

Enhancement of [*m*-methoxy ³H]MDL100907 binding to 5HT_{2A} receptors in cerebral cortex and brain stem of streptozotocin induced diabetic rats

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Abstract

5-Hydroxytryptamine_{2A} (5-HT_{2A}) receptor kinetics was studied in cerebral cortex and brain stem of streptozotocin (STZ) induced diabetic rats. Scatchard analysis with [³H] (±) 2,3dimethoxyphenyl-1-[2-(4-piperidine)-methanol] ([³H]MDL100907) in cerebral cortex showed no significant change in maximal binding (B_{max}) in diabetic rats compared to controls. Dissociation constant (K_d) of diabetic rats showed a significant decrease (p < 0.05) in cerebral cortex, which was reversed to normal by insulin treatment. Competition studies of [³H]MDL100907 binding in cerebral cortex with ketanserin showed the appearance of an additional low affinity site for 5-HT_{2A} receptors in diabetic state, which was reversed to control pattern by insulin treatment. In brain stem, scatchard analysis showed a significant increase (p < 0.05) in B_{max} accompanied by a significant increase (p < 0.05) in K_d. Competition analysis in brain stem also showed a shift in affinity towards a low affinity State for 5-HT_{2A} receptors. All these parameters were reversed to control level by insulin treatment. These results show that in cerebral cortex there is an increase in affinity of 5-HT_{2A} receptors without any change in its number and in the case of brain stem there is an increase in number of 5HT_{2A} receptors accompanied by a decrease in its affinity during diabetes. Thus, from the results we suggest that the increase in affinity of 5-HT_{2A} receptors in cerebral cortex and upregulation of 5-HT_{2A} receptors in brain stem may lead to altered neuronal function in diabetes. (Mol Cell Biochem 199: 81–85, 1999)

Key words: diabetes, serotonin, 5-HT_{2A} receptor, cerebral cortex, brain stem, streptozotocin

Introduction

5-Hydroxytryptamine (5-HT) is a neurotransmitter known to play an important role in several physiological functions. A decrease in 5-HT synthesis and turn over in central nervous system (CNS) has been reported in chronically hyperglycemic rats [1, 2]. The effect of 5-HT is mediated in different tissues by different subclasses of 5-HT receptors, each of which are coded by a distinct gene and possess distinct pharmacological properties and physiological functions [3]. Previous studies have shown that 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) triggers adrenaline release and thereby inhibits insulin secretion in conscious rats

[4, 5]. The 5-HT₂ receptor subtypes are also thought to play a major role in mediating the effects of serotonin in a wide variety of tissues. 5-HT₂ subtype is found in high concentration in frontal cortex of the brain, blood vessels, gastrointestinal and uterine smooth muscle cells [6, 7]. Alteration in the number of 5-HT₂ receptors have been reported in several central nervous system disorders and is also thought to have a major role in insulin secretion and diabetes mediated depression [7].

In the present study we investigated the kinetic parameters of 5-HT_{2A} receptors in cerebral cortex and brain stem of STZ induced diabetic rats using a selective antagonist [³H]-MDL100907. We report an increased affinity of the receptor

binding in the cerebral cortex without any change in receptor number and an increased number of receptors in the brain stem with a decrease in affinity during diabetes.

Materials and methods

All biochemicals used were of analytical grade, [^3H]-MDL100907 (82.0 Ci/mmol) was purchased from Amersham radiochemical UK, ketanserin was a generous gift from Janssen Laboratories, Belgium. Streptozotocin was purchased from Sigma chemical Co., St. Louis, USA.

Animal experiments

Adult male Wistar rats of 200–240 g body wt were used for all experiments. They were housed in separate cages under 12 h light and 12 h dark periods and were maintained on standard food pellets and water *ad libitum*. Diabetes was induced by a single intrafemoral dose (65 mg/kg body wt) of STZ prepared in citrate buffer, pH 4.5 [8, 9]. The animals were randomly divided into three groups i.e. control, diabetic and insulin treated diabetic group with 4–6 animals in each group. The insulin treated diabetic group received a daily dose (1 Unit/kg body wt) of Lente and Plain insulin. The dose was increased daily according to the blood glucose level [10]. Glucose was measured by GOD-POD glucose estimation kit (Glaxo India Ltd.)

Tissue preparation

Rats were sacrificed by decapitation on the 14th day of the experiment. The cerebral cortex and brain stem were dissected out quickly over ice according to the procedure of Glowinski and Iversen [11].

