Evaluation of mitochondria-linked PI3K/Akt/GLUT-4 signaling pathway in rodent liver and pancreatic tissues in sub-chronic model of type-2 diabetes mellitus (T2DM)

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder that continues to be a major health problem worldwide. It affects approximately 6% of the world population and is estimated that the number of diabetic individuals will increase by 54% till 2030 (Shaw et al., 2010). More than 90% of diabetic individuals have type-2 diabetes mellitus (T2DM), a consequence of a high-fat diet, sedentary lifestyle and genetic predisposition (Hotamisligil, 2006; Shaw et al., 2010). The pathophysiology of T2DM involves dysfunction of several organs including liver and pancreas (Uysal et al., 2005; Gürpınar et al., 2012). Therefore, it is imperative to evaluate the underlying mechanism of dysfunction of such tissues in T2DM condition.

The pathogenesis of T2DM involves a progressive development of insulin resistance (IR) in several tissues (Jurysta et al., 2013; Bazotte et al., 2014). It has been well documented that there is impairment in the activation of the insulin receptor substrate/phopstidylionositol-3 kinase/Akt (IRS/PI3K/Akt) signaling pathway in IR (Benomar et al., 2006). This leads to the reduction of the translocation of the glucose transporter-4 (GLUT-4) from the cytosol to the cell surface in several tissues (Furtado et al., 2002; Watson et al., 2004; Leney and Tavaré, 2009). This ultimately results in the reduction of glucose utilization in these tissues. Further, it has also been reported that the pancreatic beta-cell dysfunction in T2DM condition is associated with the impairment in PI3K/Akt signaling pathway (Kwon et al., 2014). Further, GLUT-4 is also reported in the pancreatic tissues in addition to GLUT-1 (Jurysta et al., 2013). However, there is no report on the PI3K/Akt/GLUT-4 signaling pathway in the pancreatic tissues. Therefore, the PI3K/Akt/GLUT-4 signaling pathway has been proposed to be evaluated as a functional attribute to IR relative to liver and pancreatic dysfunction in the T2DM condition.

Mitochondrial dysfunction is now generally accepted as a consistent attribute in most of the tissues in T2DM condition (Petersen et al., 2003; Lowell and Shulman, 2005; Kim et al., 2008; Johannsen and Ravussin, 2009). It is associated with aberrant electron transport chain activity in terms of increase in the activity of respiratory complex enzymes. It is also associated with oxidative stress in terms of increase in the level of lipid peroxidation and compromised antioxidant enzyme activities in tissues in T2DM condition (Garabadu and Krishnamurthy, 2013; Garabadu and Krishnamurthy, 2014). These events lead to a consequence of mitochondrial-dependent apoptosis in such tissues in T2DM condition. The Na^+-K^+-ATPase activity and mitochondrial dysfunction can be considered as a marker for metabolic crisis at cellular and sub-cellular levels respectively (Yang et al., 2009; Zarros et al., 2009). In cell-line study, mitochondrial DNA depletion by chronic exposure of ethidium bromide impairs PI3K/Akt/GLUT-4 signaling pathway in myocytes (Park et al., 2005). Moreover, the translocation of cytosolic phosphorylated Akt to mitochondria causes increase in complex-V activity and ATP production in diabetic rats (Yang et al., 2013). Thus, it is presumed that mitochondrial dysfunction is strongly associated with the derailment of PI3K/Akt/GLUT-4 signaling pathway in T2DM condition. However, till date there is no report on the PI3K/Akt/GLUT-4 signaling pathway in peripheral tissues such as liver and pancreas through pharmacological manipulation of mitochondrial dysfunction in T2DM condition.

Metformin, a well known mitochondrial complex-I inhibitor, is commonly used to mitigate IR in the treatment of T2DM (Davidson and Peters, 1997). Preclinical and clinical studies suggest that metformin ameliorates mitochondria dysfunction, defects in insulin signaling pathway and deteriorated Na^+-K^+-ATPase activity in diabetic condition (Pryor et al., 2000; Kumar and Dey, 2002; Yuan et al., 2003; Sonntag et al., 2005; Kukidome et al., 2006;

Chakraborty et al., 2011; Qiu et al., 2012). Therefore, the present study was undertaken to evaluate the effect of mitochondrial complex-I inhibitor, metformin, on T2DM-induced aberrant PI3K/Akt/GLUT-4 signaling pathway in liver and pancreatic tissues of rats. The indices such as glucose homeostasis, β-cell function, hyperinsulinemia, IR, hypercorticosteronemia and dyslipidemia were estimated in T2DM animals to evaluate the anti-diabetic activity of metformin. The activity of Na^+-K^+-ATPase is evaluated as a marker of metabolic energy crisis in discrete tissues. Furthermore, the overt effect of metformin on mitochondrial dysfunction in terms of mitochondrial function (respiratory complex enzyme activity), integrity (mitochondrial membrane potential) and mitochondrial-linked apoptosis (levels of expression of cytochrome-C, caspase-9 and caspase-3) was evaluated in both the tissues.

Experimental procedures

Animals

Male Charles Foster strain albino rats (230 ± 20 g) were purchased from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University (BHU) and were housed in polypropylene cages under controlled environmental conditions (25 ± 1 °C, 45-55% relative humidity and 12:12 h light/dark cycle). The experiments were conducted in accordance with the principles of laboratory animal care (National Research Council US Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). Experiments on animals were approved by the Institutional Animal Ethics Committee of BHU, Varanasi, India (Protocol No: Dean/11-12/CAEC/328). The animals had free access to commercial rat feed (Doodh dhara Pashu Ahar, India) and water *ad libitum* unless stated otherwise during the experiment. Animals were acclimatized for at least one week before using them for experiments and exposed only once to every experiment.

Chemicals

Streptozotocine, thiobarbituric acid (TBA), tetra methyl rhodamine methylester (TMRM) and dexamethasone were procured from Sigma (St. Louis, MO, USA). Antibodies such as phosphor-Akt^{ser473} (p-Akt), total Akt, GLUT-4, cytochrome-C, caspase-9, procaspase-3, caspase-3 and beta-actin were purchased from Abcam Plc., Cambridge, USA. All other chemicals and reagents were available commercially from local suppliers and were of analytical grade.

Induction of T2DM in animals

The T2DM was induced in overnight fasted rats by a single injection of streptozotocine (45 mg/kg, i.p.), 15 min after nicotinamide (110 mg/kg, i.p.) administration. Streptozotocine was dissolved in 0.1 M citrate buffer (pH 4.5) and nicotinamide was dissolved in physiological saline (Masiello et al., 1998).

