Evaluation of mitochondria-linked PI3K/Akt/GLUT-4 signaling pathway in rat brain tissues in sub-chronic model of T2DM

Introduction

The pathophysiology of T2DM causes dysfunction in several organs including brain (Uysal et al., 2005; Gürpınar et al., 2012). Additionally, it has been reported that the administration of insulin to central nervous system induces anxiety in animals (Sakandelidze et al., 2011) suggesting the fact that hyperinsulinemia could be a factor for the genesis of anxiety. Moreover, clinical studies demonstrate that the prevalence of anxiety symptoms is considerably higher in T2DM patients (Ball et al., 2002; Collins et al., 2009; Sharma et al., 2010; Kahl et al., 2015; Browne et al., 2015). Therefore, it is imperative to evaluate the underlie mechanism of dysfunction of brain tissue in T2DM condition.

The development of insulin resistance (IR) in the central tissues gains critical attention in the pathogenesis of T2DM (Jurysta et al., 2013; Bazotte et al., 2014). It is documented that there is impairment in the activation of the insulin receptor substrate/phopstidylionositol-3 kinase/Akt (IRS/PI3K/Akt) signaling pathway is observed 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 (Furtado et al., 2002; Watson et al., 2004; Leney et al., 2009) in several tissues including brain (Jurysta et al., 2013; Blázquez et al., 2014). Moreover, IR in terms of decrease in the translocation of GLUT-4 in several anxiety-sensitive brain regions such as hippocampus (HIP) and hypothalamus (HYP) has been reported in post-traumatic stress disorder subjected animals (Cohen et al., 2009). As anxiety is comorbid with T2DM, the PI3K/Akt/GLUT-4 signaling pathway could be considered as a functional attribute to IR for central tissues in the T2DM condition.

In our previous studies, we report the dysfunction of mitochondria in several brain regions in terms of increase in the activity of respiratory complex enzymes, increase in oxidative stress and compromised antioxidant enzyme activities in T2DM rodents (Garabadu and Krishnamurthy, 2013; Garabadu and Krishnamurthy, 2014). Mitochondrial dysfunction in diabetic animals is associated with a reduction of Na^+-K^+-ATPase activity (an enzyme implicated in neuronal excitability as well as metabolic energy production) and an increase in apoptosis (Yang et al., 2009; Zarros et al., 2009). Moreover, mitochondria-dependent apoptosis is observed in anxiety-sensitive brain regions in post-traumatic stress disorder exposed rats (Garabadu et al., 2015) suggesting the fact that mitochondrial dysfunction is common to several tissues to induce apoptotic cell death. 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-induced anxiety condition. However, till date there is no report of any mitochondria targeted drug on the PI3K/Akt/GLUT-4 signaling pathway in this T2DM-induced anxiety condition.

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, 2005; Kukidome et al., 2006; Chakraborty et al., 2011; Qiu et al., 2012). It has also been reported that metformin exerts neuroprotective effect in neurodegenerative disorders such as cerebral ischemia and Parkinson's disease (Zhu et al., 2014; Patil et al., 2014). Additionally, it exhibits anxiolytic effect in the comorbid condition of T2DM and stress (Garabadu and Krishnamurthy, 2014).

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 HIP, HYP, pre-frontal cortex (PFC) and amygdale (AMY) of rats. The indices such as glucose homeostasis, β-cell function, hyperinsulinemia, IR and hypercorticosteronemia were estimated in T2DM animals to evaluate the anti-diabetic activity of metformin. The anxiety-like behaviors of T2DM rats were estimated in several experimental animal models such as open field (OF), hole-board (HB) and elevated plus maze (EPM) tests to elaborate the neuroprotective mechanism of metformin. Further, the activity of Na^+-K^+-ATPase is evaluated as a marker of metabolic crisis at sub-cellular level in discrete brain 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 these discrete tissues.

Experimental procedures

Animals

Male Charles Foster strain albino rats (200–250 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; Garabadu and Krishnamurthy, 2013).

Experimental design

The whole study protocol consisted of single set 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. 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. After 1 hr to last dose on D-13, all the animals were subjected to access the level of anxiety in open field (OF), hole-board (HB) and elevated plus maze (EPM) test paradigms with a time lag of 15 min

between each performance. All the behavioral observations were recorded and quantified with ANY-maze™ (Version-3.72, USA) video-tracking system. Thereafter, all the animals were killed by decapitation. The blood was collected and stored immediately at -80 °C till further study. The brains were removed and microdissected (Palkovits and Brownstein, 1988) into HIP, HYP, PFC and AMY and stored immediately at -80 °C till further study.

Assessment of anxiety in different behavioral models

Open field (OF) test

An open field apparatus, made of plywood and consisting of a square (61 × 61 cm) with high walls (61 × 61 cm), similar to that of Bronstein (1972) was used to study both anxiety-like behavior and locomotor activity in rats. The entire apparatus was painted black except for 6 mm white lines that divided the floor into 16 squares. Each animal was placed in the periphery of the test apparatus and the total distance travelled was recorded for 5 min. The ambulation (the number of squares crossed by the animal), rearing (number of times the animal stood on the hind limbs) and percentage in time spent in the center of the apparatus was considered as a measure of anxiety-like behaviors and the total distance travelled in the OFT was recorded as an index of locomotor activity.

Hole-board (HB) test

The HB apparatus consisted of Perspex box (60 × 60 × 35 cm) with four equidistant holes 4 cm in diameter in the floor. The floor of the box was positioned 12 cm above the ground and divided into nine (20 × 20 cm) squares. For the HB experiments, each animal was placed in the center of the apparatus and allowed to freely explore the apparatus for 5 min. Total number of head-dipping, sniffing and squares crossed were recorded. The ratio of head-dipping/sniffing was also calculated as a measure of anxiety (Garabadu and Krishnamurthy, 2014).

Elevated plus maze (EPM) test

The EPM test was used to assess anxiety-like behaviors in a fabricated apparatus (Garabadu and Krishnamurthy, 2014). The fabricated EPM consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm) with an open roof elevated to a height of 50 cm from the floor in a dimly lit room.The rats were placed individually on the central square of the plus maze facing an enclosed arm. The percentage of entries and time spent into open arm were observed as indices of anxiety-like behaviors. The total arm entries and the speed in whole maze were recorded as an index of locomotor activity. An arm entry was defined when all four limbs of the rat were on the arm (Pellow et al., 1985).

Estimation of plasma glucose and insulin level

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 and corticosterone levels. The insulin was estimated by ELISA kit following manufacturer's instructions. The plasma glucose 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).

Estimation of plasma corticosterone level

The plasma corticosterone was quantified in a HPLC with Ultraviolet (UV) detector system (Waters, USA), according to Woodward and Emery (1987) with minor modifications using

dexamethasone as an internal standard (Garabadu et al., 2011). Briefly, 500 µL of plasma containing known quantity of dexamethasone was extracted with 5 mL of dichloromethane. The dichloromethane extract was evaporated to dryness and dissolved in 100 µL of mobile phase. Twenty microliter of extract was injected into HPLC system for quantification. Mobile phase consisted of methanol:water (70:30) at a flow rate of 1.2 ml/min and corticosterone was detected at 250 nm using UV detector (Model 2487, Waters, USA). The chromatogram was recorded and analyzed with empower software (Version 2).