5HT_{2A} receptor binding studies

5HT_{2A} receptor binding assay was done according to the modified procedure of Green *et al.* [12]. The cerebral cortex and brain stem were homogenized in 10 vol of ice cold 0.32 M sucrose in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 900 g for 10 min and the supernatant again centrifuged at 17,000 g for 1 h. The pellet was resuspended in 50 vol of 50 mM Tris HCl, pH 7.5 and recentrifuged at 17,000 g for another 1 h. The final pellet was resuspended in a minimum volume of Tris HCl, pH 7.7 containing 4 mM CaCl₂.

Binding assays were done using different concentrations i.e. 0.25–2.5 nM of [^3H]MDL100907 in Tris buffer, pH 7.7 containing CaCl₂ (4 mM), ascorbate (0.2%), and pargyline (10 μM) in a total incubation volume of 250 μl . Specific

binding was determined using 100 μM cold ketanserin. Competition studies were carried out with 0.5 nM [^3H]-MDL100907 in each tube with cold concentration varying from 10⁻⁹–10⁻⁴ M of ketanserin.

Tubes were incubated at 37°C for 30 min and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 3.0 ml of ice cold Tris buffer, pH 7.7. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

Protein determination

Protein was measured by the method of Lowry *et al.* [13] using bovine serum albumin as standard.

Receptor data analysis

The receptor data were analyzed by nonlinear regression using Graphpad Prism software, Graphpad Inc., USA. The concentration of the competing drug that competes for half the specific binding was defined as EC₅₀, which is the same as IC₅₀ [14]. The affinity of the receptor for the competing drug is designated as K_i and is defined as the concentration of the competing ligand that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors [15].

Statistics

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03).

Results

Streptozotocin administration to rats brought about a significant increase ($p < 0.001$) in blood glucose level. Treatment with insulin significantly reduced ($p < 0.001$) the blood glucose to near control value (Table 1).

Table 1. Blood glucose levels and body weight of experimental animals

	Blood glucose level (mg/dl)	Body weight (g)
Control	83.41 ± 13.39	211.42 ± 19.58
Diabetic	376.50 ± 27.28*	198.40 ± 14.79
Diabetic + insulin	124.81 ± 15.72†	200.5 ± 20.46

* $p < 0.001$ compared to control. † $p < 0.001$ compared to diabetic. Values are mean ± S.D. of 4–6 separate experiments.

Table 2. 5-Hydroxytryptamine_{2A} (5-HT_{2A}) receptor binding parameters in cerebral cortex and brain stem of control, diabetic and diabetic plus insulin treated rats

Animal status	[<i>m</i> -methoxy- ³ H]MDL100907 binding			
	Cerebral cortex		Brain stem	
	B _{max} (f moles/mg protein)	K _d (nM)	B _{max} (f moles/mg protein)	K _d (nM)
Control	230.00 ± 45.70	1.08 ± 0.11	34.77 ± 5.27	0.77 ± 0.30
Diabetic	208.66 ± 59.84	0.60 ± 0.09*	80.60 ± 7.40*	2.62 ± 0.53*
Diabetic + insulin	212.00 ± 39.58	0.95 ± 0.15†	47.22 ± 7.50†	0.90 ± 0.35†

**p* < 0.05 compared to control; †*p* < 0.05 compared to diabetic. B_{max} – Binding maximum, (fmoles/mg protein), K_d – Dissociation constant (nM). Values are mean ± S.E.M. of 4–6 separate experiments. Scatchard analysis of [³H]MDL100907 against ketanserin in cerebral cortex of control diabetic, and insulin treated diabetic rats. Incubation was done with different concentration, i.e. 0.25–2.5 nM of [³H]MDL100907 in a total incubation volume of 250 μl. One hundred μM ketanserin was used to determine the specific binding. Reaction was stopped by rapid filtration through GF/B (Whatman) filters with ice cold Tris buffer pH 7.7.

Scatchard analysis in cerebral cortex of diabetic rats did not show any significant change in B_{max} when compared to controls, but the K_d of diabetic rats showed a significant decrease (*p* < 0.05). Insulin treatment significantly (*p* < 0.05) reversed the K_d to near control level (Table 2). This shows an increase in affinity of the 5-HT_{2A} receptors during diabetes without any change in the receptor number. These results were confirmed by competition binding assay of [³H]MDL100907 against ketanserin (Fig. 1) which showed a low affinity site fitting a two-site model instead of the one-site model seen in control. In diabetic group the hill slope is also away from unity (0.66), confirming the two-site model (Table 3). Insulin treatment effectively reversed the two-site model to one-site model having a hill slope above unity (1.42). The appearance of low affinity site in diabetic group is evident from the EC₅₀ values (control EC₅₀₍₂₎ – 3.72 × 10⁻⁸, diabetic EC₅₀₍₂₎ –

1.03 × 10⁻⁶). Insulin treatment was able to reverse this change in affinity to control level (EC₅₀₍₂₎ – 6.89 × 10⁻⁸).

