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

Introduction

Several medications are developed and available for the management of hyperglycemia in T2DM. However, they do not completely restore glucose homeostasis and/or have adverse effects (Barnett et al., 2013). Hence, it can be presumed that ameliorating insulin resistance could be an important strategy in the development of new pharmacological agents for T2DM.

Insulin-sensitive tissue such as liver is typical involved, 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)-protein kinase-B (Akt) pathway (LeRoith and Gavrilova, 2006; Alessi et al., 1996; Sale and Sale, 2008; Coffer et al., 1998). In response to insulin, Akt activates intracellular vesicles containing glucose transporter-4 (GLUT-4) proteins to translocate to the cell surface and thus glucose is utilized by the tissues (Holman and Kasuga, 1997; Manning and Cantley, 2007; Bertrand et al., 2008). It is well established that there is a loss in the insulin-mediated PI3K/Akt/GLUT-4 signaling pathway which is the fundamental cause for the pathogenesis of insulin resistance in several tissues including liver during T2DM condition (Gao et al., 2015; Ren et al., 2015). As most of the currently available oral hypoglycemic drugs have therapeutic limitations, there is always a need to discover alternative targets which can facilitate the PI3K/Akt/GLUT-4 signaling pathway during T2DM condition.

Gamma amino butyric acid (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 bicuculline-sensitive GABA-A receptor ($GABA_AR$) 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 both central and peripheral tissues (Ackermann et al., 2008; Tian et al., 2011; Soltani et al., 2011). 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., 2011; Tian et al., 2004; Kanai et al., 2009). 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 the effect of $GABA_A$-mediated action on the hepatic insulin signaling pathway during T2DM condition. Therefore, we investigated the GABA mediated regulation of hepatic insulin signaling pathway in T2DM condition.

Hence, in the present objective, we explored the effect of $GABA_AR$ activation through muscimol on hepatic insulin resistance in streptozotocine-nicotinamide-induced T2DM rats. Further, the effect of $GABA_AR$ activation was evaluated in presence or absence of bicuculline and wortmannin on hepatic insulin resistance to establish a relationship between bicuculline-sensitive $GABA_AR$ activity and PI3K/Akt/GLUT-4 signaling pathway of insulin in T2DM rats.

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 (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, muscimol, 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+MUS, T2DM+MUS+BIC, T2DM+MUS+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 muscimol (MUS; 1.0 mg/kg, i.p.; Sollozo-Dupont et al., 2015), 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+MUS, T2DM+BIC, T2DM+WAT and Control group respectively. The groups T2DM+MUS+BIC and T2DM+MUS+WAT were received MUS 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 de-esterification 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

Muscimol exhibits anti-hyperglycemic activity in T2DM rats in OGTT and ITT

Table-19 and 20 depicts the effect of muscimol 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 ([$F (6, 140) = 186.0$, $P<0.05$] and [$F (6, 140) = 195.7$, $P<0.05$] respectively) and time ([$F (3, 140) = 4.1$, $P<0.05$] and [$F (3, 140) = 29.6$, $P<0.05$] respectively). However, there was no significant interaction in OGTT and ITT between group and time ([$F (18, 140) = 0.3$, $P>0.05$] and [$F (18, 140) = 0.6$, $P>0.05$] respectively). Post-hoc test showed that muscimol reduced the T2DM-induced increase in the level of plasma glucose 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. When bicuculline or wortmannin was administered along with muscimol in T2DM rats, the anti-hyperglycemic activity of muscimol was not observed at 0 time point of OGTT and ITT. 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.

Bicuculline-sensitive $GABA_A$ activation attenuates fasting blood glucose and insulin levels, insulin resistance and improved beta-cell function in T2DM rodents

Table-21 demonstrates the effect of muscimol 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 [$F(6, 35) = 58.2$; $p<0.05$], insulin [$F(6, 35) = 7.7$; $p<0.05$], HOMA-IR [$F(6, 35) = 32.2$; $p<0.05$] and HOMA-B [$F(6, 35) = 92.3$; $p<0.05$]. Post-hoc test showed that muscimol mitigated T2DM-induced increase in the levels of plasma glucose, insulin and IR, and decrease in beta-cell function (HOMA-B) in rats. When either bicuculline or wortmannin was administered along with muscimol, the anti-hyperglycemic, anti-hyperinsulinemic, anti-IR and anti-beta cell dysfunction activity of muscimol was not observed in T2DM animals. However, neither bicuculline nor wortmannin treatment caused any change to the T2DM-induced alterations in the above parameters.