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, pH 7.4, 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 HIP, HYP, PFC and AMY 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 (ε_{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 brain regions

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·mL^{-1}; and aprotinin, 5 mg·mL^{-1}) and phosphatase inhibitors (NaF, 10 mM and Na_3VO_4, 1 mM)]. The lysates were then centrifuged at $1000 \times g$ for 10 min at 4 °C. Pellets were re-suspended in NP-40 free buffer A in ice for another 10 min with occasional vortex, and re-centrifuged at $1000 \times g$ for 10 min at 4 °C. The pellets were re-suspended in buffer A and placed in ice for 1 h with occasional vortexing, and centrifuged at 16 000 $\times g$ 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 $1000 \times g$ were combined and centrifuged at 16 000 $\times g$ 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 re-probed 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 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 of data 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 ameliorates 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-8. Repeated measures of two-way ANOVA revealed that there were significant differences in the body weight among groups [F (3, 40) = 3.5; p<0.05], time [F (1, 40) = 16.7; p<0.05] and there was significant interaction between group and time [F (3, 40) = 6.9; 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 ameliorates fasting blood glucose, insulin and IR, and improved β-cell function in T2DM rats

Table-8 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) = 880.0; p<0.05], insulin [F (3, 20) = 67.2; p<0.05], HOMA-IR [F (3, 20) = 1566.0; p<0.05] and HOMA-B [F (3, 20) = 55.7; 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 exhibits anxiolytic activity in OF, HB and EPM tests

The effect of metformin on ambulation (A), rearing (B), percentage of time spent in the center (C) and total distance travelled (D) in OF test for control and T2DM rats is illustrated in Fig-6. Statistical analysis showed that there were significant differences among groups in ambulation [F (3, 20) = 13.5; p<0.05], rearing [F (3, 20) = 14.9; p<0.05] and percentage of time spent in the center [F (3, 20) = 41.9; p<0.05] during OF test. However, there was no significant difference among groups in total distance travelled [F (3, 20) = 0.8; p>0.05] during OF test. Post-hoc test revealed that metformin treatment did not cause any significant change in the ambulation, rearing and percentage of time spent in the center of control animals. The number of ambulation and rearing, and the percentage of time spent in the center were decreased in T2DM rats compared to control and MET group animals. However, metformin significantly mitigated the T2DM-induced decrease in the ambulation, rearing and percentage of time spent in the center in rats suggesting its anxiolytic-like activity.

Fig-7 depicts the effect of metformin on number of head-dip (A) and edge-sniff (B), ratio of head-dip/edge sniff (C), and number of squares crossed (D) in HB test paradigm of both control and T2DM rats. One-way ANOVA showed that there were significant differences in head-dip [F (3, 20) = 8.1; p<0.05] and edge-sniff [F (3, 20) = 7.6; p<0.05], and ratio of head-dip/edge sniff [F (3, 20) = 10.8; p<0.05] among groups. However, there was no significant difference in number of squares crossed [F (3, 20) = 0.3; p>0.05] among groups. Post-hoc test revealed that metformin treatment did not cause any significant change in either number of head-dip or edge-sniff, or ratio of head-dip/edge sniff in control rats. The number of head-dip and the ratio of head-dip/edge sniff were significantly decreased and the number of edge-sniff was significantly increased in T2DM rats compared to control and MET group animals. Metformin

treatment exhibited anxiolytic activity in rats in terms of increase in the T2DM-induced decrease in the number of head-dip and ratio of head-dip/edge sniff. Moreover, metformin attenuated the T2DM-induced increase in the number of edge-sniff in rats.

The effect of metformin on percentage entries (A) and time spent (B) into open arm, total arm entries (C) and speed in the whole maze (D) in EPM test paradigm for control and T2DM rats is illustrated in Fig-8. One-way ANOVA revealed that there were significant differences in percentage entries [$F_{(3, 20)}$ = 84.5; $p < 0.05$] and time spent [$F_{(3, 20)}$ = 27.1; $p < 0.05$] into open arm among groups. However, there were no significant differences among groups in total arm entries [$F_{(3, 20)}$ = 0.2; $p > 0.05$] and speed in the whole maze [$F_{(3, 20)}$ = 0.2; $p > 0.05$]. Post-hoc test showed that metformin did not cause any change in the percentage entries and time spent into open arm in control animals. T2DM induction caused significant decrease in the percentage entries and time spent into the open arm in rats compared to control and MET group animals. Metformin treatment attenuated the T2DM-induced decrease in the percentage entries and time spent into open arm in animals.

Metformin reverses T2DM-induced changes in the plasma level of corticosterone

Table-9 documents the effect of metformin on the level of corticosterone in plasma of control as well as T2DM rats. Statistical analysis showed that there were significant differences in the plasma level of corticosterone [$F_{(3, 20)}$ = 92.8; $p < 0.05$] in animals. Post-hoc test revealed that metformin treatment to the control animals did not cause any change in the level of corticosterone. T2DM caused significant increase in the level of corticosterone in rats compared to control and MET group animals. Metformin significantly attenuated the T2DM-induced changes in rat plasma level of corticosterone.

Metformin attenuates T2DM-induced decrease in Na$^+$-K$^+$-ATPase activity in HIP, HYP, PFC and AMY

The effect of metformin on the activity of Na$^+$-K$^+$-ATPase and Mg^{+2}-ATPase in HIP, HYP, PFC and AMY of control and T2DM rats is depicted in Table-10. Statistical analysis revealed that there were significant differences in the activity of Na$^+$-K$^+$-ATPase in HIP [F (3, 20) = 48.1; p<0.05], HYP [F (3, 20) = 38.3; p<0.05], PFC [F (3, 20) = 41.0; p<0.05] and AMY [F (3, 20) = 44.6; p<0.05] among groups. However, there were no significant differences in the activity of Mg^{+2}-ATPase in HIP [F (3, 20) = 2.7; p>0.05], HYP [F (3, 20) = 0.7; p>0.05], PFC [F (3, 20) = 0.1; p>0.05] and AMY [F (3, 20) = 0.4; p>0.05] among groups. Post-hoc test revealed that metformin significantly increased the activity of Na$^+$-K$^+$-ATPase in all the brain regions compared to control rats. 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 all the tissues.

Metformin attenuates T2DM-induced decrease in the level of expression of p-Akt in HIP, HYP, PFC and AMY

Fig-9 illustrates the effect of metformin on the level of expression of Akt and p-Akt in HIP, HYP, PFC and AMY of control and T2DM rats. Statistical analysis showed that there were significant differences in the level of expression of p-Akt and the ratio of p-Akt/Akt in HIP ([F (3, 8) = 24.0; p<0.05] and [F (3, 8) = 51.6; p<0.05] respectively), HYP ([F (3, 8) = 17.7; p<0.05] and [F (3, 8) = 51.8; p<0.05] respectively), PFC ([F (3, 8) = 20.8; p<0.05] and [F (3, 8) = 38.0; p<0.05] respectively) and AMY ([F (3, 8) = 22.6; p<0.05] and [F (3, 8) = 69.7; p<0.05] respectively) among groups. However, there were no significant differences in the level of expression of Akt in HIP [F (3, 8) = 0.1; p>0.05], HYP [F (3, 8) = 0.1; p>0.05], PFC [F (3, 8) =

0.1; p>0.05] and AMY [F (3, 8) = 0.3; 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 all the tissues.