Scatchard analysis in brain stem showed a significant increase in B_{max} (*p* < 0.05) of diabetic rats when compared to control. There was also a significant increase (*p* < 0.05) in K_d of 5-HT_{2A} receptors (Table 2). The B_{max} was significantly reversed (*p* < 0.05) to normal by insulin treatment. These results show an upregulation of 5-HT_{2A} receptors accompanied by a decrease in its affinity during diabetic state. It was further analyzed by competition binding assay studies with [³H]-MDL100907 against ketanserin, which showed a shift from one-site model to a two-site model in diabetic group (Fig. 2). In diabetic group the Hill slope is away from unity (0.54) confirming the two-site model. Insulin treatment reversed the curve to a one-site model (Hill slope-2.24; Table 4).

Discussion

The major findings of this study are that there is an increase in affinity of cerebral cortex 5-HT_{2A} receptors without any change in its number and an appearance of a low affinity site during STZ-induced diabetes. In the case of brain stem 5-HT_{2A} receptors there is an upregulation of 5-HT_{2A} receptors accompanied by a decrease in its affinity. These alterations of 5-HT_{2A} receptors in the cerebral cortex and brain stem is a compensatory mechanism for the decreased 5-HT level reported during diabetes in the brain regions [16].

It has been well documented that long term hyperglycemia in diabetic animals can lead to chronic hypofunction of central 5-HT neurons leading to decreased brain tryptophan, 5-HT and 5-Hydroxy indole acetic acid (5-HIAA) [16, 17]. The decrease in brain 5-HT level is due to the decreased uptake of tryptophan into the brain [18, 19]. One of the main determinant of brain tryptophan content is the circulating insulin level. An increase in the level of insulin can result in decreased plasma concentrations of large neutral aminoacids which compete with tryptophan for uptake into the brain [18].

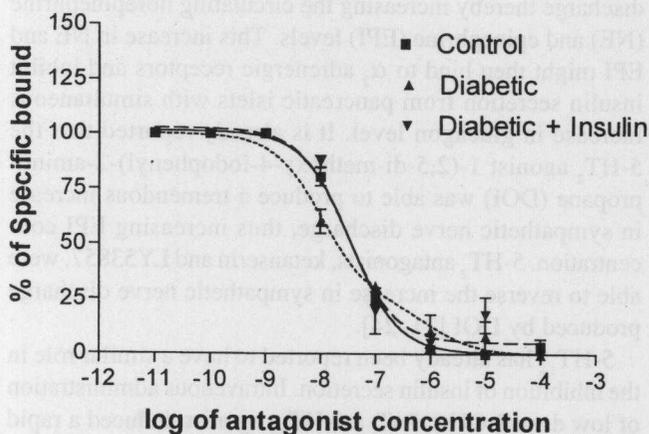


Fig. 1. Displacement of [³H]MDL100907 with ketanserin in cerebral cortex of control, diabetic and insulin treated diabetic rats. Incubation was done at 37°C for 30 min with 0.5 nM [³H]MDL100907 in each tube and cold concentration varying from 10⁻⁹–10⁻⁴ M. Reaction was stopped by rapid filtration through GF/B (Whatman) filters with ice cold Tris buffer pH 7.7. Values are representation of 4–6 separate experiments.

Table 3. Binding parameters of [³H]MDL100907 against Ketanserin in cerebral cortex of experimental animals

Animal status	Best-fit model	log(EC ₅₀)-1	log(EC ₅₀)-2	K _{i(H)}	K _{i(L)}	Hill slopes
Control	one-site	-7.452	-	2.629 × 10 ⁻⁸	-	1.098
Diabetic	two-site	-7.980	-5.986	8.154 × 10 ⁻⁹	8.05 × 10 ⁻⁷	0.669
Diabetic + insulin	one-site	-7.580	-	2.149 × 10 ⁻⁸	-	1.422

Data are from displacement curves as determined by non-linear regression analysis using the computer program PRISM and a one-site vs. two-site model. The affinity for the first and second site of the competing drug are designated as K_{i(H)} (for high affinity) and K_{i(L)} (for low affinity). EC₅₀ is the concentration of the competitor that competes for half the specific binding and it is same as IC₅₀. The equation built-into the programme is defined in terms of the log (EC₅₀).

