Evaluation of metformin on bicuculline-sensitive $GABA_A$ receptor-linked PI3K/Akt/GLUT-4 signaling in rat hepatic tissue in sub-chronic model of T2DM

Introduction

Type-2 diabetes mellitus (T2DM) is considered as a metabolic disorder of dysregulation of insulin signaling pathway in several tissues including liver (Ren et al., 2015). The previous chapter (Chapter-5) suggests that there is a derailment in bicuculline-sensitive $GABA_A$-mediated Ca^{+2}/PI3K/Akt/GLUT-4 signaling in the liver tissues of T2DM rodents. The chapter-2 documents that metformin restores T2DM-induced derailment in the hepatic PI3K/Akt/GLUT-4 signaling in rats. Hence, the present study proposed to evaluate the effect of metformin on bicuculline-sensitive $GABA_A$-mediated Ca^{+2}/PI3K/Akt/GLUT-4 signaling in T2DM animals.

Liver is considered as one of the most insulin-sensitive tissue, in part, in regulating whole body fuel metabolism (LeRoith and Gavrilova, 2006). The effect of insulin is mediated primarily through the insulin receptor substrate (IRS)-phosphoinositide3-kinase (PI3K)-proteinkinase-B(Akt) pathway (Alessi et al., 1996; Coffer et al., 1998; LeRoith and Gavrilova, 2006; Sale and Sale, 2008). In response to insulin, Akt activates intracellular vesicles containing glucose transporter-4 (GLUT-4) proteins to translocate to the plasma membrane and thus glucose utilization is occurred in the tissues (Holman and Kasuga, 1997; Manning and Cantley, 2007; Bertrand et al., 2008). A derailment in the PI3K/Akt/GLUT-4 signaling is reported as one of the predisposing cause for the pathogenesis of insulin resistance in several tissues including liver during T2DM condition (Gao et al., 2015; Ren et al., 2015). It has been reported that metformin restores several pathways including PI3K/Akt/GLUT-4 signaling in liver of T2DM exposed animals (Garabadu and Krishnamurthy, 2014). Further, it exhibits anxiolytic activity against T2DM rats (Garabadu and Krishnamurthy, 2014). However, its effect on bicuculline-sensitive gamma amino butyric acid (GABA)-A receptor ($GABA_AR$) activity has not yet explained.

The GABA exerts peripheral in addition to central effect (Ackermann et al., 2008), in tissues such as adipose and pancreas (Tian et al., 2011; Soltani et al., 2011). Additional research reports the presence of $GABA_AR$ in the peripheral tissues including liver (Minuk et al., 1987). Further, GABA exhibits tissue-specific effect on the insulin signaling pathway through $GABA_AR$ in peripheral tissues (Tian et al., 2011; Soltani et al., 2011; Minuk et al., 1987). It has been reported that GABA, through $GABA_AR$ stimulation, promotes the depolarization of membrane potentials by opening the voltage-dependent calcium channel in different cells (Tian et al., 2004; Kanai et al., 2009). Bicuculline-sensitive $GABA_AR$ activation promotes insulin secretion from pancreatic beta-cells through intracellular calcium-dependent PI3K/Akt signaling pathway in type-1 diabetic condition (Tian et al., 2011; Soltani et al., 2011). Although this is proposed to be beneficial in the management of type-1 diabetes, this could be detrimental in severity of loss in insulin sensitivity in conditions such as T2DM (Tahara et al., 2008; Garabadu and Krishnamurthy, 2013). In contrast, $GABA_AR$ stimulation attenuates peripheral insulin resistance probably through activation of the Ca^{2+}/PI3K/Akt signaling pathway in several insulin sensitive tissues including autoimmune T-cells and adrenal chromaffin cells (Tian et al., 2004; Kanai et al., 2009; Tian et al., 2011). The derailment of PI3K/Akt mediated insulin signaling pathway is also the most considered mechanism in the pathogenesis of peripheral insulin resistance in the T2DM condition (Gao et al., 2015; Ren et al., 2015). However, there is no report on $GABA_A$-mediated effect of metformin against hepatic insulin resistance during T2DM condition.