Metformin attenuates T2DM-induced changes in the translocation of GLUT-4 from cytoplasm to plasma membrane in HIP, HYP, PFC and AMY

The effect of metformin on the level of expression of cytoplasmic and membranous GLUT-4 in HIP, HYP, PFC and AMY of control and T2DM rats is depicted in Fig-10. Statistical analysis showed that there were significant differences in the level of expression of cytoplasmic and membranous GLUT-4, and the ratio of membranous/cytoplasmic GLUT-4 in HIP ([F (3, 8) = 6.8; p<0.05], [F (3, 8) = 27.5; p<0.05] and [F (3, 8) = 43.4; p<0.05] respectively), HYP ([F (3, 8) = 20.1; p<0.05], [F (3, 8) = 38.3; p<0.05] and [F (3, 8) = 116.9; p<0.05] respectively), PFC ([F (3, 8) = 6.4; p<0.05], [F (3, 8) = 18.3; p<0.05] and [F (3, 8) = 22.0; p<0.05] respectively) and AMY ([F (3, 8) = 5.2; p<0.05], [F (3, 8) = 15.9; p<0.05] and [F (3, 8) = 21.7; 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 all 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 HIP, HYP, PFC and AMY

Table-11 illustrates the effect of metformin on the activity of mitochondrial complex-I, II, IV and V, and level of MMP in HIP, HYP, PFC and AMY 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 HIP ($[F_{(3, 20)} = 15.8; p<0.05]$, $[F_{(3, 20)} = 106.4; p<0.05]$, $[F_{(3, 20)} = 153.9; p<0.05]$, $[F_{(3, 20)} = 33.0; p<0.05]$ and $[F_{(3, 20)} = 22.6; p<0.05]$ respectively); HYP ($[F_{(3, 20)} = 15.3; p<0.05]$, $[F_{(3, 20)} = 20.7; p<0.05]$, $[F_{(3, 20)} = 47.0; p<0.05]$, $[F_{(3, 20)} = 30.2; p<0.05]$ and $[F_{(3, 20)} = 28.6; p<0.05]$ respectively); PFC ($[F_{(3, 20)} = 9.6; p<0.05]$, $[F_{(3, 20)} = 50.0; p<0.05]$, $[F_{(3, 20)} = 58.7; p<0.05]$, $[F_{(3, 20)} = 39.1; p<0.05]$ and $[F_{(3, 20)} = 40.8; p<0.05]$ respectively) and AMY ($[F_{(3, 20)} = 9.6; p<0.05]$, $[F_{(3, 20)} = 5.9; p<0.05]$, $[F_{(3, 20)} = 51.4; p<0.05]$, $[F_{(3, 20)} = 30.7; p<0.05]$ and $[F_{(3, 20)} = 9.1; p<0.05]$ 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 all 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 all the tissues.

Metformin mitigates T2DM-induced changes in the level of mitochondrial NO and LPO, and activity of SOD and catalase in HIP, HYP, PFC and AMY

The effect of metformin on the level of mitochondrial NO and LPO, and activity of SOD and catalase in HIP, HYP, PFC and AMY tissues in control and T2DM rodents is illustrated in Table-12. Statistical analysis showed that there were significant differences in the level of mitochondrial NO and LPO, and activity of SOD and catalase in HIP ($[F_{(3, 20)} = 19.6$; $p<0.05]$, $[F_{(3, 20)} = 6.3$; $p<0.05]$, $[F_{(3, 20)} = 14.0$; $p<0.05]$ and $[F_{(3, 20)} = 9.9$; $p<0.05]$ respectively); HYP ($[F_{(3, 20)} = 32.3$; $p<0.05]$, $[F_{(3, 20)} = 7.5$; $p<0.05]$, $[F_{(3, 20)} = 32.5$; $p<0.05]$ and $[F_{(3, 20)} = 6.4$; $p<0.05]$ respectively); PFC ($[F_{(3, 20)} = 123.4$; $p<0.05]$, $[F_{(3, 20)} = 21.1$; $p<0.05]$, $[F_{(3, 20)} = 29.5$; $p<0.05]$ and $[F_{(3, 20)} = 115.9$; $p<0.05]$ respectively) and AMY ($[F_{(3, 20)} = 21.2$; $p<0.05]$, $[F_{(3, 20)} = 25.2$; $p<0.05]$, $[F_{(3, 20)} = 6.2$; $p<0.05]$ and $[F_{(3, 20)} = 17.7$; $p<0.05]$ 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 HIP, HYP, PFC and AMY

Fig-11 depicts the effect of metformin on the level of expression of cytochrome-C and caspase-9 in HIP, HYP, PFC and AMY in control and T2DM rats. Statistical analysis showed that there were significant differences in the level of expression of cytochrome-C and caspase-9 in HIP ($[F$

(3, 8) = 19.8; p<0.05] and [F (3, 8) = 28.5; p<0.05] respectively); HYP ([F (3, 8) = 21.6; p<0.05] and [F (3, 8) = 67.6; p<0.05] respectively); PFC ([F (3, 8) = 20.2; p<0.05] and [F (3, 8) = 23.5; p<0.05] respectively) and AMY ([F (3, 8) = 19.2; p<0.05] and [F (3, 8) = 21.8; 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 all the tissues.

The effect of metformin on the level of expression of procaspase-3 and caspase-3, and the ratio of caspase-3/procaspase-3 in HIP, HYP, PFC and AMY in control and T2DM rats is depicted in Fig-12. Statistical analysis showed that there were significant differences in the level of expression of procaspase-3 and caspase-3, and the ratio of caspase-3/procaspase-3 in HIP ([F (3, 8) = 48.1; p<0.05], [F (3, 8) = 29.3; p<0.05] and [F (3, 8) = 111.0; p<0.05] respectively); HYP ([F (3, 8) = 38.6; p<0.05], [F (3, 8) = 10.4; p<0.05] and [F (3, 8) = 26.3; p<0.05] respectively); PFC ([F (3, 8) = 21.9; p<0.05], [F (3, 8) = 10.2; p<0.05] and [F (3, 8) = 19.0; p<0.05] respectively) and AMY ([F (3, 8) = 52.7; p<0.05], [F (3, 8) = 49.4; p<0.05] and [F (3, 8) = 115.1; 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 all the tissues.

Table-8: 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)	236.5 ± 10.60	234.5 ± 6.08	243.6 ± 9.65	243.8 ± 4.14
Body weight on day-13 (g)	232.7 ± 03.02	235.0 ± 4.80	191.5 ± 5.68 [a,b]	224.8 ± 2.87 [c]
Fasting blood glucose (mmol/L)	4.8 ± 0.18	4.5 ± 0.18	24.7 ± 0.58 [a,b]	7.4 ± 0.15 [a,b,c]
Fasting blood Insulin (pmol/L)	74.7 ± 3.78	72.5 ± 3.09	125.1 ± 2.59 [a,b]	87.2 ± 2.19 [a,b,c]
HOMA-IR	2.3 ± 0.11	2.1 ± 0.15	20.3 ± 0.33 [a,b]	4.2 ± 0.23 [a,b,c]
HOMA-B	184.5 ± 10.72	184.3 ± 20.32	16.6 ± 0.62 [a,b]	60.3 ± 2.23 [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-9: Effect of metformin on the level of CORT of normal and T2DM rats.