Bicuculline-sensitive $GABA_A$ activation attenuates T2DM-induced decrease in $[Ca^{+2}]_i$ in liver tissue of rats

The effect of muscimol in absence or presence of bicuculline and wortmannin on the $[Ca^{+2}]_i$ in liver tissue is depicted in Fig-21. Statistical analysis revealed that there were significant

differences among groups in the $[Ca^{+2}]_i$ in liver tissue in rats [F (6, 35) = 15.1; $p<0.05$]. Post-hoc test showed that muscimol attenuated the T2DM-induced decrease in the level of $[Ca^{+2}]_i$ in the liver tissue of the animals. Bicuculline abolished the effect of muscimol on $[Ca^{+2}]_i$ while co-administered to T2DM rats. However, wortmannin did not cause any change to the effect of muscimol 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.

Bicuculline-sensitive $GABA_A$ activation improves insulin signaling pathway in liver of T2DM rodents

Fig-22 illustrates the effect of muscimol 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) = 22.0; $p<0.05$] and [F (6, 14) = 21.8; $p<0.05$] 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) = 0.9; $p>0.05$]. Post-hoc test showed that muscimol 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 muscimol on T2DM-induced decrease in the level of p-Akt and the ratio of p-Akt/Akt in liver were abolished when bicuculline or wortmannin 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.

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

The effect of muscimol 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-23. Statistical analysis showed that there were significant differences in the levels of expression of GLUT-4 in the cytosolic [$F(6, 14) = 18.2$; $p<0.05$] and membranous [$F(6, 14) = 10.5$; $p<0.05$] fractions, and their ratio of membranous/cytosolic GLUT-4 [$F(6, 14) = 9.9$; $p<0.05$] among groups. Post-hoc test revealed that muscimol 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 bicuculline or wortmannin was co-administered with muscimol, the effects of muscimol on the levels of expression of GLUT-4 in cytoplasmic and membranous fractions as well as their ratio in the liver tissue were abolished. 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-19: Effect of Bicuculline-sensitive $GABA_A$ receptor activation on the plasma glucose level of T2DM rats in OGTT.

	Plasma glucose level (mmol/L)			
Groups	0 min	30 min	60 min	120 min
Control	3.5 ± 0.15	6.7 ± 0.07	7.3 ± 0.08	5.3 ± 0.13
T2DM	24.3 ± 1.22 [a]	25.8 ± 1.17 [a]	26.2 ± 1.29 [a]	26.4 ± 1.33 [a]
T2DM+MUS	13.9 ± 1.09 [a, b]	14.3 ± 1.12 [a, b]	15.1 ± 1.32 [a, b]	15.3 ± 1.27 [a, b]
T2DM+MUS+BIC	24.5 ± 1.19 [a, c]	25.1 ± 1.31 [a, c]	25.9 ± 1.13 [a, c]	26.7 ± 1.14 [a, c]
T2DM+MUS+WAT	24.3 ± 1.07 [a, c]	25.7 ± 1.22 [a, c]	26.1 ± 1.21 [a, c]	26.4 ± 1.27 [a, c]
T2DM+BIC	23.9 ± 1.11 [a, c]	24.7 ± 1.25 [a, c]	25.8 ± 1.45 [a, c]	26.5 ± 1.41 [a, c]
T2DM+WAT	24.7 ± 1.42 [a, c]	25.1 ± 1.33 [a, c]	25.4 ± 1.21 [a, c]	26.1 ± 1.39 [a, c]

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

Table-20: Effect of Bicuculline-sensitive $GABA_A$ receptor activation on the plasma glucose level of T2DM rats in ITT.