Experimental design

The whole study protocol consisted of three independent sets of experiments. The animals were acclimatized for seven days and were randomly divided into four groups of six animals each namely control, metformin treated control (MET), type 2 diabetic (T2DM) and metformin administered T2DM (DMET) in each of the experiment. The experimental protocol was followed for 13 days for all experiments. The day animals received the streptozotocine and nicotinamide injection was considered as day-1 (D-1). On D-7, after 1 hr to blood collection, either metformin (25 mg/kg, *p.o.*; Yanardag et al., 2005) or vehicle was administrated to the respective group animals. This treatment schedule was continued for seven consecutive days i.e., from D-7 to D-13 of the experimental design. The experiment 1 and 2 were performed for the oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) respectively after 1 hr to last dose on D-13. In experiment 3, after 1 hr to last dose on D-13 all the animals were killed by

decapitation. The blood, liver and pancreas were collected and stored immediately at -80 °C till further study.

Oral glucose tolerance test (OGTT)

The OGTT is considered as a classical and model-based estimate of beta-cell function (Rijkelijkhuizen et al., 2009). The OGTT was performed on overnight fasted rats on 13th day of the experimental schedule. Metformin and vehicle were given 60 min prior to glucose administration (2 g/kg, i.g.). The blood samples were collected through retro-orbital puncture just before glucose load (0 min) and at 30, 60 and 120 min after glucose administration. Plasma glucose concentrations were determined with glucose GOD PAP kit (Priman Instrument Pvt. Ltd., India) based on glucose oxidase method (Wang et al., 2013).

Insulin tolerance test (ITT)

The ITT is a simple and reliable method of estimating insulin sensitivity (Duseja et al., 2007; Muniyappa et al., 2008). The ITT was performed on overnight fasted rats on 13th day of the experimental schedule. Metformin and vehicle were given 60 min prior to insulin administration (0.4 IU/kg, s.c.). The blood samples were collected through retro-orbital puncture just before glucose load (0 min) and at 30, 60 and 90 min after insulin administration. Plasma glucose concentrations were determined with glucose GOD PAP kit (Priman Instrument Pvt. Ltd., India) based on glucose oxidase method (Wang et al., 2013).

Estimation of plasma glucose and lipid profile

On D-13 of the experimental protocol, 1 ml of blood was collected through retro-orbital puncture and centrifuged at 3000 × *g* for 5 min at 4 °C to obtain plasma for measuring the glucose, insulin, triglyceride, high density lipoprotein (HDL) and low density lipoprotein (LDL) levels. The insulin was estimated by ELISA kit following manufacturer's instructions. The plasma glucose,

triglyceride, HDL and LDL were determined spectrophotometrically (BioTek Instruments Inc., Epoch®, USA) in triplicate using commercial available kits.

Insulin resistance and β-cell function

Homoeostasis model assessment (HOMA) of IR (HOMA-IR) and HOMA of β-cell function (HOMA-B) were calculated by the HOMA method using the following equations (Matthews et al., 1985): IR (HOMA-IR) ¼ (fasting glucose (mmol/l) × fasting insulin (mIU/ml))/22·5, and β-cell function (HOMA-B) ¼ (20 × fasting insulin (mIU/ml))/(fasting glucose (mmol/l) – 3·5).

Determination of Na^+-K^+-ATPase and Mg^{2+}-ATPase activities

The Na^+-K^+-ATPase activity was calculated from the difference between total ATPase activity (Na^+, K^+, Mg^{2+}-dependent ATPase) and Mg^{2+}-dependent ATPase activity. Total ATPase activity was assayed in an incubation medium consisting of 50 mM Tris-HCl, pH7.4, 120 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 240 mM sucrose, 1 mM ethylenediamine tetraacetic acid K_2-salt (K^+-EDTA), 3 mM disodium ATP and 80−100 μg protein of the homogenate in a final volume of 1 ml. Ouabain (1 mM) was added in order to determine the activity of Mg^{2+}-ATPase. The reaction was started by adding ATP and stopped after an incubation period of 20 min by addition of 2 ml mixture of 1% lubrol and 1% ammonium molybdate in 0.9 M H_2SO_4(Tsakiris, 2001). The yellow color which developed was read at 390 nm. The activity was expressed as nmol Pi/min/mg protein.

Assessment of mitochondrial function, integrity and oxidative stress

Isolation of mitochondria from rat tissue

The mitochondria were isolated from liver and pancreatic tissues by following standard protocol (Pedersen et al., 1978). The mitochondrial protein content was estimated using the method of Lowry et al. (1951).

Estimation of mitochondrial respiratory complex-I, II, IV and V activity

The activity of NADH dehydrogenase (complex-I) was measured by catalytic oxidation of NADH with potassium ferricyanide as an artificial electron acceptor at excitation and emission wavelengths for NADH were 350 nm and 470 nm, respectively (Shapiro et al., 1979). Activity of NADH dehydrogenase was expressed as nmole NADH oxidised/min/mg protein. The mitochondrial succinate dehydrogenase (SDH; complex-II) was determined by the progressive reduction of nitro blue tetrazolium (NBT) to an insoluble colored compound, diformazan at 570 nm (Sally and Margaret, 1989). The SDH activity was expressed as micromole formazan produced/min/mg protein. The activity of cytochrome oxidase (complex-IV) was measured in mitochondrial fraction in presence of reduced cytochrome c at 550 nm for 3 min (Storrie and Madden, 1990). Results were expressed as nmole cytochrome c oxidized/min/mg protein ($\varepsilon 550$ = 19.6 $mmol^{-1}cm^{-1}$). The F1-F0 synthase (complex-V) was measured by incubating mitochondrial suspension in ATPase buffer (Griffiths and Houghton, 1974) and the phosphate content was measured (Fiske and Subbarao, 1925). Results were expressed as nmole ATP hydrolyzed/min/mg protein.

Evaluation of MMP in discrete tissues

The rhodamine dye taken up by mitochondria was measured in spectrofluorometer (Hitachi, F-2500) at an excitation λ of 535 ± 10 nm and emission λ of 580 ± 10 nm (Huang, 2002). The results were expressed as fluorescence intensity/mg protein.

Estimation of lipid peroxidation (LPO) and nitric oxide (NO) level

Mitochondrial malondialdehyde (MDA) content was measured as a marker of LPO at 532 nm (Ohkawa et al., 1979). The extent of LPO was expressed as micromoles of MDA/mg protein.

The NO level was estimated as a marker for nitrosative stress (Green et al., 1982) and expressed as nmoles of NO/mg protein.

Assessment of superoxide dismutase (SOD) and catalase (CAT) activity

Superoxide dismutase (SOD) activity was determined by the reduction of NBT in presence of phenazine-methosulphate and NADH at 560 nm using n-butanol as blank (Kakkar et al., 1984). A single unit of the enzyme was expressed as 50% inhibition of NBT reduction/minute/mg protein. Decomposition of hydrogen peroxide in presence of CAT was followed at 240 nm (Beers and Sizer, 1952). The results were expressed as units (U) of CAT activity/min/mg of protein.