Groups	CORT(μg/dl)
Control	16.6 ± 1.47
MET	16.5 ± 1.53
T2DM	42.5 ± 0.76 [a,b]
DMET	22.4 ± 1.20 [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-10: Effect of metformin on Na^+-K^+-ATPase and Mg^{+2}-ATPase activity in HIP, HYP, PFC and AMY of both normaland T2DM rats.

Tissue	Control	MET	T2DM	DMET
Na^+-K^+-ATPase activity (nmol Pi/min/mg protein)				
HIP	170.2 ±6.35	209.2 ± 4.98 [a]	126.2 ± 5.60 [a,b]	193.1 ± 3.38 [a,b,c]
HYP	187.4 ± 7.12	216.0 ± 6.57 [a]	127.6 ± 5.69 [a,b]	177.1 ± 3.93 [b,c]
PFC	195.0 ± 8.34	228.6 ± 4.61 [a]	124.7 ± 5.91 [a,b]	149.4 ± 9.18 [a,b,c]
AMY	195.8±8.34	228.5 ± 8.34 [a]	115.5 ± 7.14 [a,b]	141.3 ± 6.98 [a,b,c]
Mg^{+2}-ATPase activity (nmol Pi/min/mg protein)				
HIP	578.9 ± 12.19	584.5 ± 08.12	547.9 ± 15.10	554.8 ± 06.07
HYP	563.3 ± 15.62	539.0 ± 06.57	550.8 ± 12.29	553.7 ± 11.95
PFC	538.9 ± 18.89	541.8 ± 09.95	546.9 ± 12.11	549.7 ± 07.87
AMY	535.2 ± 17.75	560.1 ± 16.56	548.2 ± 14.67	547.4 ± 15.23

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-11: Effect of metformin on activity of mitochondrial complex-I, II, IV and V, and the level of MMP in HIP, HYP, PFC and AMY of both normal and T2DM rats.

Tissue	Control	MET	T2DM	DMET
Complex-I(nmol NADHoxidized/min/mg protein)				
HIP	6.2 ± 0.58	6.1 ± 0.79	11.5 ± 0.56 [a,b]	9.3 ± 0.67 [a,b,c]
HYP	6.3 ± 0.49	6.2 ± 0.82	10.5 ± 0.68 [a,b]	10.8 ± 0.55 [a,b]
PFC	4.3 ± 0.15	4.0 ± 0.35	6.1 ± 0.21 [a,b]	6.1 ± 0.58 [a,b]
AMY	6.8 ± 0.44	5.7 ± 0.82	10.5 ± 0.83 [a,b]	7.8 ± 0.45 [c]
Complex-II(µmol formazan produced/min/mg protein)				
HIP	0.5 ± 0.03	0.5 ± 0.02	1.0 ± 0.02 [a,b]	0.9 ± 0.03 [a,b]
HYP	0.5 ± 0.04	0.5 ± 0.04	0.9 ± 0.01 [a,b]	0.8 ± 0.07 [a,b]
PFC	0.5 ± 0.07	0.5 ± 0.06	1.5 ± 0.06 [a,b]	1.4 ± 0.11 [a,b]
AMY	0.4 ± 0.05	0.4 ± 0.05	0.6 ± 0.05 [a,b]	0.6 ± 0.04 [a,b]
Complex-IV(nmol cytochrome coxidized/min/mg protein)				
HIP	0.9 ± 0.02	1.0 ± 0.05	1.8 ± 0.03 [a,b]	1.5 ± 0.03 [a,b,c]
HYP	1.2 ± 0.08	1.1 ± 0.09	2.1 ± 0.07 [a,b]	1.9 ± 0.06 [a,b]
PFC	1.5 ± 0.11	1.5 ± 0.12	3.0 ± 0.12 [a,b]	3.0 ± 0.11 [a,b]
AMY	1.4 ± 0.06	1.3 ± 0.06	2.1 ± 0.05 [a,b]	1.7 ± 0.04 [a,b,c]
Complex-V(nmol ATPhydrolyzed/mg protein)				
HIP	08.0 ± 0.67	08.8 ± 0.60	17.5 ± 1.1 [a,b]	13.1 ± 0.45 [a,b,c]
HYP	17.4 ± 1.44	19.1 ± 1.29	37.9 ± 2.47 [a,b]	37.0 ± 2.55 [a,b]
PFC	13.4 ± 1.11	14.7 ± 0.99	29.2 ± 1.90 [a,b]	29.1 ± 1.40 [a,b]
AMY	13.9 ± 1.15	15.3 ± 1.04	30.3 ± 1.98 [a,b]	22.3 ± 1.03 [a,b,c]
MMP				
HIP	558.4 ± 8.15	546.5 ± 12.83	393.7 ± 21.66 [a,b]	479.9 ± 17.64 [a,b,c]
HYP	544.0 ± 15.81	519.7 ± 15.82	394.1 ± 08.44 [a,b]	393.8 ± 18.10 [a,b]
PFC	545.0 ± 12.33	510.9 ± 18.96	365.3 ± 17.52 [a,b]	368.4 ± 07.02 [a,b]
AMY	552.7 ± 20.22	552.2 ± 40.67	369.4 ± 18.70 [a,b]	474.8 ± 30.05 [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-12: Effect of metformin on mitochondrial levels of NO and LPO, and activity of SOD and CAT in HIP, HYP, PFC and AMY of both normal and T2DM rats.

