.001, p<0.05, p<0.01 respectively) (Table 1).

HbA1c level was increased significantly in STZ-NA group as compared to healthy controls, (p<0.001).Treatment with metformin was ameliorated HbA1c level in DMet group versus STZ-NA, (p<0.01). Cholesterol increased significantly in STZ-NA group compared to control group (p<0.001). In the DE and DEMet group, cholesterol level was decreased sinificantly compared to STZ-NA group (p<0.001). TG level in STZ-NA group did not change signficantly compared to control group, (p>0,05). Apo A-I and HDL levels was decreased in STZ-NA group compared to control group, (p<0.001 and p<0.001, respectively). Metformin treatment

increased HDL level in DMet and DEMet groups compared to STZ-NA group (p<0.001 and p<0.001 nrespectively) (Table 1).

Plasma LCAT activity in the STZ-NA group was lower than in the control group (p<0.001). In plasma LCAT activity, there was no difference between STZ-NA group and other groups (p>0.05) (Table 2). Liver NO was decreased significantly in STZ-NA group compared to control group, (p<0.001). In liver NO, there was no difference between STZ-NA group and other groups. STZ-NA group showed a reduced NOS activity compared to control group (p<0.01). It was observed that NOS activities in DE and DEMet groups was increased significantly as compared to STZ-NA group, (p<0.001 and p<0.005, nrespectively) (Table 2).

DISCUSSION

This is the first study giving information about measured NO, NOS and LCAT activities in STZ-NA induced diabetic rats and also including data for metformin, exercise and combined exercise-metformin treatment in STZ-NA induced diabetic rats. In recent years, STZ and nicotinamide are used to induce type 2 diabetes mellitus in rats. The diabetic animal model induced by administration of STZ and nicotinamide shows hyperglycemia and reduced glucose intolarance, but still able to secrete insulin in the presence of glucose (Li et al., 2011). In our study, blood glucose levels were increased in STZ-NA group compared to the controls although there were no differences between the glucose levels of the control rats at the beginning and at the end of the study. The same increase is reported by many researchers (Saravanan and Pari, 2006; Novelli et al., 2001; Novelli et al., 2004).

In type 2 diabetes mellitus, increased physical activity decreases fasting plasma glucose and insulin levels, suggesting increased insulin sensitivity (Lehman et al., 2001). In our study, serum glucose levels decreased after exercise intervention in DE group (p<0.05). This result supports Reaven's results demostrating "exercise decreases glucose concentrations in animal models of type 2 diabetes" (Reaven and Chang, 1981). The reduction of glucose levels in exercise group may be associated with a compensatory function in pancreatic β-cells. In a swim exercise model of type 2 diabetic rats, regular exercise increased the rates of β-cell proliferation and β-cell mass compared with sedentary control animals (Yi et al., 2009). Also Choi et al. (2006) showed that exercise ameliorates glucose homeostasis by improving β-cell function and mass as well as reducing insulin resistance in 90% of pancreatectomized rats. Like insulin, exercise increases glucose uptake into skeletal muscle. Exercise increase glucose uptake into skeletal, via a pathway independent to that of insulin. Glucose can not passively diffuse into a cell and must be transported through the cell membrane by glucose transporters

Table 1. Blood glucose, HbA1c and some lipid profiles of the studied groups.

Variable	Control	STZ-NA	DMet	DE	DEMet
Glucose (mg/dl)	82.63±8.3	120.25±2.6***	104.25±7.0[###]	113.25±6.1[#]	110.50±3.7[##]
HbA1c (mg/dl)	3.61±0.4	4.69±0.4***	3.90±0.5[##]	4.26±0.4[n.s]	4.26±0.6[n.s]
Apo A-I (mg/dl)	3.30±0.8	1.53±0.2***	1.54±0.2[n.s]	1.21±0.4[n.s]	1.34±0.4[n.s]
HDL (mg/dl)	52.00±4.3	44.50±5.1**	54.25±5.1[###]	46.13±3.1[n.s]	47.75±6.1[###]
Cholesterol (mg/dl)	54.13±8.6	71.38±6.3***	67.50±5.3[n.s]	59.38±4.1[###]	57.13±5.3[###]
TG (mg/dl)	60.50±7.2	67.38±2.9[n.s]	61.63±8.3[n.s]	62.88±4.1[n.s]	60.00±4.8[n.s]

***, $P<0.001$ versus control group, **, $P<0.01$ versus control group, [###], $p<0.001$ versus STZ-NA group, [##], $p<0.01$ versus STZ-NA group, [#], $p<0.05$ versus STZ-NA group, [n.s], $p>0.05$ versus STZ-NA group.

Table 2. Liver NO levels, serum NOS levels and plasma LCAT activity of the studied groups.