Immunoblotting

The preparation of cellular membrane fraction was performed as described previously (Nishiumi and Ashida, 2007). In brief, after washed three times with PBS, tissues were lysed with buffer A [Tris, pH 8.0, 50 mM; dithiothreitol, 0.5 mM; NP-40, 0.1% (v/v); protease inhibitors (phenylmethylsulphonyl fluoride, 1 mM; leupeptin, 5 $mg \cdot mL^{-1}$; and aprotinin, 5 $mg \cdot mL^{-1}$) and phosphatase inhibitors (NaF, 10 mM and Na_3VO_4, 1 mM)]. The lysates were then centrifuged at 1000 × *g* for 10 min at 4 °C. Pellets were resuspended in NP-40 free buffer A in ice for another 10 min with occasional vortex, and recentrifuged at 1000 × *g* for 10 min at 4 °C. The pellets were resuspended in buffer A and placed in ice for 1 h with occasional vortexing, and centrifuged at 16 000 × *g* for 20 min at 4 °C. The supernatant was collected as the plasma membrane fraction and stored at -80 °C until use. The supernatants from the first and second spins at 1000 × *g* were combined and centrifuged at 16 000 × *g* for 20 min at 4 °C. The resultant supernatant was collected and used as the cytosol fraction. Protein concentrations were determined according to Bradford (1976) in each fraction. A standard plot was generated using

bovine serum albumin. An aliquot of each sample were electrophoresed in 10% SDS-PAGE gels for Akt, p-Akt, GLUT-4, cytochrome-C, caspase-9, procaspase-3 and caspase-3 proteins, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. The membrane was incubated overnight with rabbit anti-Akt (Abcam Plc., Cambridge, USA), anti-p-Akt (Abcam Plc., Cambridge, USA), anti-GLUT-4 (Abcam Plc., Cambridge, USA), anti-cytochrome-C (Abcam Plc., Cambridge, USA), anti-caspase-9 (Abcam Plc., Cambridge, USA), anti-procaspase-3 (Abcam Plc., Cambridge, USA) and anti-caspase-3 (Abcam Plc., Cambridge, USA) polyclonal primary antibody at a dilution of 1:1000, 1:1000, 1:1000, 1:500; 1:500; 1:500 and 1:500 respectively. After detection with the desired antibodies against the proteins of interest the membrane was stripped with stripping buffer (25 mM Glycine pH 2.0, 2% SDS for 30 min at room temperature) and reprobed overnight with rabbit anti β-actin (Santa Cruz Biotechnology Inc.; Santa Cruz, California, USA) polyclonal primary antibody at a dilution of 1:500 to confirm equal loading of protein. Further, membrane was probed with corresponding secondary antibodies. Immunoreactive band of proteins were detected by chemiluminescence using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, USA). Quantification of the results was performed by densitometric scan of films. The immunoreactive area was determined by densitometric analysis using Biovis gel documentation software.

Data Analysis

All the data were mean ± standard error of the mean (SEM). The statistical significance for time-course effect on the plasma glucose level in OGTT and ITT, and body weight in T2DM study were evaluated by using repeated measure two-way analysis of variance (ANOVA) with Bonferroni post hoc test. All other statistical analyses were done using one-way ANOVA with

Newman–Keuls post hoc analysis to monitor significance among groups. $p<0.05$ was considered as significant.

Results

Metformin improves glucose tolerance and insulin sensitivity in OGTT and ITT respectively

Table-1 and 2 depicts the effect of metformin on plasma glucose level of normal and T2DM rats at different time points after administration of glucose and insulin respectively. Repeated measures of two-way ANOVA revealed that there were significant differences for plasma glucose level in OGTT and ITT among groups ([$F(3, 80) = 498.8$; $p<0.05$] and [$F(3, 80) = 1391.0$; $p<0.05$] respectively), time ([$F(3, 80) = 25.1$; $p<0.05$] and [$F(3, 80) = 25.7$; $p<0.05$] respectively) and there was a significant interaction between group and time ([$F(9, 80) = 6.3$; $p<0.05$] and [$F(9, 80) = 5.7$; $p<0.05$] respectively). Post-hoc analysis showed that before loading of either glucose or insulin to the animals (0 min), metformin did not cause any change in the level of plasma glucose in the vehicle treated rats. However, metformin showed a significant decrease in the plasma glucose level in T2DM rats. Metformin did not cause any change in the level of plasma glucose of normal rats at any point of time after administration of either glucose or insulin. The level of plasma glucose was increased at all time points after administration of either glucose or insulin to the animals compared to control and MET group rats. Metformin significantly decreased the plasma glucose level in T2DM rats at all time points after administration of either glucose or insulin.

Metformin mitigates T2DM-induced decrease in body weight in rats

The effect of metformin on change in the body weight of normal and T2DM rats at D-1 and D-13 of the experimental design is illustrated in Table-3. Repeated measures of two-way ANOVA

revealed that there were significant differences in the body weight among groups [F (3, 40) = 3.1; $p<0.05$], time [F (1, 40) = 16.5; $p<0.05$] and there was significant interaction between group and time [F (3, 40) = 6.7; $p<0.05$]. Post-hoc test revealed that metformin treatment to the normal rats did not cause any change in the body weight. There was a significant decrease in the body weight of T2DM rats compared to control and metformin treated normal rats. Metformin treatment improved the T2DM-induced decrease in the body weight in the animals.

Metformin attenuates fasting blood glucose, insulin and IR, and improves β-cell function in T2DM rats

Table-3 demonstrates the effect of metformin on the level of fasting blood glucose and insulin, and the extent of IR, and the function of pancreatic β-cell in normal as well as T2DM rats. Statistical analysis revealed that there were significant differences among groups for the level of fasting blood glucose [F (3, 20) = 539.5; $p<0.05$], insulin [F (3, 20) = 65.8; $p<0.05$], HOMA-IR [F (3, 20) = 1068.6; $p<0.05$] and HOMA-B [F (3, 20) = 55.3; $p<0.05$]. Post-hoc test showed that metformin treatment to normal rats did not cause any significant change in the levels of fasting blood glucose, insulin, HOMA-IR and HOMA-B. The levels of fasting blood glucose, insulin and HOMA-IR were significantly increased and the level of HOMA-B was significantly reduced in T2DM rats compared to control and MET group rats. Metformin attenuated the changes in the levels of fasting blood glucose, insulin, HOMA-IR and HOMA-B in T2DM rats.

Metformin reverses T2DM-induced changes in the plasma level of TC, TG, HDL and LDL

Table-4 documents the effect of metformin on the level of TC, TG, HDL and LDL in plasma of control as well as T2DM rats. Statistical analysis showed that there were significant differences in the plasma level of TC [F (3, 20) = 57.5; $p<0.05$], TG [F (3, 20) = 16.3; $p<0.05$], HDL [F (3, 20) = 49.1; $p<0.05$] and LDL [F (3, 20) = 19.7; $p<0.05$] in animals. Post-hoc test revealed that

metformin treatment to the control animals did not cause any change in the level of all these biochemical parameters. T2DM caused significant increase in the level of TC, TG and LDL in rats compared to control and MET group animals. However, the level of HDL was significantly decreased in the plasma of T2DM animals compared to control and MET group rats. Metformin significantly attenuated the T2DM-induced changes in all these biochemical parameters in plasma of rats.