Tissue	Control	MET	T2DM	DMET
NO(µM MDA/mg of protein)				
HIP	1.1 ± 0.06	1.2 ± 0.13	2.2 ± 0.15 [a,b]	1.9 ± 0.13 [a,b]
HYP	0.9 ± 0.04	1.0 ± 0.14	2.0 ± 0.13 [a,b]	2.0 ± 0.09 [a,b]
PFC	1.1 ± 0.03	1.0 ± 0.07	2.1 ± 0.04 [a,b]	2.0 ± 0.06 [a,b]
AMY	1.1 ± 0.04	1.1 ± 0.05	2.1 ± 0.21 [a,b]	2.0 ± 0.07 [a,b]
LPO (nmol of MDA/mg of protein)				
HIP	0.5 ± 0.01	0.5 ± 0.05	0.7 ± 0.05 [a,b]	0.6 ± 0.01 [c]
HYP	0.6 ± 0.05	0.6 ± 0.02	0.8 ± 0.04 [a,b]	0.8 ± 0.04 [a,b]
PFC	0.9 ± 0.03	1.0 ± 0.09	1.9 ± 0.11 [a,b]	1.6 ± 0.15 [a,b]
AMY	0.6 ± 0.03	0.7 ± 0.03	1.0 ± 0.03 [a,b]	0.7 ± 0.04 [c]
SOD (Units/min/mg of protein)				
HIP	0.5 ± 0.03	0.4 ± 0.02	0.3 ± 0.02 [a,b]	0.4 ± 0.03 [c]
HYP	0.4 ± 0.01	0.4 ± 0.02	0.2 ± 0.01 [a,b]	0.2 ± 0.01 [a,b]
PFC	0.4 ± 0.01	0.3 ± 0.01	0.2 ± 0.02 [a,b]	0.2 ± 0.01 [a,b]
AMY	0.7 ± 0.02	0.7 ± 0.01	0.3 ± 0.03 [a,b]	0.6 ± 0.13 [c]
CAT (Units/min/mg of protein)				
HIP	2.3 ± 0.10	2.2 ± 0.17	1.5 ± 0.11 [a,b]	2.0 ± 0.04 [c]
HYP	3.8 ± 0.05	3.5 ± 0.06	2.8 ± 0.31 [a,b]	2.8 ± 0.25 [a,b]
PFC	2.7 ± 0.03	2.6 ± 0.10	0.6 ± 0.16 [a,b]	0.6 ± 0.10 [a,b]
AMY	2.5 ± 0.09	2.5 ± 0.12	1.5 ± 0.10 [a,b]	1.9 ± 0.13 [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).
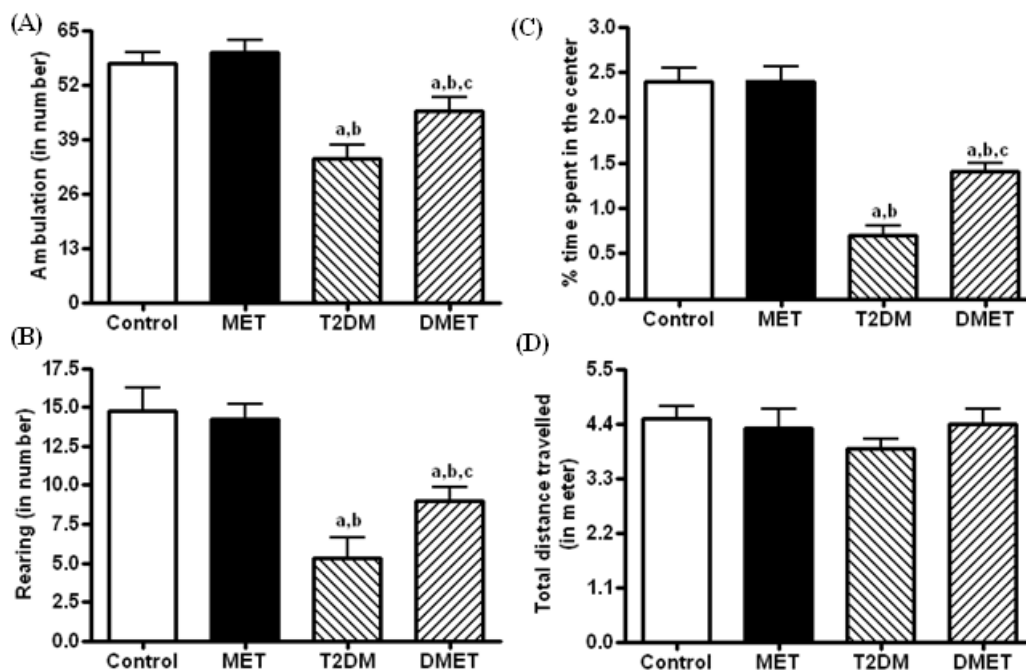