	Plasma glucose level (mmol/L)			
Groups	0 min	30 min	60 min	90 min
Control	4.2 ± 0.15	2.3 ± 0.21	2.9 ± 0.34	2.9 ± 0.28
T2DM	26.1 ± 1.13 [a]	19.7 ± 1.07 [a]	21.7 ± 1.04 [a]	22.3 ± 1.22 [a]
T2DM+MUS	14.3 ± 1.25 [a, b]	10.5 ± 1.21 [a, b]	11.3 ± 1.14 [a, b]	11.1 ± 1.18 [a, b]
T2DM+MUS+BIC	25.7 ± 1.09 [a, c]	21.1 ± 1.11 [a, c]	20.9 ± 1.14 [a, c]	21.1 ± 1.23 [a, c]
T2DM+MUS+WAT	26.3 ± 1.43 [a, c]	21.2 ± 1.23 [a, c]	20.8 ± 1.22 [a, c]	21.1 ± 1.29 [a, c]
T2DM+BIC	25.2 ± 0.98 [a, c]	18.9 ± 1.18 [a, c]	20.5 ± 1.09 [a, c]	20.3 ± 1.05 [a, c]
T2DM+WAT	25.6 ± 1.12 [a, c]	19.4 ± 1.24 [a, c]	20.3 ± 0.98 [a, c]	20.4 ± 1.03 [a, c]

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

Table-21: Effect of muscimol fasting blood glucose and insulin level, HOMA-IR and HOMA-B indices in T2DM rats.

Groups	Fasting blood Glucose (mmol/L)	Fasting blood Insulin (pmol/L)	HOMA-IR	HOMA-B
Control	4.5 ± 0.22	75.4 ± 3.42	2.5 ± 0.51	179.7 ± 13.22
T2DM	25.5 ± 1.29 [a]	123.4 ± 10.41 [a]	21.3 ± 1.49 [a]	15.8 ± 4.86 [a]
T2DM+MUS	15.3 ± 1.17 [a, b]	83.4 ± 8.73 [a, b]	10.1 ± 0.53 [a, b]	45.3 ± 3.25 [a, b]
T2DM+MUS+BIC	25.3 ± 1.08 [a, c]	125.3 ± 9.85 [a, c]	20.5 ± 1.57 [a, c]	14.3 ± 4.21 [a, c]
T2DM+MUS+WAT	26.1 ± 1.03 [a, c]	124.8 ± 8.89 [a, c]	21.7 ± 1.43 [a, c]	13.9 ± 5.33 [a, c]
T2DM+BIC	24.9 ± 1.11 [a, c]	123.7 ± 7.94 [a, c]	19.4 ± 1.51 [a, c]	15.2 ± 3.41 [a, c]
T2DM+WAT	25.1 ± 1.23 [a, c]	123.9 ± 8.93 [a, c]	19.5 ± 1.44 [a, c]	15.6 ± 4.39 [a, c]