Metformin attenuates T2DM-induced decrease in Na^+-K^+-ATPase activity in liver and pancreatic tissues

The effect of metformin on the activity of Na^+-K^+-ATPase and Mg^{+2}-ATPase in liver and pancreas of control and T2DM rats is depicted in Table-5. Statistical analysis revealed that there were significant differences in the activity of Na^+-K^+-ATPase in liver [$F(3, 20) = 9.9$; $p<0.05$] and pancreas [$F(3, 20) = 12.9$; $p<0.05$] among groups. However, there were no significant differences in the activity of Mg^{+2}-ATPase in liver [$F(3, 20) = 0.2$; $p>0.05$] and pancreas [$F(3, 20) = 0.02$; $p>0.05$] among groups. Post-hoc test revealed that metformin did not cause any change in the activity of Na^+-K^+-ATPase in liver and pancreas compared to control animals. T2DM induction decreased the activity of Na^+-K^+-ATPase in all the tissues compared to control and MET group rats. Metformin treatment reversed the T2DM-induced decrease in the activity of Na^+-K^+-ATPase in both the tissues.

Metformin attenuates T2DM-induced decrease in the level of expression of p-Akt in liver and pancreatic tissues

Fig-2 illustrates the effect of metformin on the level of expression of Akt (A) and p-Akt (C) in liver and pancreas of T2DM rats. Statistical analysis showed that there were significant differences in the level of expression of p-Akt (D) and the ratio of p-Akt/Akt (E) in liver ([F (3,

8) = 15.1; $p<0.05$] and [F (3, 8) = 24.6; $p<0.05$] respectively) and pancreas ([F (3, 8) = 8.4; $p<0.05$] and [F (3, 8) = 8.9; $p<0.05$] respectively) among groups. However, there were no significant differences in the level of expression of Akt (B) in liver [F (3, 8) = 0.8; $p>0.05$] and pancreas [F (3, 8) = 0.6; $p>0.05$] among groups. Post-hoc test revealed that metformin treatment did not cause any change in either the level of expression of p-Akt or the ratio of p-Akt/Akt in any tissue compared to control group. The level of expression of p-Akt and the ratio of p-Akt/Akt were significantly decreased in all the tissues in the T2DM group rats compared to control and MET group animals. Metformin treatment significantly reversed the T2DM-induced decrease in level of expression of p-Akt and the ratio of p-Akt/Akt in both the tissues.

Metformin attenuates T2DM-induced changes in the translocation of GLUT-4 from cytoplasm to plasma membrane in liver and pancreatic tissues

The effect of metformin on the level of expression of cytoplasmic (A) and membranous (C) GLUT-4 in liver and pancreas of T2DM rats is depicted in Fig-3. Statistical analysis showed that there were significant differences in the level of expression of cytoplasmic (B) and membranous (D) GLUT-4, and the ratio of membranous/cytoplasmic (E) GLUT-4 in liver ([F (3, 8) = 6.8; $p<0.05$], [F (3, 8) = 12.6; $p<0.05$] and [F (3, 8) = 20.6; $p<0.05$] respectively) and pancreas ([F (3, 8) = 8.2; $p<0.05$], [F (3, 8) = 24.9; $p<0.05$] and [F (3, 8) = 16.1; $p<0.05$] respectively) among groups. Post-hoc test showed that metformin treatment did not cause any change in the level of expression of cytoplasmic and membranous GLUT-4, and the ratio of membranous/cytoplasmic GLUT-4 in any tissue in rats compared to control group animals. T2DM induction caused significant increase and decrease in the level of expression of cytoplasmic and membranous GLUT-4 respectively in both tissues compared to control and MET group rats. Moreover, there was a significant decrease in the ratio of membranous/cytoplasmic GLUT-4 in all tissues in

T2DM animals compared to control and MET group rats. Metformin treatment significantly attenuated the T2DM-induced changes in the level of expression of cytoplasmic and membranous GLUT-4, and the ratio of membranous/cytoplasmic GLUT-4 in all the tissues.

Metformin ameliorates T2DM-induced changes in the activity of mitochondrial complex-I, II, IV and V, and level of MMP in liver and pancreatic tissues

Table-6 illustrates the effect of metformin on the activity of mitochondrial complex-I, II, IV and V, and level of MMP in liver and pancreatic tissues in control and T2DM rodents. Statistical analysis showed that there were significant differences in the activity of mitochondrial complex-I, II, IV and V, and level of MMP in liver ([F (3, 20) = 27.8; $p<0.05$], [F (3, 20) = 24.0; $p<0.05$], [F (3, 20) = 44.6; $p<0.05$], [F (3, 20) = 31.7; $p<0.05$] and [F (3, 20) = 6.6; $p<0.05$] respectively) and pancreas ([F (3, 20) = 36.9; $p<0.05$], [F (3, 20) = 28.1; $p<0.05$], [F (3, 20) = 47.9; $p<0.05$], [F (3, 20) = 33.3; $p<0.05$] and [F (3, 20) = 12.4; $p<0.05$] respectively) among groups. Post-hoc test revealed that metformin treatment did not cause any change in the activity of mitochondrial complex-I, II, IV and V, and level of MMP in all the tissues of animals compared to control rats. T2DM significantly increased the activities of all mitochondrial complex enzymes in animals compared to control and MET group rats. Moreover, the level of MMP was significantly decreased in both the tissues of T2DM group rats compared to control and MET group rodents. Metformin treatment mitigated the T2DM-induced changes in all these parameters except mitochondrial complex-II activity in both the tissues.

Metformin mitigates T2DM-induced changes in the level of mitochondrial NO and LPO, and activity of SOD and catalase in liver and pancreatic tissues

The effect of metformin on the level of mitochondrial NO and LPO, and activity of SOD and catalase in liver and pancreatic tissues in control and T2DM rodents is illustrated in Table-7.

Statistical analysis showed that there were significant differences in the level of mitochondrial NO and LPO, and activity of SOD and catalase in liver ([F (3, 20) = 21.8; $p<0.05$], [F (3, 20) = 5.5; $p<0.05$], [F (3, 20) = 5.2; $p<0.05$] and [F (3, 20) = 14.3; $p<0.05$] respectively) and pancreas ([F (3, 20) = 21.3; $p<0.05$], [F (3, 20) = 6.8; $p<0.05$], [F (3, 20) = 6.8; $p<0.05$] and [F (3, 20) = 14.6; $p<0.05$] respectively) among groups. Post-hoc test revealed metformin treatment did not cause any change in the level of mitochondrial NO and LPO and, activity of SOD and catalase in any tissue of the rats compared to control group rodents. The level of mitochondrial NO and LPO were significantly increased and activity of SOD and catalase were significantly decreased in T2DM-induced rats compared to control and MET group animals. Metformin treatment significantly attenuated the T2DM-induced changes in the oxidative stress markers except NO in all the tissues compared to control and MET group rats.

