Metformin attenuates hepatic insulin resistance in type-2 diabetic rats through PI3K/Akt/GLUT-4 signalling independent to bicuculline-sensitive GABA_A receptor stimulation

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ABSTRACT

Context: Metformin attenuates type-2 diabetes mellitus (T2DM)-induced hepatic dysfunction and altered PI3K/Akt/GLUT-4 signalling in experimental studies. However, its effect on bicuculline-sensitive gamma amino butyric acid (GABA)-A receptor (GABA_A) receptor (GABA_A)-mediated calcium-dependent PI3K/Akt/GLUT-4 signalling in liver challenged to T2DM has not been established.

Objective: The effectiveness of metformin on bicuculline-sensitive GABA_A receptor-mediated hepatic insulin signalling was carried out in presence or absence of bicuculline (2.0 mg/kg, i.p.) in experimental T2DM rats.

Materials and methods: The whole experimental design was divided into three independent sets of experiments. Each set comprised seven groups of male rats each. T2DM was induced in the animals by administering streptozotocin (45 mg/kg, i.p.) and nicotinamide (110 mg/kg, i.p.) at a time lag of 15 min except control group rats in three experiments. Metformin and/or bicuculline or wortmannin were administered once daily for one week from seventh day of streptozotocin injection in all the experimental sets.

Results: Metformin attenuated T2DM-induced hyperglycaemia in glucose (40%) and insulin (50%) tolerance tests in rats. Metformin also attenuated T2DM-induced hyperglycaemia (40%), hyperinsulinaemia (30%), insulin resistance (50%) and β-cell dysfunction (300%) in the animals. Metformin did not attenuate T2DM-induced decrease in rat hepatic intracellular calcium. Further, metformin mitigated T2DM-induced decrease in hepatic phosphorylated Akt and GLUT-4 translocation in the animals. The anti-diabetic activity of metformin was abolished by wortmannin but not with bicuculline co-administration in T2DM animals.

Discussion and conclusion: These results suggest that metformin ameliorated T2DM-induced hepatic insulin resistance through bicuculline-sensitive GABA_A receptor-dependent PI3K/Akt/GLUT-4 signalling pathway in animals.

Introduction

Type-2 diabetes mellitus (T2DM) is prevalent in 90% of the diabetic population (Whiting et al. 2011). Glucose tolerance, insulin resistance and/or insufficiency are considered as clinical manifestations of T2DM (Lazar 2005; Donath & Shoelson 2011). Several medications are developed and used in the management of hyperglycaemia in T2DM condition. However, they do not completely reverse glucose homeostasis and/or have adverse effects (Barnett et al. 2013). Hence, it can be speculated that ameliorating insulin resistance could be an important strategy in the development of new pharmacological agents for T2DM.

Liver is considered as one of the most insulin-sensitive tissues, in part, in regulating whole body fuel metabolism (LeRoith & 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; Coffier et al. 1998; LeRoith & Gavrilova 2006; Sale & Sale 2008). Phosphorylation of Akt translocates intracellular vesicles containing glucose transporter-4 (GLUT-4) proteins to the plasma membrane. This facilitates the glucose utilization in the tissues (Holman & Kasuga 1997; Manning & Cantley 2007; Bertrand et al. 2008). In insulin resistance there is derailing in the PI3K/Akt/GLUT-4 signalling in several tissues including liver (Gao et al. 2015; Ren et al. 2015). It has been reported that metformin restores several pathways including PI3K/Akt/GLUT-4 signalling in liver of T2DM exposed animals (Garabadu & Krishnamurthy 2014). Further, it exhibits anxiolytic activity against T2DM rats (Garabadu & Krishnamurthy 2014). However, its effect on bicuculline-sensitive γ-amino butyric acid (GABA)-A receptor (GABA_A) activity has not yet been explained.