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

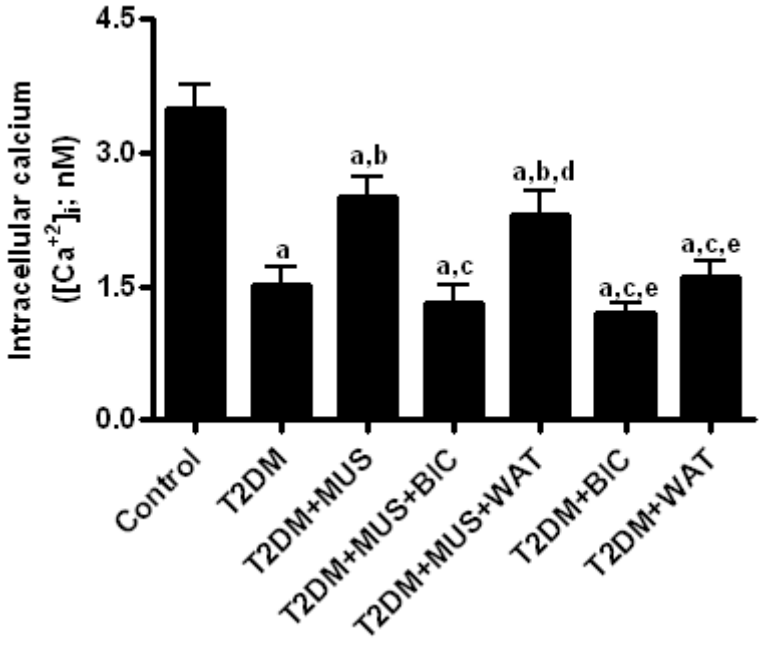

Figure 21: Effect of muscimol in presence/absence of bicuculline or wortmannin on T2DM-induced changes in the level of hepatic intracellular calcium. All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to T2DM, [c]p<0.05 compared to T2DM+MUS, [d]p<0.05 compared to T2DM+MUS+BIC and [e]p<0.05 compared to T2DM+MUS+WAT (one-way ANOVA followed by Student–Newman–Keuls test).

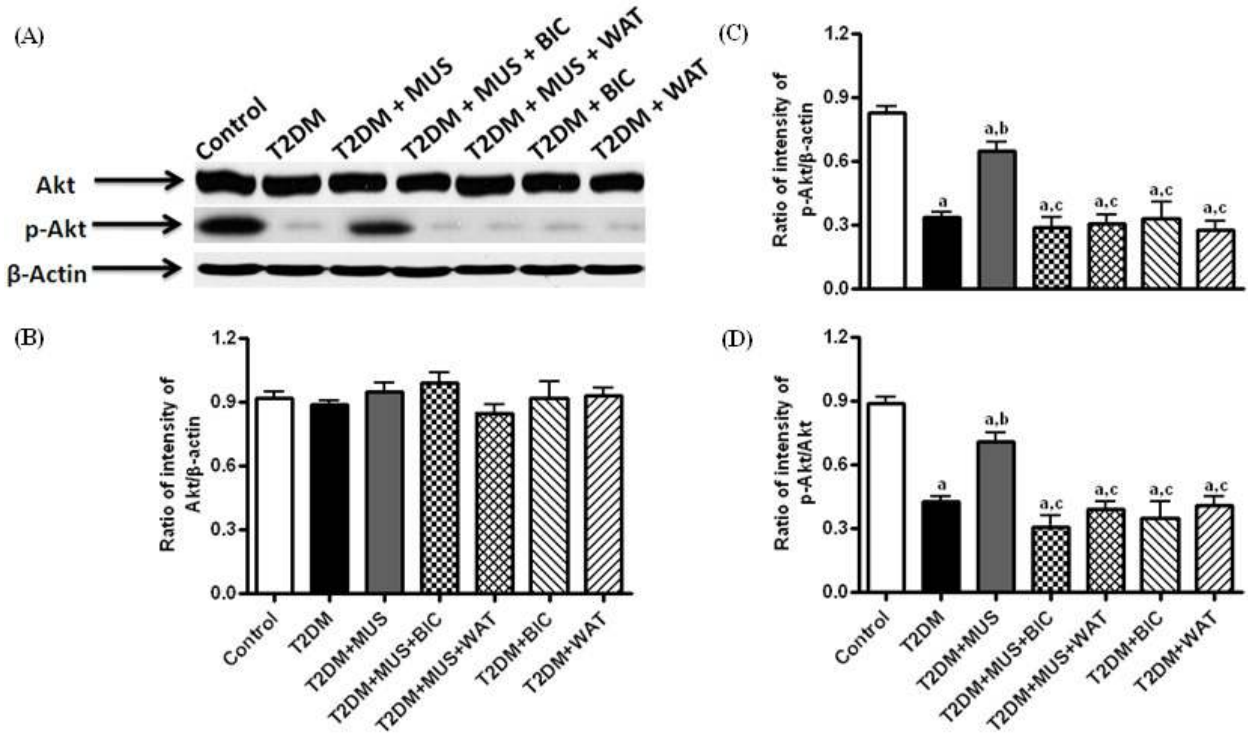

Figure 22: The effect of muscimol in presence/absence of bicuculline or wortmannin on the level of expression of Akt (B) and p-Akt (C), and ratio of p-Akt to Akt (D) in liver of T2DM rats. The blots are representative of Akt and p-Akt in liver (A). The results in the histogram are expressed as the ratio 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+MUS [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