Metformin attenuates T2DM-induced increase in the mitochondrial-linked apoptosis in liver and pancreatic tissues

Fig-4 depicts the effect of metformin on the level of expression of cytochrome-C (A) and caspase-9 (C) in liver and pancreas of T2DM rats. Statistical analysis showed that there were significant differences in the level of expression of cytochrome-C (B) and caspase-9 (D) in liver ([F (3, 8) = 29.6; $p<0.05$] and [F (3, 8) = 17.1; $p<0.05$] respectively) and pancreas ([F (3, 8) = 26.0; $p<0.05$] and [F (3, 8) = 28.1; $p<0.05$] respectively) among groups. Post-hoc test showed that there was no significant change in the level of expression of cytochrome-C and caspase-9 in all the tissues of metformin treated rats compared control group rodents. T2DM significantly increased the level of expression of cytochrome-C and caspase-9 in all the tissues in rats compared to control and MET group animals. Metformin treatment attenuated the T2DM-induced increase in the level of expression of cytochrome-C and caspase-9 in both the tissues.

The effect of metformin on the level of expression of procaspase-3 (A) and caspase-3 (C) in liver and pancreas of T2DM rats is depicted in Fig-5. Statistical analysis showed that there were significant differences in the level of expression of procaspase-3 (B) and caspase-3 (D), and the ratio of caspase-3/procaspase-3 (E) in liver ([$F(3, 8) = 22.4$; $p<0.05$], [$F(3, 8) = 67.0$; $p<0.05$] and [$F(3, 8) = 143.4$; $p<0.05$] respectively) and pancreas ([$F(3, 8) = 26.6$; $p<0.05$], [$F(3, 8) = 48.8$; $p<0.05$] and [$F(3, 8) = 98.7$; $p<0.05$] respectively) among groups. Post-hoc test revealed that metformin did not cause any change either in the level of expression of procaspase-3 and caspase-3 or the ratio of caspase-3/procaspase-3 in any tissue of control group rats. T2DM induction caused significant decrease and increase in the level of expression of procaspase-3 and caspase-3 in all the tissues respectively. Moreover, the ratio of caspase-3/procaspase-3 was significantly decreased in all the tissues in T2DM subjected rats compared to control and MET group animals. Metformin treatment significantly attenuated the T2DM-induced changes in the level of expression of procaspase-3 and caspase-3, and the ratio of caspase-3/procaspase-3 in both tissues.

Table-1: Effect of metformin on the plasma glucose level of normal and T2DM rats in OGTT.

	Plasma glucose level (mmol/L)			
Groups	0 min	30 min	60 min	120 min
Control	4.0 ± 0.12	7.1 ± 0.09	6.9 ± 0.14	5.5 ± 0.19
MET	3.8 ± 0.13	7.1 ± 0.17	6.9 ± 0.20	5.2 ± 0.22
T2DM	24.9 ± 1.16 **a,b**	26.1 ± 1.40 **a,b**	26.3 ± 1.31 **a,b**	26.2 ± 0.99 **a,b**
DMET	7.5 ± 0.39 **a,b,c**	18.2 ± 1.23 **a,b,c**	17.8 ± 1.66 **a,b,c**	17.9 ± 1.05 **a,b,c**

All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to metformin control group (MET) and [c]p<0.05 compared to T2DM (repeated measures of two-way ANOVA followed by Bonferroni post hoc test).

Table-2: Effect of metformin on the plasma glucose level of normal and T2DM rats in ITT.

	Plasma glucose level (mmol/L)			
Groups	0 min	30 min	60 min	90 min
Control	4.1 ± 0.17	2.5 ± 0.28	3.2 ± 0.37	2.9 ± 0.32
MET	4.2 ± 0.22	2.3 ± 0.26	3.0 ± 0.22	2.8 ± 0.32
T2DM	25.5 ± 0.68 **a,b**	19.5 ± 0.73 **a,b**	25.3 ± 0.95 **a,b**	24.6 ± 0.98 **a,b**
DMET	16.7 ± 0.69 **a,b,c**	14.4 ± 0.40 **a,b,c**	18.8 ± 0.51 **a,b,c**	18.4 ± 0.74 **a,b,c**

All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to MET and [c]p<0.05 compared to T2DM (repeated measures of two-way ANOVA followed by Bonferroni post hoc test).

Table-3: Effect of metformin on body weight, fasting blood glucose and insulin level, HOMA-IR and HOMA-B indices of normal and T2DM rats.

Parameters	Control	MET	T2DM	DMET
Body weight on day-1 (g)	237.5 ± 10.90	233.7 ± 6.08	243.7 ± 9.65	244.8 ± 4.14
Body weight on day-13 (g)	231.7 ± 03.05	238.0 ± 4.80	193.5 ± 5.68 **a,b**	221.8 ± 2.87 **c**
Fasting blood glucose (mmol/L)	4.7 ± 0.10	4.7 ± 0.15	25.2 ± 0.82 **a,b**	7.7 ± 0.14 **a,b,c**
Fasting blood Insulin (pmol/L)	75.2 ± 3.85	72.7 ± 3.06	125.3 ± 2.70 **a,b**	87.0 ± 2.11 **a,b,c**
HOMA-IR	2.3 ± 0.15	2.2 ± 0.15	20.2 ± 0.47 **a,b**	4.3 ± 0.11 **a,b,c**
HOMA-B	186.5 ± 10.91	184.5 ± 20.42	16.8 ± 0.96 **a,b**	60.2 ± 2.74 **a,b,c**

All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to MET and [c]p<0.05 compared to T2DM (repeated measures of two-way ANOVA followed by Bonferroni post hoc test for body weight and one-way ANOVA followed by Student–Newman–Keuls test for other analysis).

Table-4: Effect of metformin on the levels of TC, TG, HDL and LDLof normal and T2DM rats.

Groups	TC (mmol/l)	TG (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
Control	1.4 ± 0.04	0.8 ± 0.03	1.7 ± 0.06	0.8 ± 0.04
MET	1.4 ± 0.05	0.8 ± 0.05	1.7 ± 0.03	0.8 ± 0.05
T2DM	2.1 ± 0.06 [a,b]	1.2 ± 0.05 [a,b]	1.0 ± 0.04 [a,b]	1.2 ± 0.05 [a,b]
DMET	1.4 ± 0.03 [a,b,c]	1.0 ± 0.05 [a,b,c]	1.3 ± 0.06 [a,b,c]	1.0 ± 0.02 [a,b,c]

All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to MET and [c]p<0.05 compared to T2DM (one-way ANOVA followed by Student–Newman–Keuls test).

Table-5: Effect of metformin on Na^+-K^+-ATPase and Mg^{+2}-ATPase activity in liver and pancreas of both normal and T2DM rats.