Hence, the present study explored the $GABA_AR$-mediated effect of metformin on hepatic insulin resistance in streptozotocine-nicotinamide-induced T2DM rats. Further, the effect of

metformin was evaluated in presence or absence of bicuculline and wortmannin on hepatic insulin resistance to elaborate the detailed underlying mechanism of metformin.

Materials and methods

Animals

Male adult Charles Foster rats (200 ± 20 g) were procured from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University (BHU) and housed in polypropylene cages under controlled environmental conditions with a 12 hr light/dark cycle. The experimental animals had free access to food and water *ad libitum*. The experiment was conducted in accordance with the principles of laboratory animal care (NIH, 2011) guidelines. Experiments on animals were approved by the Institutional Animal Ethics Committee of BHU, Varanasi, India (Protocol No: Dean/11-12/CAEC/328).

Chemicals and reagents

Streptozotocine, metformin, bicuculline and wortmannin were purchased from Sigma (St. Louis, MO, USA). Antibodies such as phosphor-Akt^{ser473} (p-Akt), total Akt, GLUT-4 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 0.1M citrate buffer (pH 4.5) and nicotinamide was dissolved in physiological saline (Garabadu and Krishnamurthy, 2013; Masiello et al., 1998).

Experimental design

The whole study protocol was divided into three sets of individual experiments. The animals were acclimatized for seven days and randomly divided into seven groups of six animals each

namely Control, T2DM, T2DM+MET, T2DM+MET+BIC, T2DM+MET+WAT, T2DM+BIC and T2DM+WAT in all experimental sets. 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 (MET; 25.0 mg/kg, p.o.; Garabadu and Krishnamurthy, 2014), bicuculline (BIC; 2.0 mg/kg, i.p.; Sollozo-Dupont et al., 2015), wortmanin (WAT; 15.0 µm/kg, i.v.; Wen et al., 2015) or vehicle was administrated to T2DM+MET, T2DM+BIC, T2DM+WAT and Control group respectively. The groups T2DM+MET+BIC and T2DM+MET+WAT were received MET and either BIC or WAT at a time lag of 30 min respectively. 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 and liver were collected and stored immediately at -80 °C till further study.

Oral glucose tolerance test (OGTT)

Oral glucose tolerance test (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. Drug or vehicle was 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)

Insulin tolerance test (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. Drug or vehicle was given 60 min prior to insulin administration (0.4 IU/kg, s.c.). The blood samples were collected through retro-orbital puncture just before insulin 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).

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 (Matthews et al., 1985) using the following equations: IR (HOMA-IR) = ¼ (fasting glucose (mmol/l) × fasting insulin (µIU/ml))/22·5, and β-cell function (HOMA-B) = ¼ (20 × fasting insulin (µIU/ml))/(fasting glucose (mmol/l) – 3·5).

Intracellular free calcium estimation

Briefly, liver tissue was harvested and agitated in an ice-cold Hank' balanced salt solution (HBSS), containing 200 UI/mL collagenase (Type IA, Sigma), albumin (0.5 mg/mL) and 2.7 mM $CaCl_2$, for 60–120 min at 37 °C, in a water-saturated atmosphere of 95% air and 5% CO_2 for the collagenase withdrawal. Thereafter, the cells were loaded with Krebs/Hepes buffer solution (mM: 143.3 Na^+; 4.7 K^+; 2.5 Ca^{2+}; 1.3 Mg^{2+}; 125.6 Cl^-; 25 HCO^{3-}; 1.2 H_2PO^{4-}; 1.2 SO_4^{2-}; 11.7 glucose and 10 HEPES; pH 7.4) followed by 10 µM acetoxymethylester fura-2 (AM form, dissolved in dimethylsulfoxide; Molecular Probes, USA) and 5 µl (10% in DMSO) pluronic acid, and agitated to attain a complete deesterification of the probe. The cell number was adjusted to 0.8 million cells/mL. The alteration in the fluorescence intensity was monitored in Hitachi

fluorescence spectrophotometer (model F-2500) by alternative excitation at 340/380 nm. The emission was estimated at 510 nm and the ratio of the emitted light from the 2 wavelengths (R) was used as a measurement of $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985). The intensity of fluorescence was calculated automatically. The Rmax and Rmin values were determined by addition of digitonin (50 μM) and Mn^+ (2 mM) + EGTA (5 mM), respectively. The $[Ca^{2+}]_i$ levels were expressed as nM and calculated by using the following formula: $[Ca^{2+}]_i = 224 \times [(R - Rmin)/(Rmax - R)]$.