Variable	Control	STZ-NA	DMet	DE	DEMet
NO (µmol/mg protein)	0.78±0.2	0.58±0.1***	0.60±0.1[n.s]	0.51±0.1[n.s]	0.52±0.1[n.s]
NOS (pmol/ml)	85.3±8.4	75.9±4.9**	80.1±5.5[n.s]	84.1±5.2[##]	81.8±4.1[#]
LCAT (µmol/ml/saat)	6.28±1.3	4.29±1.1***	5.08±0.7[n.s]	4.44±0.9[n.s]	4.51±1.2[n.s]

***, $P<0.001$ versus control group, **, $P<0.01$ versus control group, [###], $p<0.001$ versus STZ-NA group, [##], $p<0.01$ versus STZ-NA group, [#], $p<0.05$ versus STZ-NA group, [n.s], $p>0.05$ versus STZ-NA group.

(GLUTS). GLUT4 is the major glucose transporter isoform expressed in skeletal muscle, and has a large capacity to increase glucose transport across the cell membrane through facilitative diffusion. The mechanism (s) through which exercise stimulates GLUT4 translocation and glucose uptake appear to arise from local factors within skeletal muscle such as NO. There is building evidence that NO is important for the regulation of glucose uptake into skeletal muscle of rodents and humans during contraction/exercise independent of blood flow, and likely to be associated with signaling GLUT4 translocation (Merry and McConell, 2009). Exercise, from a metabolic perspective, may promote increased plasma arginine levels by increasing amino acid mobilization from skeletal muscle, thus normalizing cellular production of NO (Newsholme et al., 2010).

Current therapeutic strategies for type 2 diabetes involve insulin and oral antidiabetic agents via stimulating pancreatic insulin secretion, reducing hepatic glucose production, delaying digestion and absorption of intestinal carbohydrate or improving insulin action (Tahara et al., 2008). In our study, we used metformine as antidiabetic agent. It has been reported that metformin has multiple activities, including suppression of hepatic glucose, production and improvement of peripheral insulin sensitivity, in type 2 diabetic patients. So, it reduces hyperglycemia (Tang and Reed, 2001; Saravanan and Pari, 2006; Okamoto et al., 2008; Anurag and Anuradha, 2002). In the present study, we observed that metformin rapidly decreased blood glucose levels. In our study, serum glucose levels of the intervention groups (Groups III, IV and V) were significantly lower than the STZ-NA group (Group III) (p<0.05, p<0.001, p<0.01, respectively).

In our study, metformin treatment reduced HbA1c levels, similar to the decrease in blood glucose levels. On the other hand, we did not observe the same decrease in both of the DE group (Group III) and DEMet groups (Group V). This can be explained by the theory of Valeri et al. (2004) describing that, levels of HbA1c are probably determined by factors other than glucose metabolism, including the rate of haemoglobin glycation and red cell survival. There is need of new studies regarding the corelation between exercise and HbA1c levels.

Diabetes is associated with profound alterations in plasma lipid and lipoprotein profile. Hyperlipidemia is recognized by elevated levels of cholesterol, TG and phospholipids; and changes in lipoprotein composition (Saravanan and Pari, 2006). In our study, STZ-NA application significantly increased cholesterol and TG levels while it decreased HDL levels. The increase in serum lipids in the diabetics is mainly due to the increased mobilization of free fatty acids from peripheral deposit, since insulin inhibits hormone sensitive lipase. Treatment of hyperlipidemia in diabetes involves improving glycemic control, exercise and drugs (Tang and Reed, 2001). It is reported that exercise (Lampman and Schteingart, 1991), and metformin (Saravanan and Pari, 2006; Katakam et al., 2000) each have beneficial effects on lipid regulation in type 2 diabetes. In our study, HDL levels increased by metformin treatment. This increase also had been observed in DEMet group (Group V). While, exercise, had no effect on HDL levels. The increase in combined group could be explained by the

effect of metformin treatment. On the other hand, metformin treatment had no effect on cholesterol levels while exercise decreases it. However, neither metformin treatment nor exercise had effect on TG levels. Maybe the reason of this ineffectiveness was due to the shortness of the experimental period. There was no enough time for the appearance of the decrease.