Tissue	**Control**	**MET**	**T2DM**	**DMET**
Na^+-K^+-ATPase activity (nmol Pi/min/mg protein)				
Liver	44.5 ± 3.18	41.9 ± 2.25	26.0 ± 2.30 [a,b]	35.2 ± 2.63[c]
Pancreas	40.0 ± 3.34	37.5 ± 4.22	13.1 ± 1.97 [a,b]	24.8 ± 3.89 [a,b,c]
Mg^{+2}-ATPase activity (nmol Pi/min/mg protein)				
Liver	122.6 ± 08.92	132.5 ± 07.57	129.2 ± 07.16	127.6 ± 10.87
Pancreas	109.9 ± 09.13	110.3 ± 02.66	111.8 ± 08.05	111.6 ± 08.91

All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to MET and [c]p<0.05 compared to T2DM (one-way ANOVA followed by Student–Newman–Keuls test).

Table-6: Effect of metformin on activity of mitochondrial complex-I, II, IV and V, and the level of MMP in liver and pancreas of both normal and T2DM rats.

Tissue	Control	MET	T2DM	DMET
Complex-I(nmol NADHoxidized/min/mg protein)				
Liver	14.1 ± 1.67	13.8 ± 1.43	44.2 ± 4.06 [a,b]	34.5 ± 3.45 [a,b,c]
Pancreas	10.9 ± 1.13	11.5 ± 0.79	44.2 ± 4.07 [a,b]	34.5 ± 3.45 [a,b,c]
Complex-II(µmol formazan produced/min/mg protein)				
Liver	17.4 ± 1.24	18.2 ± 1.89	32.0 ± 1.47 [a,b]	29.8 ± 1.55 [a,b]
Pancreas	17.1 ± 1.16	18.2 ± 1.87	32.3 ± 1.19 [a,b]	29.8 ± 1.56 [a,b]
Complex-IV(nmol cytochrome coxidized/min/mg protein)				
Liver	4.5 ± 0.32	4.5 ± 0.35	9.1 ± 0.37 [a,b]	7.2 ± 0.31 [a,b,c]
Pancreas	6.3 ± 0.45	6.3 ± 0.49	12.8 ± 0.51 [a,b]	10.0 ± 0.35 [a,b,c]
Complex-V(nmol ATPhydrolyzed/mg protein)				
Liver	24.4 ± 2.02	26.7 ± 1.81	53.1 ± 3.46 [a,b]	39.3 ± 1.62 [a,b,c]
Pancreas	19.5 ± 1.61	21.4 ± 1.45	42.4 ± 2.77 [a,b]	32.0 ± 1.06 [a,b,c]
MMP				
Liver	716.8 ± 39.48	716.6 ± 53.57	519.7 ± 15.37 [a,b]	683.4 ± 26.27 [c]
Pancreas	703.2 ± 43.85	663.3 ± 25.48	427.3 ± 39.19 [a,b]	539.8 ± 30.78 [a,b,c]

All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to MET and [c]p<0.05 compared to T2DM (one-way ANOVA followed by Student–Newman–Keuls test).

Table-7: Effect of metformin on mitochondrial levels of NO and LPO, and activity of SOD and CAT in Liver and Pancreas of both normal and T2DM rats.

Tissue	Control	MET	T2DM	DMET
NO(µM MDA/mgprotein)				
Liver	2.6 ± 0.09	2.5 ± 0.12	4.7 ± 0.47 [a,b]	4.6 ± 0.16 [a,b]
Pancreas	3.3 ± 0.12	3.2 ± 0.15	6.0 ± 0.61 [a,b]	5.8 ± 0.20 [a,b]
LPO (nmol of MDA/mg of protein)				
Liver	0.7 ± 0.05	0.7 ± 0.06	1.0 ± 0.10 [a,b]	0.8 ± 0.05 [c]
Pancreas	0.7 ± 0.04	0.7 ± 0.06	1.0 ± 0.10 [a,b]	0.8 ± 0.04 [c]
SOD (Units/min/mg of protein)				
Liver	1.0 ± 0.08	0.9 ± 0.11	0.6 ± 0.04 [a,b]	0.9 ± 0.08 [c]
Pancreas	1.0 ± 0.08	1.0 ± 0.09	0.6 ± 0.04 [a,b]	1.0 ± 0.09 [c]
CAT (Units/min/mg of protein)				
Liver	6.4 ± 0.56	6.1 ± 0.57	2.4 ± 0.17 [a,b]	3.9 ± 0.59 [a,b,c]
Pancreas	6.5 ± 0.48	6.1 ± 0.57	2.4 ± 0.16 [a,b]	4.1 ± 0.65 [a,b,c]

All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to MET and [c]p<0.05 compared to T2DM (one-way ANOVA followed by Student–Newman–Keuls test).

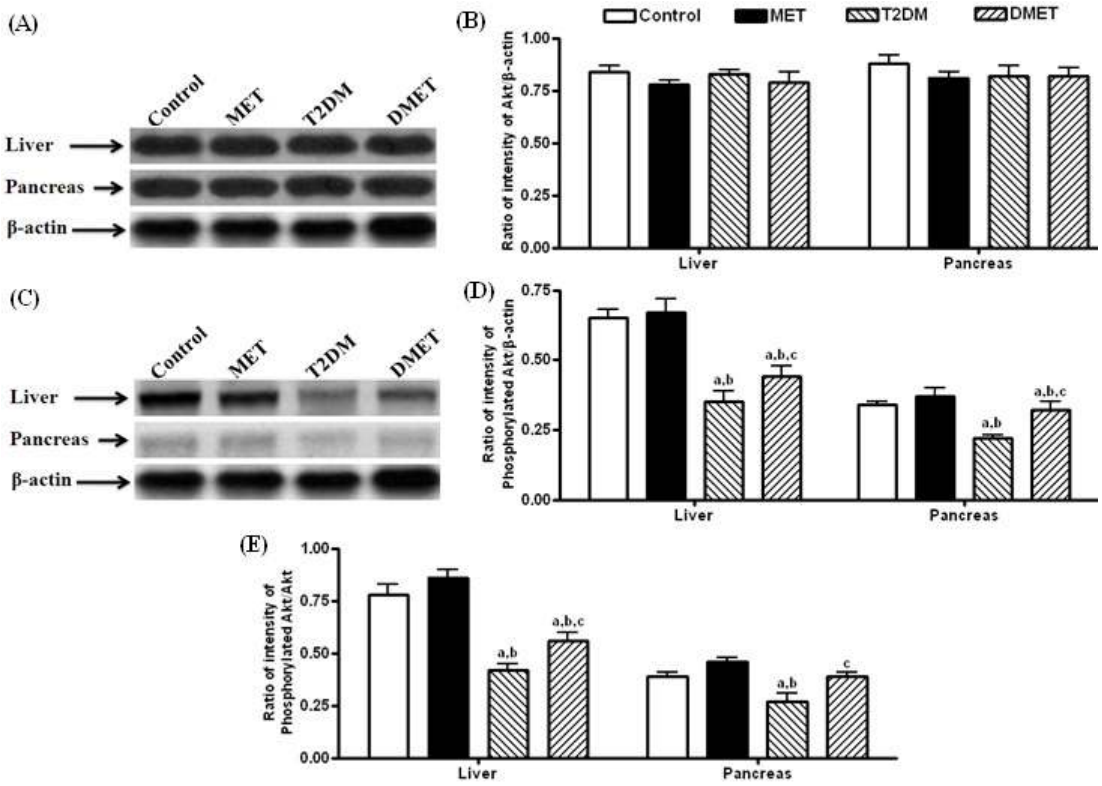

Figure 2: The effect of metformin on the level of expression of Akt (B) and p-Akt (D), and ratio of p-Akt to Akt (E) in liver and pancreas of control and T2DM rats. The blots are representative of Akt (A) and p-Akt (C) in liver and pancreas. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of Akt or p-Akt to β-actin. All values are mean ± SEM of three separate sets of independent experiments. $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to MET and $^{c}p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