Western blot technique

The preparation of cellular membrane fraction was performed as described previously (Nishiumi and Ashida, 2007). In brief, 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)] and centrifuged at 1000 × *g* for 10 min at 4 °C. Pellets were re-suspended in NP-40 free buffer A in ice for another 10 min and re-centrifuged at 1000 × *g* for 10 min at 4 °C. The pellets were re-suspended in buffer A and placed in ice for 1 h 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 and GLUT-4 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) and anti-GLUT-4 (Abcam Plc., Cambridge, USA) polyclonal primary antibody at a dilution of 1:1000. 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 plasma glucose level in OGTT and ITT in T2DM study was 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 exhibits anti-hyperglycemic activity in T2DM rats in OGTT and ITT

Table-22 and 23 depicts the effect of metformin on the T2DM-induced hyperglycemia in rats exposed to OGTT and ITT protocol respectively. Statistical analysis revealed that there were significant differences in OGTT and ITT among group ([$F(6, 140) = 198.2$, $P<0.05$] and [$F(6, 140) = 194.1$, $P<0.05$] respectively) and time ([$F(3, 140) = 3.9$, $P<0.05$] and [$F(3, 140) = 24.9$, $P<0.05$] respectively). However, there was no significant interaction in OGTT and ITT between

group and time ([F (18, 140) = 0.4, P>0.05] and [F (18, 140) = 0.4, P>0.05] respectively). Post-hoc test showed that metformin reduced the T2DM-induced hyperglycemia in animals before glucose or insulin load. This effect was also observed after administration of glucose or insulin at different time points in OGTT or ITT respectively. On 0 time point of OGTT and ITT, wortmannin administration abolished the therapeutic effect of metformin on hyperglycemia. However, bicuculline did not cause any change to the therapeutic activity of metformin at this point of time. Further, this effect was maintained in animals at all other time points in OGTT and ITT. However, bicuculline or wortmannin *per se* did not change the T2DM-induced hyperglycemia at any time point of OGTT and ITT.

Metformin attenuates fasting blood glucose and insulin levels, insulin resistance and improves beta-cell function in T2DM rodents

Table-24 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 T2DM rats. Statistical analysis revealed that there were significant differences among groups for the level of fasting blood glucose [F (6, 35) = 52.7; p<0.05], insulin [F (6, 35) = 7.7; p<0.05], HOMA-IR [F (6, 35) = 33.9; p<0.05] and HOMA-B [F (6, 35) = 83.8; p<0.05]. Post-hoc test showed that metformin mitigated T2DM-induced increase in the levels of plasma glucose, insulin and IR, and decrease in beta-cell function (HOMA-B) in rats. When wortmannin was administered along with metformin, the anti-hyperglycemic, anti-hyperinsulinemic, anti-IR and anti-beta cell dysfunction activity of metformin was not observed in T2DM animals. While bicuculline administration along with metformin did not cause any change to these therapeutic effects in T2DM rats. Moreover, neither bicuculline nor wortmannin treatment caused any change to the T2DM-induced alterations in the above parameters.

Metformin does not alter T2DM-induced decrease in $[Ca^{+2}]_i$ in rat liver tissue

The effect of metformin in absence or presence of bicuculline and wortmannin on the $[Ca^{+2}]_i$ in liver tissue is depicted in Fig-24. Statistical analysis revealed that there were significant differences among groups in the $[Ca^{+2}]_i$ in liver tissue in rats [$F (6, 35) = 14.0$; $p<0.05$]. Post-hoc test showed that metformin did not alter the T2DM-induced decrease in the level of $[Ca^{+2}]_i$ in the liver tissue of the animals. Neither bicuculline nor wortmannin caused any change to the effect of metformin on $[Ca^{+2}]_i$ while co-administered to T2DM rats. Moreover, neither bicuculline nor wortmannin alone caused any change to the T2DM-induced decrease in the level of $[Ca^{+2}]_i$ in the liver tissue of the rats.