Figure 6: The effect of metformin on ambulation (A), rearing (B), percentage of time spent in the center (C) and total distance travelled (D) in control and T2DM rats during open field test on day-13. All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to metformin control group (MET) and [c]p<0.05 compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].
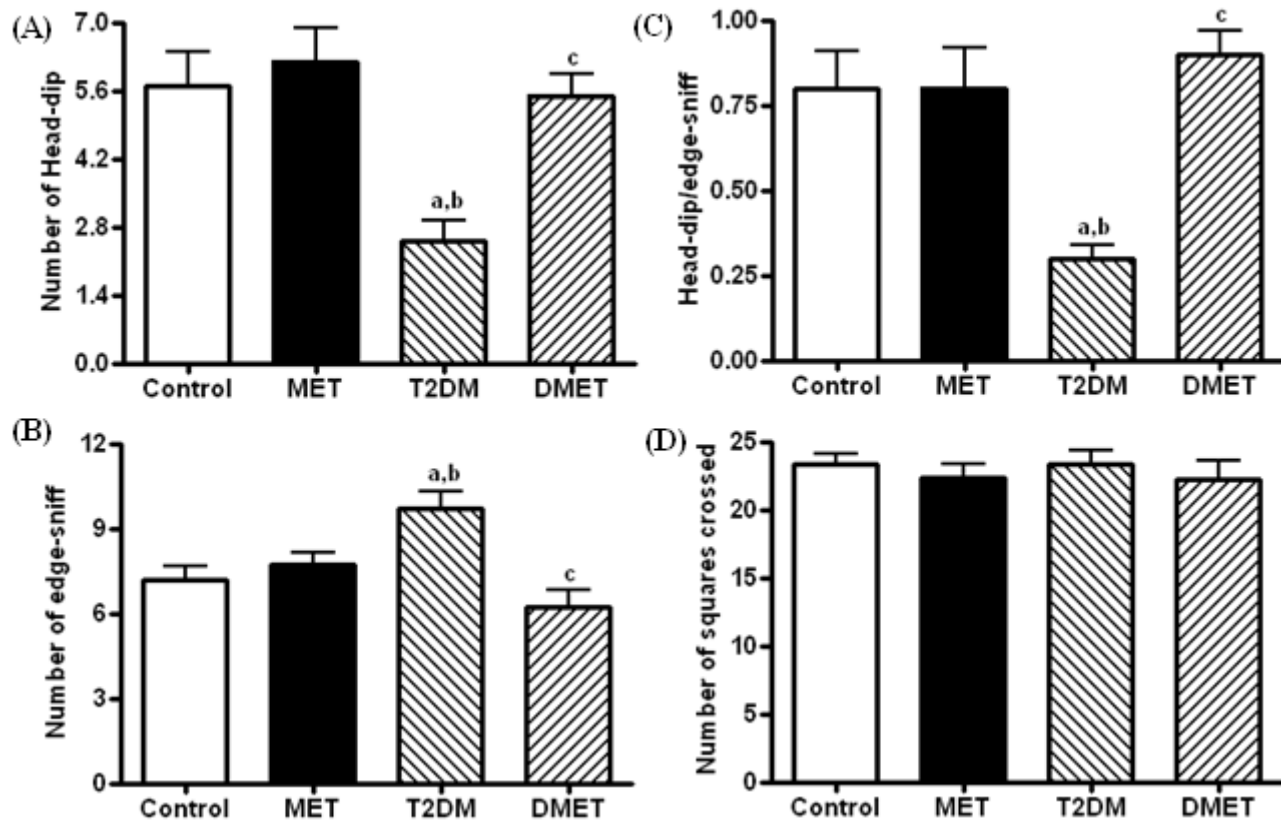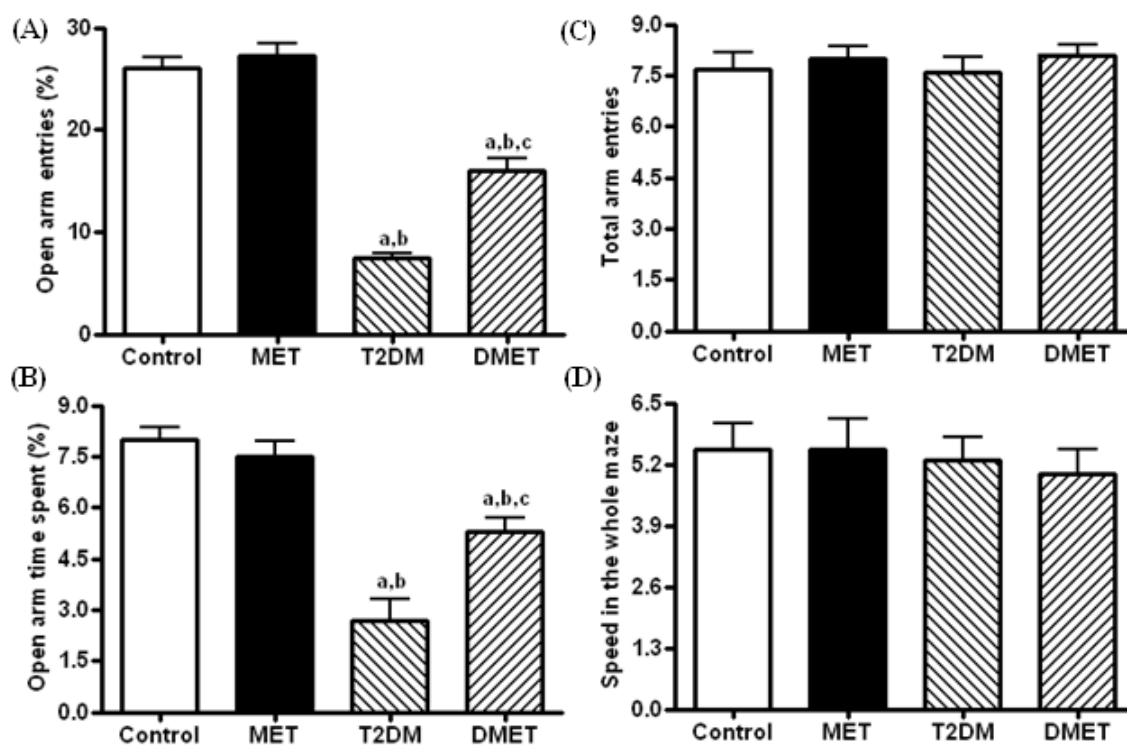
Figure 7: The effect of metformin on number of head-dips (A) and edge-sniffs (B), ratio of head-dip/edge sniff (C), and number of squares crossed (D) in control and T2DM rats during hole-board test. All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to MET and [c]p<0.05 compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].
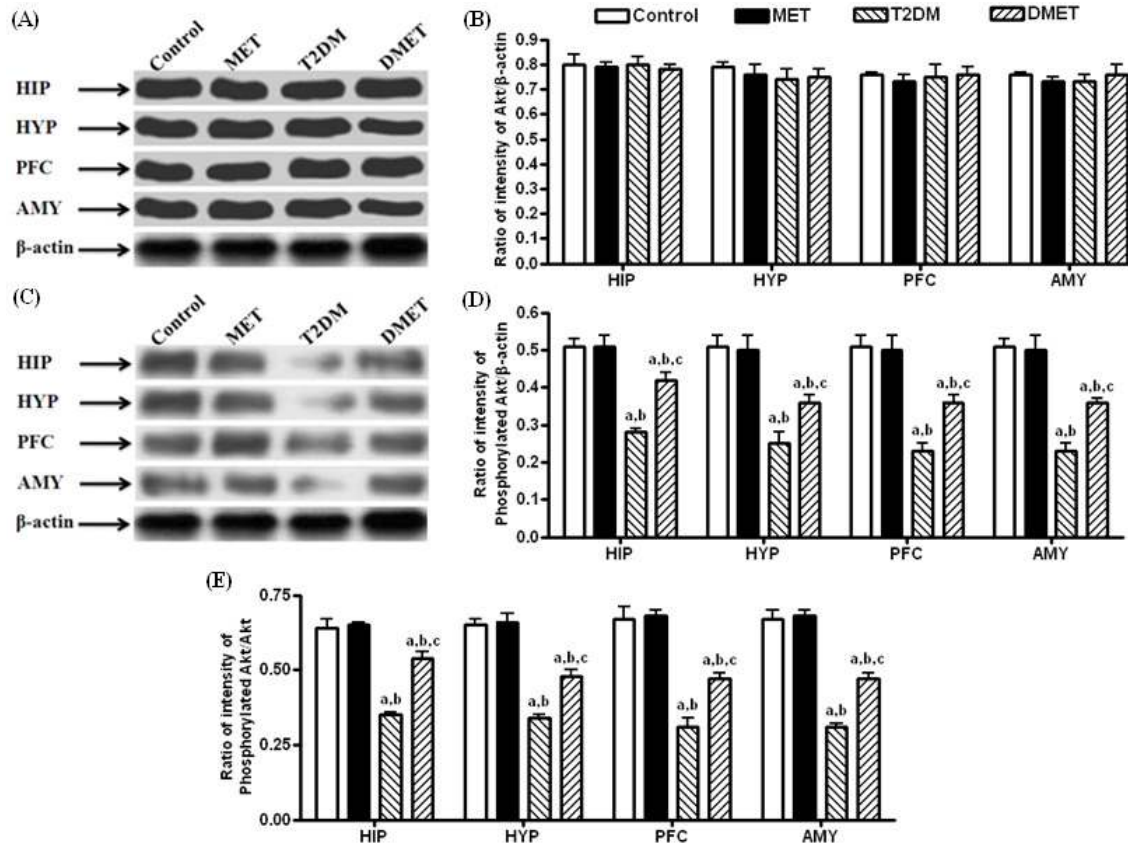
Figure 8: The effect of metformin on percentage entries (A) and time spent (B) into open arm, total arm entries (C) and speed in the whole maze (D) in control and T2DM rats during elevated plus maze test. All values are mean ± SEM (n = 6). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to MET and [c]$p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