It is generally accepted that, improved glycoregulation will result in normalization of elevated lipid levels (Iızuka, 1989). LCAT is an important enzyme involved in HDL metabolism. LCAT increases HDL size by transferring 2-acyl groups from lecithin or phosphatidylethanolamine to free cholesterol. LCAT activity therefore results in the generation of cholesteryl esters that are retained in the core of HDL particles. Hyperglycemia results in non-enzymatic glycation of various proteins. Glycation of HDL on serine residues in its most abundant protein moiety, Apo A-I, causes conformational changes eventually resulting in reduced LCAT activity (Nakhjavani et al., 2008). It is well known that HDL and LCAT activity responses to glycemic control in diabetic patients depending on the type of treatment (Iızuka, 1989). In the present study, LCAT activity was significantly decreased in STZ-NA group compared to the control group. There were no statistical differences between STZ-NA group and the treatment groups although LCAT activity was increased in the treatment groups. Probably, the duration of treatment was not adequate for the response of diabetic control. LCAT contributes to maintaining a con-centration gradient of free cholesterol between the cell membrane and plasma, and defective LCAT activity might result in an increase in the plasma cholesterol concentration. In our study, similar to the LCAT activity, the Apo A-I levels were not increased probably again due to the insufficient experimental period. The decrease in LCAT reactivity could affect the reverse cholesterol transport of HDL and thereby contribute to the athero-sclerotic process in diabetics (Kızıltunc et al., 1997). In our study, the cholesterol levels were decreased in treatment groups. High plasma TG and changes in LCAT are probably due to the abnormalities in HDL composition (Kingwell et al., 2002). In our study, when compared to control group there was a decrease in the levels of HDL and Apo A-I in STZ-NA group, supporting that data obtained by decrease in the activity of LCAT in type 2 diabetes can have negative influences on HDL and Apo A-I levels (Nobecourt et al., 2007).

Contrary to our study, Lehman et al. (2001) showed that physical activity in patients with type 2 diabetes led to an overall increase in LCAT. Exercise causes pro-duction of reactive oxygen species (ROS) (Santangelo et al., 2003). Abnormally high levels of free radicals, lipid peroxidation and simultaneous decline in antioxidant defense mechanism can lead to damage of cellular organelles and enzymes (Saravanan and Pari, 2006).

In the present study, when compared with control group, STZ-NA group showed a significant decrease in

liver NO levels. Decreased NO function is multifactorial, involving both impaired endothelial production of NO and accelerated NO inactivation. The diminished NO synthesis associated with type 2 diabetes has been attributed to uncoupling of receptor-mediated signal transduction (Angeline et al., 2011), to a relative deficiency of L-arginine and to a decrease availability of eNOS cofactor, BH_4 (Wu and Meininger, 2009; Angeline et al., 2011). The reduced NO bioavailability is caused largely by decreased NO production, which may result from accumulation of asymmetric dimethylarginine (ADMA) or reduced bioavailability of L-arginine (Gates et al., 2007). ADMA is major endogenous inhibitor of NOS and therefore NO bioavailability. Higher levels of ADMA are associated with insulin resistance and diabetes (Kanazawa et al., 2010).

The liver is the main organ of oxidative and detoxifying process. In many diseases, biomarkers of oxidative stress are elevated in the liver at an early stage (Dias et al., 2005). NO is rapidly inactivated by O_2 and it has been reported that an enhanced formation of O_2 radical may be involved in the accelerated breakdown of NO. Metformin has antioxidant activity which is independent of its effect on insulin activity. The protective effect of metformin against oxidative stress may prevent the breakdown of nitric oxide. Moreover in the recent studies, metformin is reported to increase NO activity without increasing nitric oxide expression in type 2 diabetic n-STZ model (Majithiya and Balaraman, 2006). This increase in NO activity may be due to the reduction in oxidative stress by metformin. In our study, STZ-NA administration decreased NO and NOS levels. But any of the treatment skills did not increase NO levels while exercise has positive effect on NOS levels. The deficiency on the increase of NO levels may be attributed to the insufficient dose of metformin or the insufficient time for the emergence of increase.

Increase in the formation of advanced glycation end products (AGEs) due to hyperglycemia have the capacity to suppress the expression of constitutive nitric oxide synthase activity and also quench NO, contributing to decreased levels of NO in diabetes (Mohan and Das, 2000). Free fatty acids, elevated in insulin resistant states, also inhibit eNOS activity and causes decreases in NO production. Sartoretto et al. (2005) showed that metformin treatment ameliorated total NOS activity without correcting the increased blood glucose. In the present study, metformin did not ameliorate the serum NOS activity while it decreases blood glucose. On the other hand, exercise caused an increase in both exercise and exercise and metformine groups, by supporting Kurowska, who reported that acute exercise increases NOS activity and NO release in rat experiments (Kurowska, 2002). Our study showed a significant increase in serum NOS level in DE and DEMet groups compared to STZ-NA group.

In conclusion, these results suggested that both

administration of metformin and exercise intervention decreased serum glucose levels independently. However, the surprising result that was found was that exercise intervention did not optimize the result of metformin treatment. The other finding of this study was that exercise intervention increased NOS level but exercise combined with metformin was not better than exercise alone. Thus, it appears from the present study that metformin and exercise combination has no effect. Longitudinal studies are required to confirm our results.

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