Metformin improves insulin signaling pathway in liver of T2DM rodents

Fig-25 illustrates the effect of metformin in absence or presence of bicuculline and wortmannin on the levels of expression of Akt and p-Akt, and their ratio in the liver tissue as a marker of insulin signaling pathway. Statistical analysis revealed that there were significant differences among groups in the level of expression of p-Akt and the ratio of p-Akt/Akt in liver tissue in rats ([$F (6, 14) = 21.9$; $p<0.05$] and [$F (6, 14) = 20.2$; $p<0.05$] respectively). However, there were no significant differences among groups in the level of expression of Akt in the liver tissue of the animals [$F (6, 14) = 0.7$; $p>0.05$]. Post-hoc test showed that metformin attenuated the T2DM-induced decrease in the level of p-Akt and the ratio of p-Akt/Akt in the liver tissue of the rodents. The effects of metformin on T2DM-induced decrease in the level of p-Akt and the ratio of p-Akt/Akt in liver were abolished when wortmannin but not bicuculline was co-administered along with it. In addition, neither bicuculline nor wortmannin were able to cause any change to the T2DM-induced decrease in the level of p-Akt and the ratio of p-Akt/Akt in the liver tissue.

Metformin attenuates T2DM-induced alteration in GLUT-4 translocation in liver

The effect of metformin in absence or presence of bicuculline and wortmannin on the levels of expression of GLUT-4 in cytosolic and membranous fraction, and their ratio in the liver tissue, to elaborate the rate of translocation of GLUT-4 from cytoplasm to plasma membrane, are depicted in Fig-26. Statistical analysis showed that there were significant differences in the levels of expression of GLUT-4 in the cytosolic [$F (6, 14) = 47.9$; $p<0.05$] and membranous [$F (6, 14) = 26.6$; $p<0.05$] fractions, and their ratio of membranous/cytosolic GLUT-4 [$F (6, 14) = 75.6$; $p<0.05$] among groups. Post-hoc test revealed that metformin attenuated the T2DM-induced increase and decrease in the cytoplasmic and membranous fractions in the liver tissue of the animals respectively. Further, it attenuated the T2DM-induced decrease in the ratio of membranous/cytoplasmic GLUT-4 in the liver tissue in rats. When wortmannin was co-administered with metformin, the effects of metformin on the levels of expression of GLUT-4 in cytoplasmic and membranous fractions as well as their ratio in the liver tissue were abolished. However, when bicuculline was co-administered with metformin, the therapeutic effect of metformin was not altered. Moreover, either bicuculline or wortmannin were not able to cause any significant alteration in the T2DM-induced changes in the levels of GLUT-4 in cytoplasm and plasma membrane fraction, and their ratio in the liver tissue of the animals.

Table-22: Effect of metformin on bicuculline-sensitive $GABA_A$ receptor mediated alterations in plasma glucose level of T2DM rats in OGTT.

	Plasma glucose level (mmol/L)			
Groups	0 min	30 min	60 min	120 min
Control	3.7 ± 0.13	6.6 ± 0.05	7.3 ± 0.08	5.3 ± 0.13
T2DM	25.1 ± 1.22 [a]	26.7 ± 1.27 [a]	26.5 ± 1.23 [a]	26.4 ± 1.43 [a]
T2DM+MET	12.5 ± 1.07 [a, b]	14.4 ± 1.13 [a, b]	15.2 ± 1.33 [a, b]	15.4 ± 1.28 [a, b]
T2DM+MET+BIC	12.7 ± 1.15 [a, b]	13.9 ± 1.31 [a, b]	15.9 ± 1.12 [a, b]	16.1 ± 1.13 [a, b]
T2DM+MET+WAT	25.3 ± 1.07 [a, c, d]	25.5 ± 1.24 [a, c, d]	26.1 ± 1.23 [a, c, d]	26.3 ± 1.23 [a, c, d]
T2DM+BIC	24.9 ± 1.13 [a, c, d]	24.8 ± 1.28 [a, c, d]	25.2 ± 1.25 [a, c, d]	25.9 ± 1.31 [a, c, d]
T2DM+WAT	24.7 ± 1.32 [a, c, d]	25.1 ± 1.31 [a, c, d]	25.4 ± 1.25 [a, c, d]	26.3 ± 1.33 [a, c, d]