Figure 9: 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 hippocampus (HIP), hypothalamus (HYP), pre-frontal cortex (PFC) and amygdale (AMY) of control and T2DM rats. The blots are representative of Akt (A) and p-Akt (C) in HIP, HYP, PFC and AMY. 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].
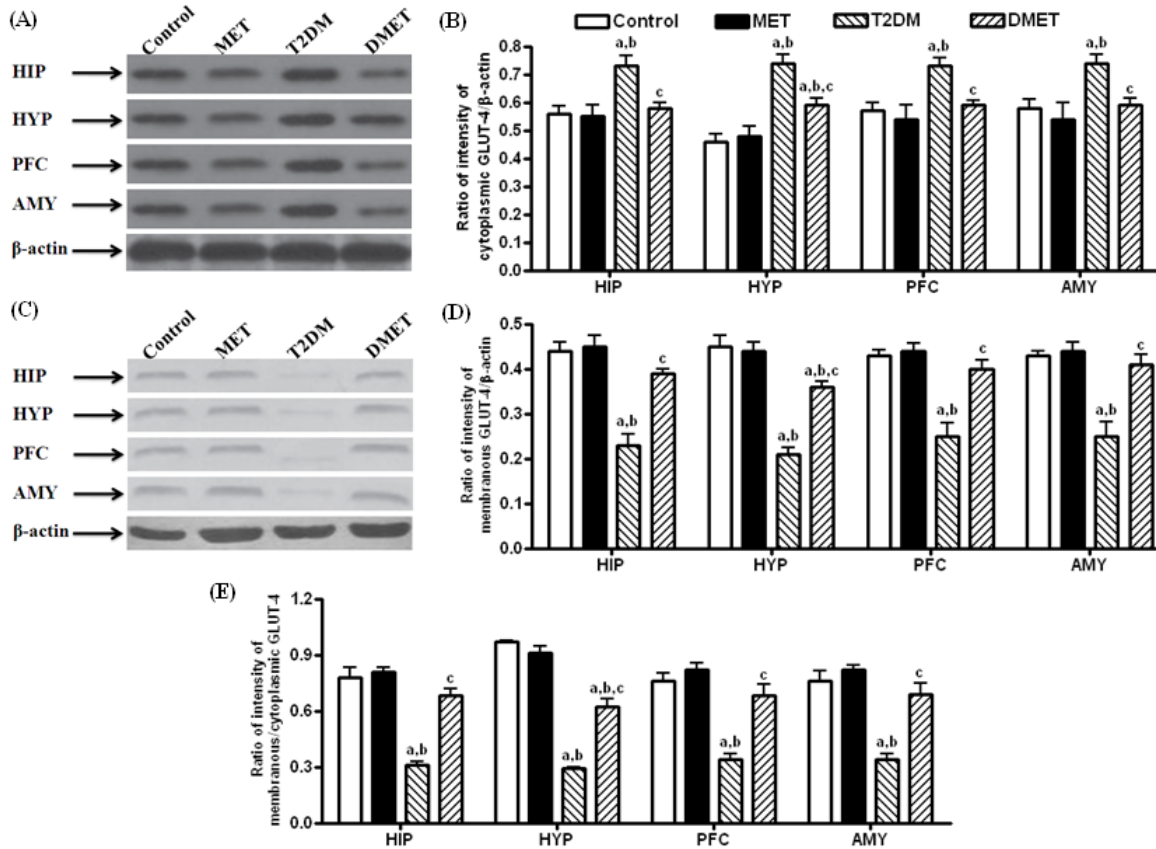
Figure 10: The effect of metformin on the level of expression of cytoplasmic (B) and membranous (D), and ratio of p-membranous to cytoplasmic (E) GLUT-4 in HIP, HYP, PFC and AMY of control and T2DM rats. The blots are representative of cytoplasmic (A) and membranous (C) GLUT-4 in HIP, HYP, PFC and AMY. 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].
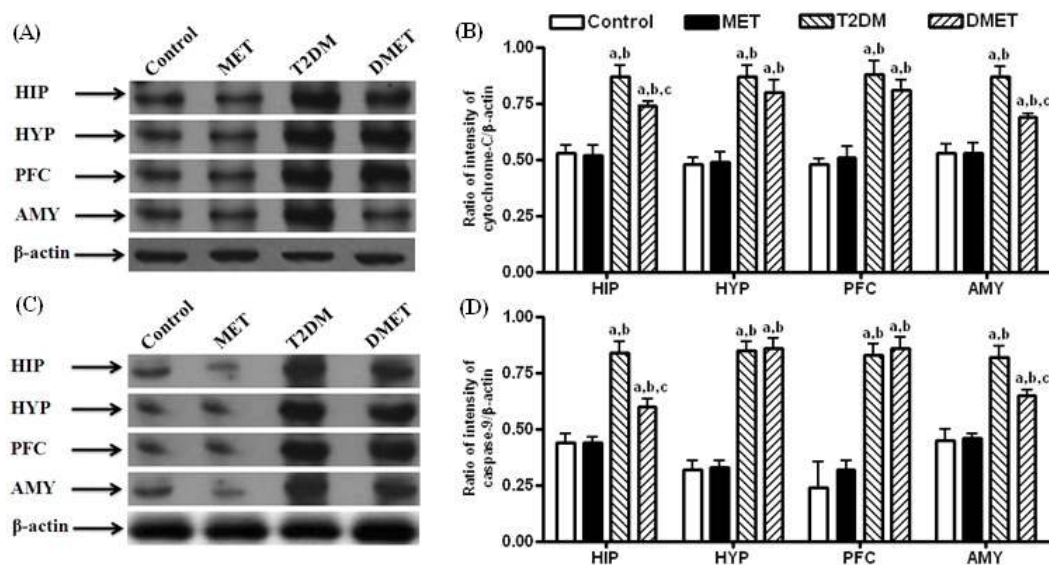
Figure 11: The effect of metformin on the level of expression of cytochrome-C (B) and caspase-9 (D) in HIP, HYP, PFC and AMY of control and T2DM rats. The blots are representative of cytochrome-C (A) and caspase-9 (C) in HIP, HYP, PFC and AMY. The results in the histogram are expressed as the ratio of 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].
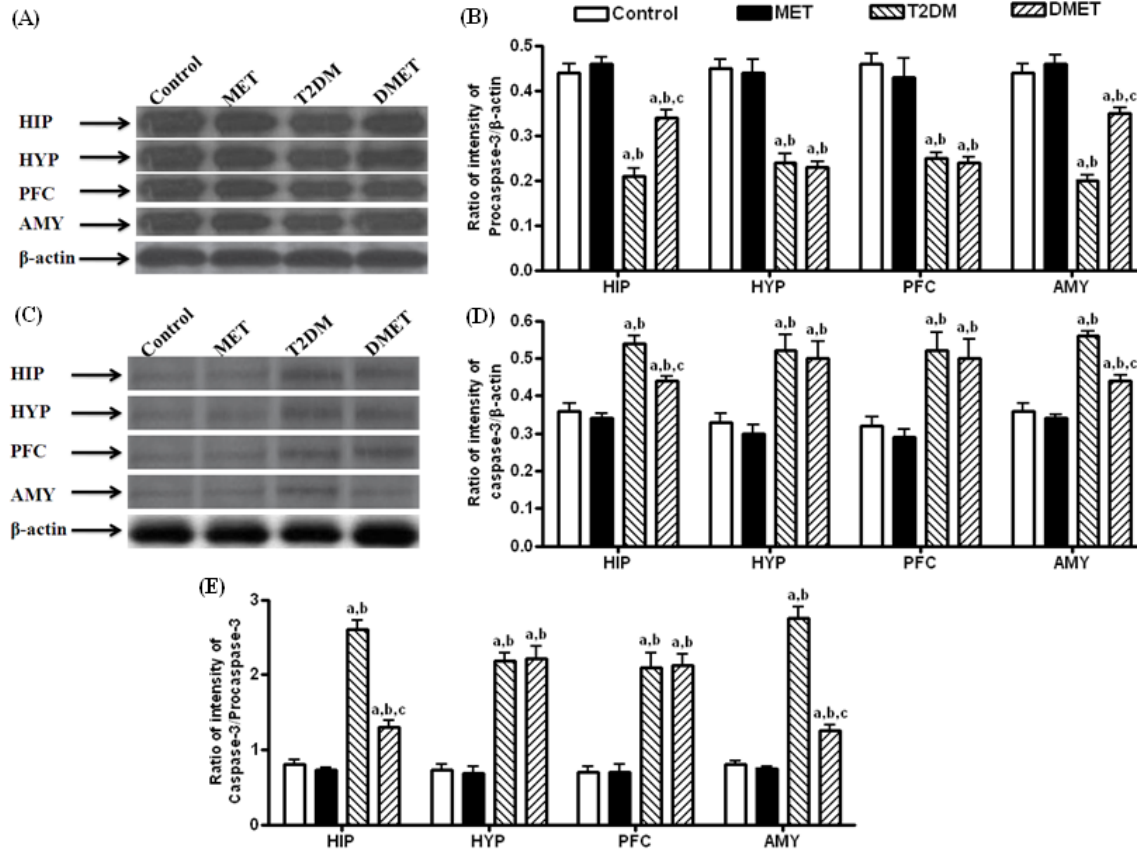
Figure 12: The effect of metformin on the level of expression of procaspase-3 (B) and caspase-3 (D), and ratio of caspase-3 to procaspase-3 (E) in HIP, HYP, PFC and AMY of control and T2DM rats. The blots are representative of procaspase-3 (A) and caspase-3 (C) in HIP, HYP, PFC and AMY. The results in 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 mitochondrial complex-I inhibitor restored the derailed PI3K/Akt/GLUT-4 signaling pathway and mitochondria-dependent apoptosis in T2DM exposed rat brain. Metformin also exhibited anti-diabetic and anxiolytic activity in these animals. These observations emphasize the fact that mitochondria targeted drugs could be potential alternative candidates in the management of T2DM-induced central complications.