The GABA exerts effect on peripheral tissues such as adipose and pancreas (Soltani et al. 2011; Tian et al. 2011) in addition to brain (Ackermann et al. 2008). Additional research reports the presence of GABA_A in the peripheral tissues including liver (Minuk et al. 1987). Further, GABA exhibits tissue-specific effect on the insulin signalling pathway through GABA_A receptor in peripheral tissues (Minuk et al. 1987; Soltani et al. 2011; Tian et al. 2011). It has been reported that GABA, through GABA_A 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_A receptor promotes insulin secretion from pancreatic β-cells through intracellular calcium-dependent PI3K/Akt signalling pathway in type-1 diabetic condition (Soltani et al. 2011; Tian 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 & Krishnamurthy 2013). In contrast, GABA\_A-R stimulation attenuates peripheral insulin resistance probably through activation of the Ca\(^{2+}\)/PI3K/Akt signalling pathway in several insulin sensitive tissues including autoimmune T-cells and adrenal chromaffin cells (Kanai et al. 2009; Tian et al. 2004, 2011). The derailing of PI3K/Akt-mediated insulin signalling 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\_A-R-mediated effect of metformin on hepatic insulin resistance in streptozotocin-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 h light/dark cycle. The experimental animals had 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**

Streptozotocin, metformin, bicuculline and wortmannin were purchased from Sigma (St. Louis, MO). Antibodies such as phospho-Akt (p-Akt), total Akt, GLUT-4 and β-actin were purchased from Abcam Plc., Cambridge, MA. 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 streptozotocin (45 mg/kg, i.p.), 15 min after nicotinamide (110 mg/kg, i.p.) administration. Streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5) and nicotinamide was dissolved in physiological saline (Masiello et al. 1998; Garabadu & Krishnamurthy 2013).

**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 the experiments. The day animals received the streptozotocin 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 & Krishnamurthy 2014), bicuculline (BIC; 2.0 mg/kg, i.p.) (Sollozo-Dupont et al. 2015), wortmannin (WAT; 15.0 μg/kg, i.v.) (Ren 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 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 h to last dose on D-13. In experiment 3, after 1 h 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 until further study.

**Oral glucose tolerance test (OGTT)**

Oral glucose tolerance test (OGTT) is considered as a classical and model-based estimate of β-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 (Primam Instrument Pvt. Ltd., New Delhi, 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; Munipappula 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 (Primam 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-β) were calculated by the HOMA method (Matthews et al. 1985) using the following equations: IR (HOMA-IR) = [fasting glucose (mmol/L) × fasting insulin (μIU/mL)]/22.5, and β-cell function (HOMA-β) = [20 × fasting insulin (μIU/mL)]/(fasting glucose (mmol/L) – 3.5). The insulin was estimated by ELISA kit (Cayman Chemical, MI) following manufacturer’s instructions.

**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% CO₂ for the collagenase withdrawal. Thereafter, the cells were loaded with Krebs/Hepes buffer solution (mM: 143.3 Na⁺; 4.7 K⁺; 2.5 Ca²⁺; 1.3 Mg²⁺; 125.6 Cl⁻; 25 HCO₃⁻; 1.2 H₂PO₄⁻; 1.2 SO₄²⁻; 11.7 glucose and 10 HEPEs; pH 7.4) followed by 10 μM acetoxyanethylester fura-2 (AM form, dissolved in dimethylsulfoxide; Molecular Probes, Eugene, OR) and 5 μL (10% in DMSO) pluronic acid, and agitated to attain a complete desterification 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 intensity of fluorescence was calculated automatically. The Rₘₐₓ and Rₘᵟᵣᵱ values were determined by addition of digitonin (50 μM) and Mn²⁺ (2 mM) + EGTA (5 mM), respectively. The [Ca²⁺]ᵢ levels were expressed as nM and calculated by using the following formula: [Ca²⁺]ᵢ = 224 × [(R – Rₘᵟᵱ)/(Rₘₐₓ – R)].

Western blot technique

The preparation of cellular membrane fraction was performed as described previously (Nishiumi & 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/mL; and aprotinin, 5 mg/mL) and phosphatase inhibitors (NaF, 10 mM and Na₃VO₄, 1 mM)] and centrifuged at 1000 g for 10 min. The pellets were resuspended in NP-40 free buffer A in ice for another 10 min 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 1000g were combined and centrifuged at 16,000g 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), anti-p-Akt (Abcam Plc., Cambridge) and anti-Glut-4 (Abcam Plc., Cambridge) 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 reprobed overnight with rabbit anti-β-actin (Santa Cruz Biotechnology Inc.; Santa Cruz, CA) 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, Piscataway, NJ). Quantification of the results was performed by densitometric scan of films. The immunoreactive area was determined by densitometric analysis using Biovis gel documentation software, Upland, CA.