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

Table-23: Effect of metformin on bicuculline-sensitive $GABA_A$ receptor mediated changes in the plasma glucose level of T2DM rats in ITT.

	Plasma glucose level (mmol/L)			
Groups	0 min	30 min	60 min	90 min
Control	4.5 ± 0.13	2.6 ± 0.23	2.7 ± 0.24	2.8 ± 0.25
T2DM	26.1 ± 1.25 [a]	19.9 ± 1.14 [a]	21.3 ± 1.02 [a]	22.5 ± 1.25 [a]
T2DM+MET	14.1 ± 1.11 [a, b]	10.7 ± 1.27 [a, b]	11.1 ± 1.13 [a, b]	11.1 ± 1.16 [a, b]
T2DM+MET+BIC	14.7 ± 1.03 [a, b]	11.1 ± 1.21 [a, b]	10.9 ± 1.12 [a, b]	11.1 ± 1.24 [a, b]
T2DM+MET+WAT	26.4 ± 1.44 [a, c, d]	21.4 ± 1.13 [a, c, d]	21.8 ± 1.21 [a, c, d]	21.5 ± 1.25 [a, c, d]
T2DM+BIC	25.2 ± 0.78 [a, c, d]	19.9 ± 1.15 [a, c, d]	20.4 ± 1.05 [a, c, d]	20.6 ± 1.06 [a, c, d]
T2DM+WAT	25.5 ± 1.13 [a, c, d]	19.8 ± 1.21 [a, c, d]	20.3 ± 0.78 [a, c, d]	20.4 ± 1.73 [a, c, d]

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

Table-24: Effect of metformin on bicuculline-sensitive $GABA_A$ mediated alterations in the fasting blood glucose and insulin level, HOMA-IR and HOMA-B indices of T2DM rats.

Groups	Fasting blood Glucose (mmol/L)	Fasting blood Insulin (pmol/L)	HOMA-IR	HOMA-B
Control	4.8 ± 0.19	75.2 ± 3.22	2.7 ± 0.32	175.4 ± 13.41
T2DM	25.7 ± 1.23 [a]	122.4 ± 10.21 [a]	21.2 ± 1.29 [a]	15.5 ± 4.85 [a]
T2DM+MET	15.3 ± 1.17 [a, b]	81.4 ± 8.71 [a, b]	10.3 ± 0.33 [a, b]	45.2 ± 3.22 [a, b]
T2DM+MET+BIC	15.5 ± 1.14 [a, b]	80.8 ± 9.15 [a, b]	10.5 ± 1.53 [a, b]	44.8 ± 4.41 [a, b]
T2DM+MET+WAT	26.4 ± 1.43 [a, c, d]	121.8 ± 8.29 [a, c, d]	21.4 ± 1.44 [a, c, d]	13.2 ± 5.23 [a, c, d]
T2DM+BIC	25.9 ± 1.21 [a, c, d]	122.2 ± 7.74 [a, c, d]	19.2 ± 1.52 [a, c, d]	13.2 ± 3.42 [a, c, d]
T2DM+WAT	25.8 ± 1.21 [a, c, d]	121.6 ± 8.73 [a, c, d]	19.1 ± 1.41 [a, c, d]	13.6 ± 4.29 [a, c, d]