Substantial literature supports that the co-morbidity of anxiety disorders and T2DM is a topic of both clinical and policy interest (Roy-Byrne et al., 2008). Similar to earlier reports, in the present study metformin attenuated T2DM-induced anxiety in different animal models without alteration in locomotor activity (Sakandelidze et al., 2011). Interestingly, presence of anxiety is reported as a contributor for an additional time of hospitalization of diabetic patients (Ball et al., 2002). Given the implications of co-morbidity between anxiety and T2DM, it is crucial to investigate whether there is a shared relationship underlying these conditions.

The primary basis of T2DM pathogenesis is unknown; however, there is a general agreement that IR followed by β-cell dysfunction is a major event in the development of the disease (Tahara et al., 2008). In our study, metformin attenuated hyperglycemia, hyperinsulinemia and IR. Our findings as well as those shown by other authors have demonstrated that animals, when injected with a low dose of streptozotocine and intermittent dose of nicotinamide, display several attributes of IR including hyperglycaemia, hyperinsulinaemia and decreased β-cell function (HOMA-B; Tahara et al., 2008; Garabadu and Krishnamurthy, 2014). We report for the first time that metformin at the sub-cellular level attenuated T2DM-induced impairment in the PI3K/Akt/GLUT-4 signaling pathway in terms of increase in the level of p-Akt in cytoplasma and GLUT-4 at the plasma membrane of brain

tissues. The effect of metformin is also observed in other tissues similar to that of our findings (Jurysta et al., 2013; Blázquez et al., 2014). This indicates that the tissue specific effect of metformin on IR is following similar signaling pathway in central tissues in this model of T2DM-induced anxiety condition. It has been reported that IR exists in selected brain regions of animals subjected to predator scent stress paradigm, a type of anxiety disorder (Cohen et al., 2009), suggesting the fact that tissue specific IR may be the common link in the pathogenesis of comorbid condition of T2DM and anxiety.

One of the major metabolic effects of hypercortisolaemia and a key feature in the development of the metabolic syndrome is IR in brain tissues (Haas and Biddinger, 2009; van Donkelaar et al., 2014). Similar to our earlier result, the present study showed hypercortisolaemia in T2DM-induced rats with IR (Garabadu and Krishnamurthy, 2014) and metformin attenuated these alterations. Moreover, metformin improved T2DM-induced reduction in body weight in the animals (Tahara et al., 2008) indicating that the presence of poor glycemic control may contribute towards loss in body weight in T2DM rats. In support to our results, another study has reported that increased corticosterone level as a consequence of hyperactivity of hypothalamic-pituitary-adrenal cortex-axis causes conversion of glycerol to glucose through a substrate push mechanism and conversion of pyruvate to glucose through greater acetyl-CoA allosteric activation of pyruvate carboxylase flux (Perry et al., 2014). These results suggest that hypercortisolaemia may contribute towards the tissues specific IR in the comorbid condition of T2DM and anxiety.

Mitochondrial dysfunction is considered as one of the predisposing factor in IR and metabolic syndrome (Petersen et al., 2003; Lowell and Shulman, 2005; Johannsen and Ravussin, 2009). Therefore, in the present study we explored the mitochondrial function and integrity, and

mitochondrial-linked apoptosis in central tissues of T2DM-induced anxiety rats. We report that there was a significant increase in the activity of all the mitochondrial respiratory complex enzyme activities in all the selected tissues similar to that of earlier studies (Garabadu and Krishnamurthy, 2013; Garabadu and Krishnamurthy, 2014). These results suggest mitochondria dysfunction in most of the rat tissues subjected to T2DM. This effect could be attributed due to hypercortisolaemia (Perry et al., 2014). Therefore, it can be presumed that tissue specific IR could be due to mitochondrial dysfunction caused by hypercortisolaemia. Our present study demonstrated that metformin treatment attenuated T2DM-induced over activation of respiratory enzymes except mitochondrial complex-II in all of the tissues. In support of our study, it has already been reported that metformin is considered as a mitochondrial complex-I inhibitor (Pryor et al., 2000; Kumar and Dey, 2002; Yuan et al., 2003; Sonntag, 2005; Kukidome et al., 2006; Chakraborty et al., 2011; Qiu et al., 2012) and probably this inhibitory potential may improve the overt mitochondrial function in such tissues in this model.

In the present study, metformin ameliorated the T2DM-induced increase in the extent of lipid peroxidation and failure in antioxidant system (decreased activity of SOD and catalase) in central 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). There was a loss in the mitochondrial integrity in terms of decrease in the MMP in all the tissues in T2DM subjected animals similar to that of previous studies (Asensio-Lopez et al., 2014; Chung et al., 2015). Additionally, metformin mitigated the increase in the extent of mitochondria-linked apoptosis as evidenced from its ability to decrease of the elevated level of expression cytochrome-C, caspase-9 and caspase -3 considered to be of markers of mitochondria-linked apoptosis in all the tissues similar to that of earlier studies

(Asensio-Lopez et al., 2014; Chung et al., 2015). It has also been documented that the mitochondrial dysfunction in diabetic animals is associated with a reduction of Na^+-K^+-ATPase activity. The dysfunction of Na^+-K^+-ATPase activity is associated with increased mitochondria-linked apoptosis (Yang et al., 2009; Zarros et al., 2009). In our study, metformin ameliorated the reduced activity of Na^+-K^+-ATPase in all of the tissues. It has been documented that metformin increased the diabetes-induced decrease in the activity of Na^+-K^+-ATPase in the erythrocytes (Chakraborty et al., 2011). Moreover, there exist a bidirectional relationship between mitochondria function and insulin signaling pathway in tissues (Wen et al., 2015). Hence, based on these observations, it can be assumed that the beneficial effect of metformin on diabetes-induced IR, apoptotic cell death, reduced Na^+-K^+-ATPase activity and eventually anxiety could be due to its regulation of mitochondrial function and integrity.

In conclusion, there is a derangement in PI3K/Akt/GLUT-4 signaling pathway along with mitochondrial dysfunction in all the tissues of T2DM exposed animals. Metformin improved PI3K/Akt/GLUT-4 signaling pathway and also restored mitochondrial function in these animals. Further, it mitigated hypercortisolaemia in these rats. Additionally, at sub-cellular level, it ameliorated the tissue-specific IR, mitochondrial dysfunction, mitochondrial oxidative stress and mitochondria-linked apoptotic pathway in all of these tissues of T2DM exposed rodents. These results emphasize the fact that mitochondrial complex-I inhibitor could be a potential candidate in the management of co-occurring condition of T2DM and anxiety. Additionally, it can be postulated that mitochondria targeted drugs would be alternative in the pharmacotherapy of comorbid condition of T2DM and anxiety.