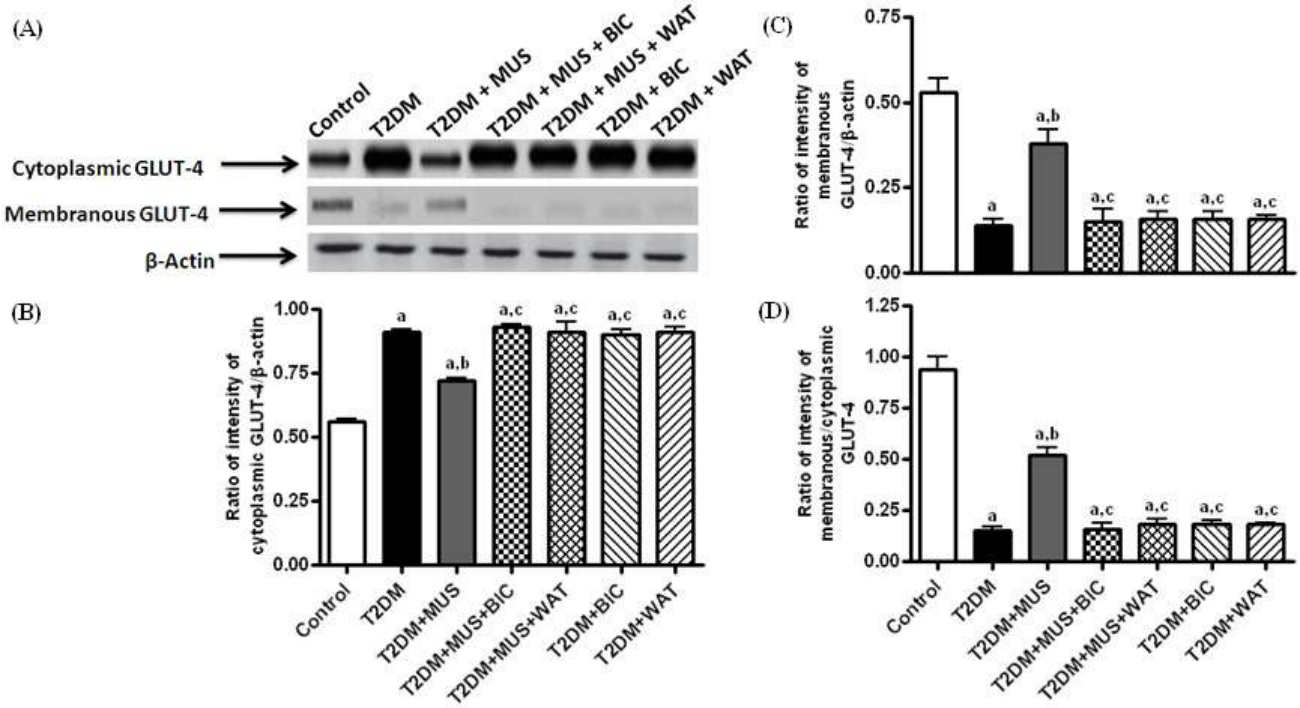

Figure 23: The effect of muscimol in presence/absence of bicuculline or wortmannin on the level of expression of cytoplasmic (B) and membranous (C), and ratio of membranous to cytoplasmic (D) GLUT-4 in liver of T2DM rats. The blots are representative of cytoplasmic and membranous (A) GLUT-4 in liver. 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+MUS [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

Discussion

We for the first time report that $GABA_AR$ activation by muscimol attenuated hepatic insulin resistance in streptozotocine-nicotinamide-induced T2DM rats. Bicuculline abolished the anti-diabetic activity of muscimol suggesting that the later mediates its action through bicuculline-sensitive $GABA_AR$ activity. Moreover, wortmannin blocked the beneficial effects of muscimol in T2DM animals indicating that bicuculline-sensitive $GABA_AR$ activation and PI3K/Akt/GLUT-4 signaling pathway of insulin are interlinked. These observations may be extrapolated to the fact that bicuculline-sensitive $GABA_AR$ could be a potential target in the management of T2DM.