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

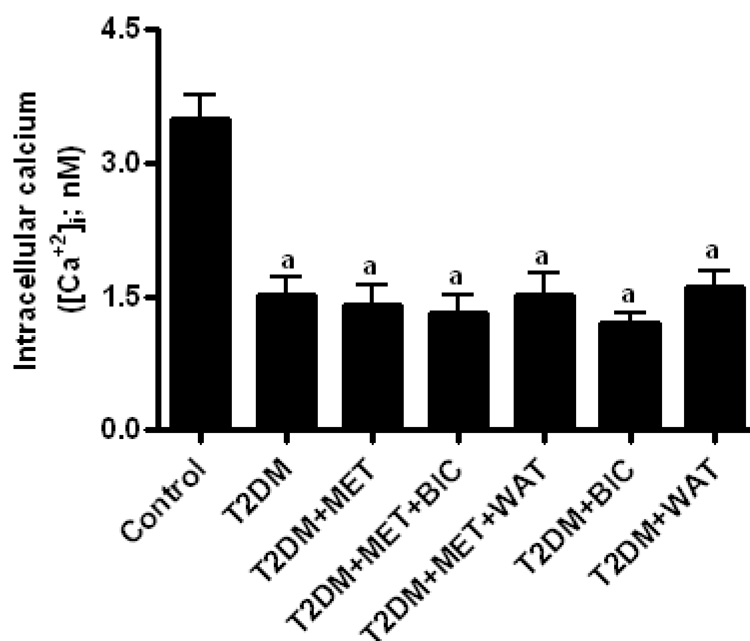

Figure 24: Effect of metformin in presence/absence of bicuculline or wortmannin on T2DM-induced changes in the level of hepatic intracellular calcium. All values are mean ± SEM (n = 6). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to T2DM, [c]$p<0.05$ compared to T2DM+MET [d]$p<0.05$ compared to T2DM+MET+BIC and [e]$p<0.05$ compared to T2DM+MET+WAT (one-way ANOVA followed by Student–Newman–Keuls test).