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

Wortmannin but not bicuculline abolished the anti-hyperglycaemic activity of metformin in T2DM rats in OGTT and ITT

Tables 1 and 2 depict the effect of metformin on the T2DM-induced changes in plasma glucose level of T2DM rats in ITT.

### Table 1. Effect of metformin in presence/absence of bicuculline or wortmannin on T2DM-induced changes in plasma glucose level of T2DM rats in OGTT.

| 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 ± 1.22ᵇ | 26.7 ± 1.27ᵃ | 26.5 ± 1.23ᵇ | 26.4 ± 1.43ᵃ |
| T2DM + MET | 12.5 ± 1.07ᵇ | 14.4 ± 1.13ᵇ | 15.2 ± 1.33ᵇ | 15.4 ± 1.28ᵇ |
| T2DM + MET + BIC | 12.7 ± 1.15ᵇ | 13.9 ± 1.31ᵇ | 15.9 ± 1.12ᵇ | 16.1 ± 1.13ᵇ |
| T2DM + MET + WAT | 25.3 ± 1.07ᶜ,d | 25.5 ± 1.24ᶜ,d | 26.1 ± 1.23ᶜ,d | 26.3 ± 1.23ᶜ,d |
| T2DM + BIC | 24.9 ± 1.13ᶜ,d | 24.8 ± 1.28ᶜ,d | 25.2 ± 1.25ᶜ,d | 25.9 ± 1.31ᶜ,d |
| T2DM + WAT | 24.7 ± 1.32ᶜ,d | 25.1 ± 1.31ᶜ,d | 25.4 ± 1.25ᶜ,d | 26.3 ± 1.33ᶜ,c,d |

All values are mean ± SEM (n = 6).

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### Table 2. Effect of metformin in presence/absence of bicuculline or wortmannin on T2DM-induced changes in the plasma glucose level of T2DM rats in ITT.

| 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 ± 1.25ᵇ | 19.9 ± 1.1ᵃ | 21.3 ± 1.0²ᵇ | 22.5 ± 1.25ᵃ |
| T2DM + MET | 14.1 ± 1.11ᵇ | 10.7 ± 1.23ᵇ | 11.1 ± 1.13ᵇ | 11.1 ± 1.16ᵇ |
| T2DM + MET + BIC | 14.7 ± 1.03ᶜ,d | 11.1 ± 1.21ᶜ;d | 10.9 ± 1.12ᶜ;d | 11.1 ± 1.24ᶜ,d |
| T2DM + MET + WAT | 26.4 ± 1.44ᶜ,d | 21.4 ± 1.13ᶜ,d | 21.8 ± 1.21ᶜ,d | 21.5 ± 1.25ᶜ,d |
| T2DM + BIC | 25.2 ± 0.78ᶜ,d | 19.9 ± 1.15ᶜ,d | 20.4 ± 1.05ᶜ,d | 20.6 ± 1.06ᶜ,d |
| T2DM + WAT | 25.5 ± 1.13ᶜ,d | 19.8 ± 1.21ᶜ,d | 20.3 ± 0.78ᶜ,d | 20.4 ± 1.73ᶜ,c,d |

All values are mean ± SEM (n = 6).

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*p < 0.05 compared to control,

*p < 0.05 compared to T2DM,

*p < 0.05 compared to T2DM + MET and

*p < 0.05 compared to T2DM + MET + BIC (repeated measures of two-way ANOVA followed by Bonferroni post hoc test).

### Notes

- 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.
Metformin did not alter T2DM-induced decrease in [Ca\(^{2+}\)] in rat liver tissue

The effect of metformin in absence or presence of bicuculline and wortmannin on the [Ca\(^{2+}\)] in liver tissue is depicted in Figure 1. Statistical analysis revealed that there were significant differences among groups in the [Ca\(^{2+}\)] 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\(^{2+}\)], in the liver tissue of the animals. Neither bicuculline nor wortmannin caused any change to the effect of metformin on [Ca\(^{2+}\)], 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\(^{2+}\)], in the liver tissue of the rats.