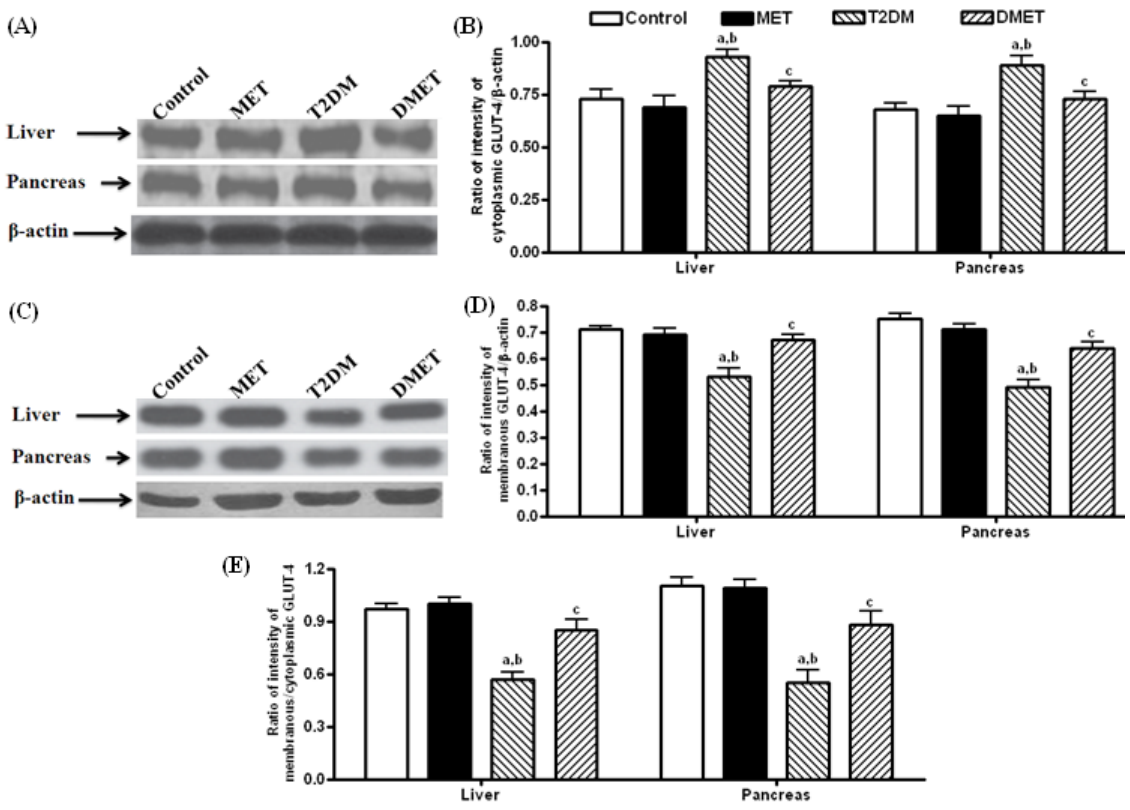

Figure 3: The effect of metformin on the level of expression of cytoplasmic (B) and membranous (D), and ratio of p-membranous to cytoplasmic (E) GLUT-4 in liver and pancreas of control and T2DM rats. The blots are representative of cytoplasmic (A) and membranous (C) GLUT-4 in liver and pancreas. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of cytoplasmic or membranous GLUT-4 to β-actin. All values are mean ± SEM of three separate sets of independent experiments. $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to MET and $^{c}p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

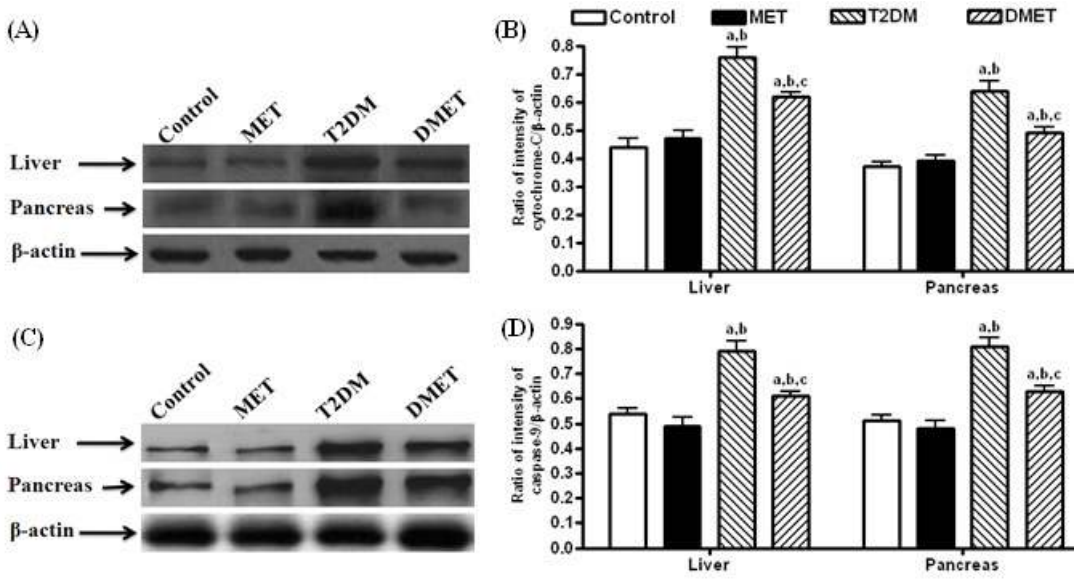

Figure 4: The effect of metformin on the level of expression of cytochrome-C (B) and caspase-9 (D) in liver and pancreas of control and T2DM rats. The blots are representative of cytochrome-C (A) and caspase-9 (C) in liver and pancreas. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of cytochrome-C or caspase-9 to β-actin. All values are mean ± SEM of three separate sets of independent experiments. [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to MET and [c]$p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