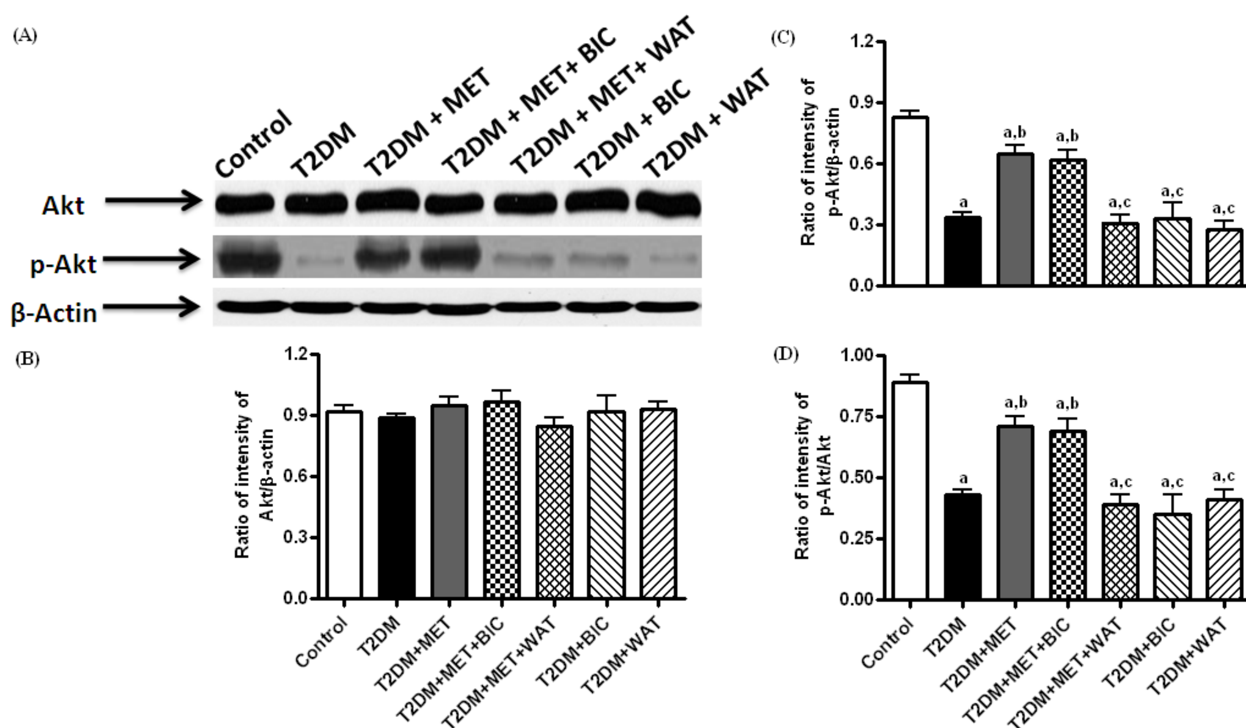

Figure 25: The effect of metformin in presence/absence of bicuculline or wortmannin on the level of expression of Akt (B) and p-Akt (C), and ratio of p-Akt to Akt (D) in liver of T2DM rats. The blots are representative of Akt and p-Akt in liver (A). The results in the histogram are expressed as the ratio 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 T2DM and [c]$p<0.05$ compared to T2DM+MET [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

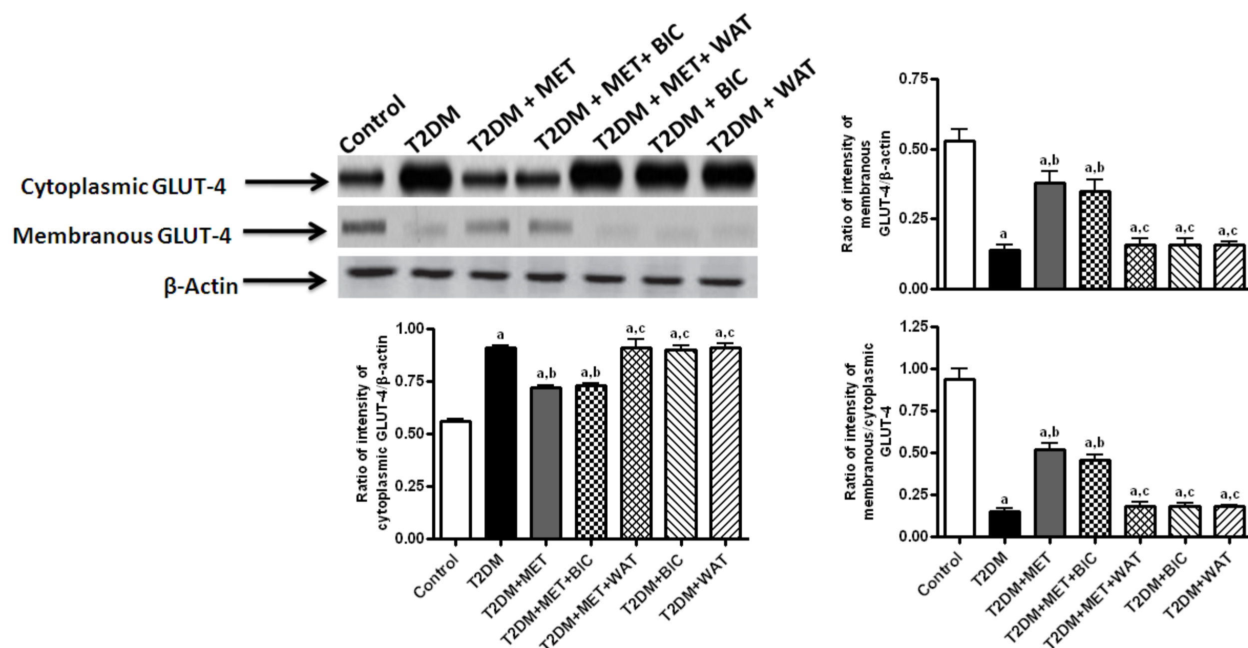

Figure 26: The effect of metformin in presence/absence of bicuculline or wortmannin on the level of expression of cytoplasmic (B) and membranous (C), and ratio of p- membranous to cytoplasmic (D) GLUT-4 in liver of T2DM rats. The blots are representative of cytoplasmic and membranous (A) GLUT-4 in liver. 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 T2DM and $^{c}p<0.05$ compared to T2DM+MET [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

Discussion

We for the first time report that metformin attenuated hepatic insulin resistance through $GABA_A$R-independent mechanism in streptozotocine-nicotinamide-induced T2DM rats. Bicuculline did not abolish the anti-diabetic activity of metformin suggesting that the later does not mediate its action through bicuculline-sensitive $GABA_A$R activity. Moreover, wortmannin blocked the beneficial effects of metformin in T2DM animals. These observations may be extrapolated to the fact that metformin exhibits anti-diabetic activity through bicuculline-sensitive $GABA_A$R-independent mechanism in T2DM condition.