Table 3. Effect of metformin in presence/absence of bicuculline or wortmannin on T2DM-induced changes 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 | 155.5 ± 4.85^a |
| T2DM + MET      | 15.3 ± 1.17^a                 | 81.4 ± 8.71^b                 | 10.3 ± 0.33^b | 45.2 ± 3.22^ab |
| T2DM + MET + BIC| 15.5 ± 1.14^b,c,d             | 80.8 ± 9.35^b                 | 10.5 ± 1.53^b | 44.8 ± 4.41^b |
| T2DM + MET + WAT| 26.4 ± 1.43^b,c,d             | 121.8 ± 8.29^b,c,d           | 21.4 ± 1.44^c,d| 13.2 ± 5.23^c,d |
| T2DM + BIC      | 25.9 ± 1.21^c,d               | 122.2 ± 7.34^c,d             | 19.2 ± 1.52^c,d| 13.2 ± 3.42^c,d |
| T2DM + WAT      | 25.8 ± 1.21^c,d               | 121.6 ± 8.72^c,d             | 19.1 ± 1.41^d | 13.6 ± 4.29^d |

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

Figure 1. The 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). ^p < 0.05 compared to control, ^p < 0.05 compared to T2DM, ^p < 0.05 compared to T2DM + MET, ^p < 0.05 compared to T2DM + MET + BIC and ^p < 0.05 compared to T2DM + MET + WAT (one-way ANOVA followed by Student–Newman–Keuls test).

Wortmannin but not bicuculline abolished the therapeutic effect of metformin on insulin signalling pathway in liver of T2DM rodents

Table 3 demonstrates the effect of metformin on the level of fasting blood glucose and insulin, and the extent of IR, and the function of pancreatic β-cell in T2DM rats. Statistical analysis revealed that there were no 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-β) in rats. When wortmannin was administered along with metformin, the anti-hyperglycaemic, anti-hyperinsulinaemic, 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.

Figure 2 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 signalling 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.

Wortmannin but not bicuculline abolished the therapeutic effect of metformin on 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 Figure 3. 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 cytosolic and membranous fractions in the liver tissue of the animals respectively. Further, it attenuated the T2DM-induced decrease in the ratio of membranous/cytosolic 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.

**Discussion**

We for the first time report that metformin attenuated hepatic insulin resistance through GABA_\text{A}_R-independent mechanism in streptozotocin-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_\text{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\(_AR\)-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-\(\beta\) index (Tahara et al. 2008; Garabadu & 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\(_AR\) 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\(_AR\) activation attenuates \(\beta\)-cell dysfunction probably through downstream facilitation of PI3K/Akt signalling 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 signalling pathway by alternate pathways other than GABA\(_AR\) 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 & 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\(_AR\) 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 hyperinsulinaemia in rats and this effect was abolished when wortmannin was co-administered. These observations emphasize the fact that metformin exerts therapeutic effect against T2DM-induced hepatic insulin resistance through GABA\(_AR\)-independent mechanism.

We have estimated the \([\text{Ca}^{2+}]_i\) as a functional measure of bicuculline-sensitive GABA\(_AR\) receptor activity in the liver tissues. Metformin did not alter the T2DM-induced decrease in the \([\text{Ca}^{2+}]_i\) in the animals. The level of \([\text{Ca}^{2+}]_i\) 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 facilitates glucose utilization through GLUT-4 translocation. Only wortmannin when co-administered individually with metformin, blocked the anti-hyperglycaemic 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 signalling 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.

Conclusion

In the present study, metformin attenuated hepatic insulin resistance in T2DM rodents. In addition, it ameliorated \(\beta\)-cell dysfunction in these rats. Further, it improved the hepatic PI3K/Akt/GLUT-4 signalling pathway in T2DM rodents. Thus, it can be speculated that metformin exerts beneficial effect against T2DM-induced hepatic insulin resistance through bicuculline sensitive GABA\(_AR\)-independent mechanism.

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Disclosure statement

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