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). Muscimol attenuated beta-cell dysfunction in the T2DM rats and this effect was abolished when bicuculline was co-administered with the former. The result indicates that the bicuculline-sensitive $GABA_A$ receptor activation improves beta-cell function in T2DM condition. Moreover, wortmannin also blocked the therapeutic effect of muscimol on T2DM-induced beta-cell dysfunction. Similar to our results, reports suggest that GABA through bicuculline-sensitive $GABA_AR$ 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, we report the beneficial effect of bicuculline-sensitive $GABA_AR$ activation against T2DM-induced beta-cell dysfunction in animals. Hence, it can be speculated that bicuculline-sensitive $GABA_AR$ activation may exhibit

therapeutic activity through amending the derailed PI3K/Akt signaling pathway in pancreatic beta-cells in T2DM condition.

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). Muscimol attenuated the T2DM-induced insulin resistance in these animals. However, the therapeutic effect of muscimol was blocked when bicuculline was co-administered to these rats suggesting that bicuculline-sensitive $GABA_A$ receptor stimulation attenuates peripheral insulin resistance in T2DM condition. Moreover, wortmannin abolished the beneficial effect of muscimol in the T2DM rats. Furthermore, muscimol significantly attenuated T2DM-induced hyperinsulinemia in rats and this effect was abolished when bicuculline or wortmannin were co-administered. It is interesting to note that $GABA_AR$ stimulation decreased the hyperinsulinemic state in the rats which was contrary to the observations in type-1 diabetic animals (Soltani et al., 2011). This discrepancy could be due to the differential expression of $GABA_AR$ in respect to the condition of diabetes.

We have estimated the $[Ca^{+2}]_i$ as a functional measure of bicuculline-sensitive $GABA_A$ receptor activity in the liver tissues. Muscimol partially reversed 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 was co-administered with muscimol indicating that bicuculline-sensitive $GABA_AR$ activity may also regulate the level of $[Ca^{+2}]_i$ in the hepatocytes. Moreover, when wortmannin was administered along with muscimol, there was no change in the level of $[Ca^{+2}]_i$ in the liver tissues of T2DM rodents. These observations indicate the fact that $GABA_AR$ stimulation may

contribute in the increase in the $[Ca^{+2}]_i$ however there may be no effect of PI3K/Akt signaling pathway on the $[Ca^{+2}]_i$ in the liver tissues. Similar to our results it has been reported in other studies that $[Ca^{+2}]_i$ stimulates PI3K/Akt signaling pathway in pancreatic tissues in type-1 diabetic condition (Soltani et al., 2011; Purwana et al., 2014). 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. Both bicuculline and wortmannin when co-administered individually with muscimol, blocked the anti-hyperglycemic effect of the $GABA_A$ agonist in terms of the decrease in the level of expression of p-Akt and in the extent of translocation of GLUT-4 in liver of T2DM rats. These observations suggest that $GABA_AR$ stimulation may facilitate the PI3K/Akt/GLUT-4 signaling pathway through calcium-dependent mechanism in the hepatocytes in the T2DM condition. Similar effects have been reported in other cell types (Tian et al., 2004; Kanai et al., 2009). Based on these results it can be assumed that bicuculline-sensitive $GABA_AR$ stimulation could be a potential target in the management of T2DM.

In conclusion, the present study documents that the bicuculline-sensitive $GABA_AR$ stimulation attenuated hepatic insulin resistance in T2DM rodents. In addition, it ameliorated beta-cell dysfunction in these rats. The results suggest that bicuculline-sensitive $GABA_AR$ stimulation may facilitate the hepatic PI3K/Akt/GLUT-4 signaling pathway in T2DM rodents probably through calcium-dependent mechanism. Hence, bicuculline-sensitive $GABA_AR$ could be a potential alternative target in the pharmacotherapy of T2DM. These observations emphasize the fact that the bicuculline-sensitive $GABA_AR$ activation could be a common target in the management of both type-1 and type-2 diabetes mellitus. The drugs that activate bicuculline-

sensitive $GABA_AR$ could have wide range of therapeutic potential in the overt management of diabetes mellitus.