The pathogenesis of T2DM is still obscure; however, there is a general agreement that beta-cell dysfunction is a major event in the development of this pandemic in addition to insulin resistance (Koning et al., 2008). Similar to earlier reports, the T2DM animals in present study displayed a significant beta-cell dysfunction in OGTT and this effect was further evident in them from biochemical estimation in terms of decrease in HOMA-B index (Tahara et al., 2008; Garabadu and Krishnamurthy, 2013). Metformin attenuated beta-cell dysfunction in the T2DM rats and this effect was not abolished when bicuculline was co-administered with the former. The result indicates that metformin improves beta-cell function in T2DM condition in an independent manner, not mediated through bicuculline-sensitive $GABA_A$ receptor activation. Moreover, wortmannin blocked the therapeutic effect of metformin on T2DM-induced beta-cell dysfunction. Similar to our results, reports suggest that GABA through bicuculline-sensitive $GABA_A$R activation attenuates beta-cell dysfunction probably through downstream facilitation of PI3K/Akt signaling pathway in the type-1 diabetic condition (Soltani et al., 2011; Tersey et al., 2015). However, in the current study metformin ameliorated T2DM-induced beta-cell dysfunction in animals by amending the derailed PI3K/Akt signaling pathway by alternate pathways other than $GABA_A$R activation.

Insulin resistance is the cardinal attribute among the diagnostic features of T2DM. The present study clearly showed the presence of insulin resistance in T2DM rats by ITT test and this was further evident in these animals from the biochemical estimation in terms of increase in HOMA-IR index similar to that of earlier reports (Tahara et al., 2008; Garabadu and Krishnamurthy, 2013). Metformin attenuated the T2DM-induced insulin resistance in these animals. However, the therapeutic effect of metformin was not blocked when bicuculline was co-administered to these rats suggesting that bicuculline-sensitive $GABA_A$ receptor is not the medium of therapeutic activity of metformin against hepatic insulin resistance in T2DM condition. Moreover, wortmannin abolished the beneficial effect of metformin in the T2DM rats. Furthermore, metformin significantly attenuated T2DM-induced hyperinsulinemia in rats and this effect was abolished when wortmannin was co-administered. These observation emphasize the fact that metformin exerts therapeutic effect against T2DM-induced hepatic insulin resistance through $GABA_AR$-independent mechanism.

We have estimated the $[Ca^{+2}]_i$ as a functional measure of bicuculline-sensitive $GABA_A$ receptor activity in the liver tissues. Metformin did not alter the T2DM-induced decrease in the $[Ca^{+2}]_i$ in the animals. The level of $[Ca^{+2}]_i$ in T2DM liver cells was not altered when bicuculline or wortmannin was co-administered with metformin. Further studies elaborated the results that muscimol partially reversed the T2DM-induced decrease in the level of Akt and GLUT-4 translocation into plasma membrane of hepatocytes in the animals suggesting that muscimol encourages glucose utilization through GLUT-4 translocation. Only wortmannin when co-administered individually with metformin, blocked the anti-hyperglycemic effect of metformin, decreased the level of expression of p-Akt and the extent of translocation of GLUT-4 in liver of T2DM rats. These observations further validate the fact that action of metformin does not

involve $GABA_AR$ stimulation but facilitate the PI3K/Akt/GLUT-4 signaling pathway in the hepatocytes during T2DM condition. Based on these results it can be assumed that metformin exhibits therapeutic activity through a bicuculline-sensitive $GABA_AR$-independent mechanism against T2DM condition.