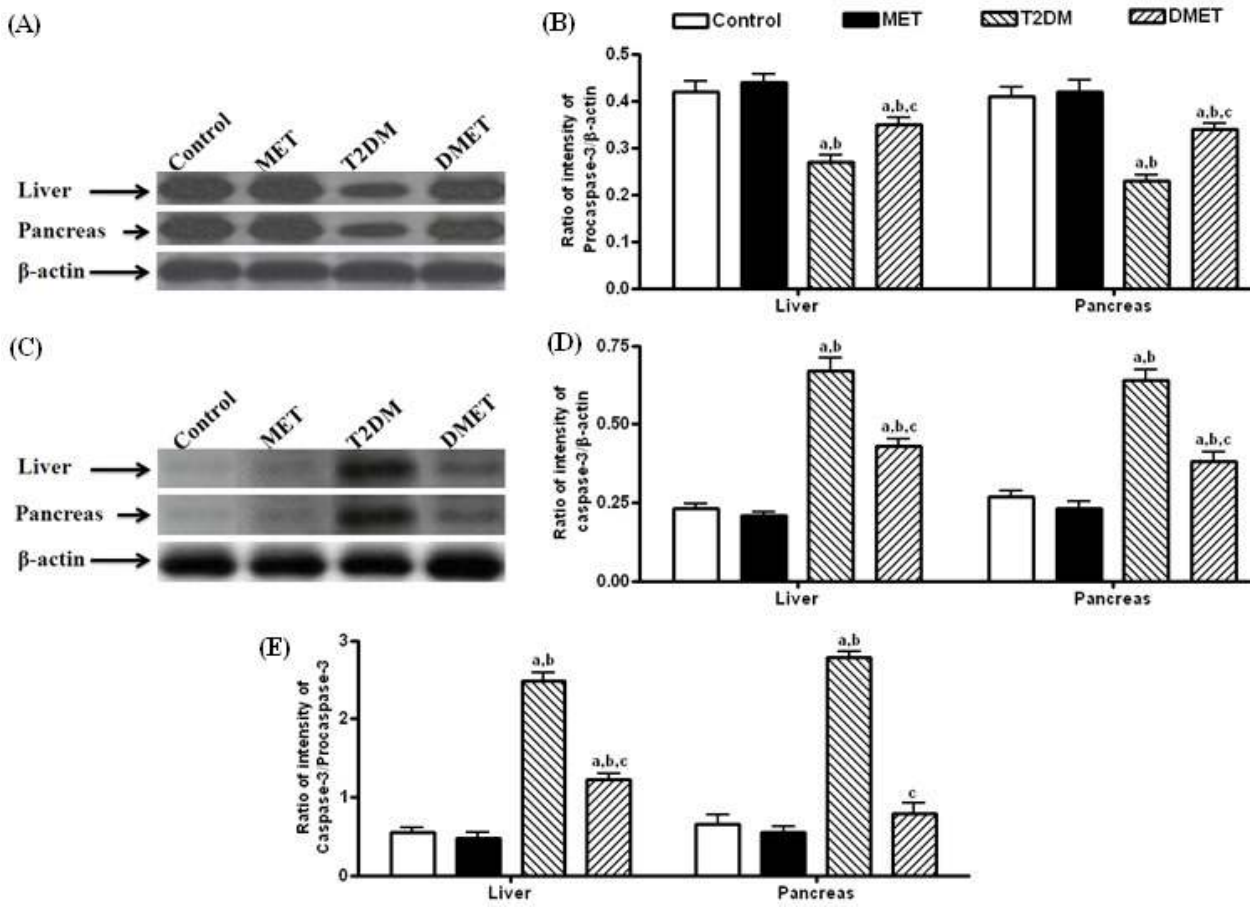

Figure 5: The effect of metformin on the level of expression of procaspase-3 (B) and caspase-3 (D), and ratio of caspase-3 to procaspase-3 (E) in liver and pancreas of control and T2DM rats. The blots are representative of procaspase-3 (A) and caspase-3 (C) in liver and pancreas. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of procaspase-3 or caspase-3 to β-actin. All values are mean ± SEM of three separate sets of independent experiments. $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to MET and $^{c}p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

Discussion

In the present study, we for the first time demonstrate that metformin ameliorated altered liver and pancreatic PI3K/Akt/GLUT-4 signaling pathway in T2DM condition. Metformin exhibited anti-diabetic activity by mitigating the disturbed glucose homeostasis, β-cell dysfunction and hyperinsulinemia in addition to dyslipidemia in T2DM rats. These observations suggest the fact that mitochondria targeted drugs could be therapeutic alternatives in the management of T2DM.

In the present study, metformin attenuated hyperglycemia, hyperinsulinemia, peripheral IR (HOMA-IR), beta-cell dysfunction (HOMA-B) and dyslipidemia in addition to the improvement in glucose and insulin tolerance in T2DM animals similar to earlier reports (Tahara et al., 2008; Garabadu and Krishnamurthy, 2014). Metformin also improved cellular level metabolic crisis in T2DM condition in terms of increase in the activity of Na^+-K^+-ATPase in both the tissues. Similar to our findings, Chakraborty et al. (2011) has reported that metformin attenuated T2DM-induced decrease in the activity of Na^+-K^+-ATPase in erythrocytes. At sub-cellular level, metformin restored T2DM-induced altered PI3K/Akt/GLUT-4 signaling pathway in terms of increase in the level of p-Akt and the translocation of GLUT-4 in both the tissues. It is interesting to note that we for the first time report the effect of mitochondria complex-I inhibitor on PI3K/Akt/GLUT-4 signaling pathway in pancreatic tissue even though such activity has already been documented in other tissues (Jurysta et al., 2013; Blázquez et al., 2014). Further, metformin attenuated T2DM-induced altered mitochondria function in terms of increase in the mitochondrial respiratory complex activities except complex-II in both the tissues. Furthermore, the mitochondria complex-I inhibitor improved mitochondrial integrity in liver and pancreas of T2DM rats. Metformin mitigated the T2DM-induced increase in the extent of lipid peroxidation and failure in antioxidant system (decreased activity of SOD and catalase) in both

tissues similar to that of earlier findings (Chakraborty et al., 2011). However, it was not able to mitigate the T2DM-induced increase in the level of NO in any of the tissues as observed in other report (Chakraborty et al., 2011). Additionally, the mitochondrial complex-I inhibitor mitigated mitochondria-dependent apoptosis as evidenced from its ability to decrease the elevated level of expression of cytochrome-C, caspase-9 and caspase-3 in these tissues similar to that of earlier reports (Asensio-Lopez et al., 2014; Chung et al., 2015). These observations suggest that mitochondrial complex-I inhibitor restores PI3K/Akt/GLUT-4 signaling pathway in peripheral tissues during T2DM.

In conclusion, metformin improved PI3K/Akt/GLUT-4 signaling pathway in liver and pancreas of T2DM animals. Further, it ameliorated T2DM-induced mitochondrial dysfunction, oxidative stress and apoptosis in both the tissues. These results emphasize the fact that mitochondrial complex-I could be a therapeutic target in the drug development for the management of T2DM. Additionally, it can be postulated that mitochondria targeted drugs could be the potential therapeutic candidates in the pharmacotherapy of T2DM.