Table 4. Binding parameters of [³H]MDL100907 against Ketanserin in brain stem of experimental animals

Animal status	Best-fit model	log(EC ₅₀)-1	log(EC ₅₀)-2	K _{i(H)}	K _{i(L)}	Hill slopes
Control	one-site	-9.475	-	2.288 × 10 ⁻¹⁰	-	2.543
Diabetic	two-site	-9.305	-7.143	4.458 × 10 ⁻¹⁰	6.475 × 10 ⁻⁸	0.546
Diabetic + insulin	one-site	-9.372	-	3.043 × 10 ⁻¹⁰	-	2.247

Data are from displacement curves as determined by non-linear regression analysis using the computer program PRISM and a one-site vs. two-site model. The affinity for the first and second site of the competing drug are designated as K_{i(H)} (for high affinity) and K_{i(L)} (for low affinity). EC₅₀ is the concentration of the competitor that competes for half the specific binding and it is same as IC₅₀. The equation built-into the programme is defined in terms of the log(EC₅₀).

STZ selectively destroys pancreatic β-cells and causes hypoinsulinemia leading to hyperglycemia [8, 9]. This decrease in the circulating insulin can increase the competition of other aminoacids with tryptophan for uptake into brain thereby decreasing the level of tryptophan and 5-HT in the brain of diabetic rats. In our experiments, treatment of diabetic rats with insulin effectively reversed the altered 5-HT_{2A} receptors to control. The increase in circulating insulin favors the

increased uptake of tryptophan into the brain which in turn increases the brain 5-HT levels thereby bringing a decrease in the 5-HT_{2A} receptors.

It is reported that the upregulation of 5-HT_{2A} receptors during diabetes is a secondary effect of hypoinsulinemia [20]. This upregulation of the receptor can have a possible role in the regulation of insulin secretion. The increased affinity and increase in number of 5-HT_{2A} receptors in cerebral cortex and brain stem respectively can increase the sympathetic nerve discharge thereby increasing the circulating norepinephrine (NE) and epinephrine (EPI) levels. This increase in NE and EPI might then bind to α₂ adrenergic receptors and inhibit insulin secretion from pancreatic islets with simultaneous increase in glucagon level. It is already reported that the 5-HT₂ agonist 1-(2,5-di-methoxy-4-iodophenyl)-2-amino-propane (DOI) was able to produce a tremendous increase in sympathetic nerve discharge, thus increasing EPI concentration. 5-HT₂ antagonists, ketanserin and LY53857, were able to reverse the increase in sympathetic nerve discharge produced by DOI [21–24].

5-HT_{1A} has already been reported to have a similar role in the inhibition of insulin secretion. Intravenous administration of low dose 8-OH-DPAT, a 5-HT_{1A} agonist, induced a rapid and transient hyperglycemia, the amplitude of which was dose-dependent [25]. The hyperglycemia was caused by an increase in EPI release which mediates its effect through α₂ adrenergic receptors. Idazoxan pretreatment prevented the hyperglycemic response of 8-OH-DPAT [26]. In addition to the central 5-HT₂ receptors the peripheral 5-HT₂ receptors may also play a major role in regulation of insulin, since the

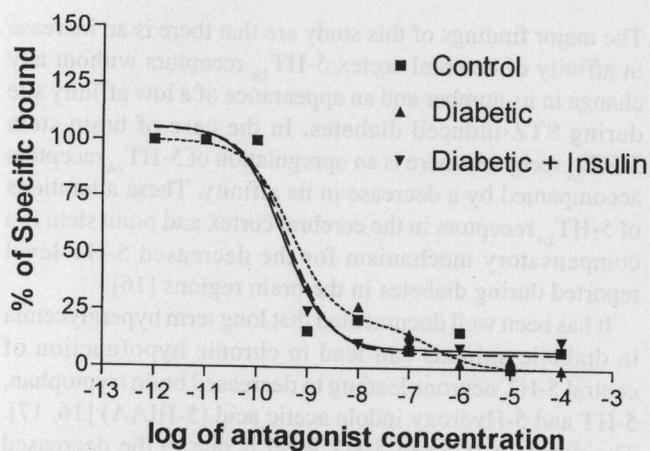


Fig. 2. Displacement of [³H]MDL100907 with ketanserin in brain stem of control, diabetic and insulin treated diabetic rats. Incubation was done at 37°C for 30 min. with 0.5 nM [³H]MDL100907 in each tube and cold concentration varying from 10⁻⁹–10⁻⁴ M. Reaction was stopped by rapid filtration through GF/B (Whatman) filters with ice cold Tris buffer pH 7.7. Values are representation of 4–6 separate experiments.

pancreatic islets contain a large amount of endogenous serotonin [27–29]. Our preliminary studies on 5-HT_{2A} receptors in pancreatic islets have shown alterations in binding parameters during diabetes (data not shown). McDonald [30] have reported increased expression of 5-HT₂ receptor mRNA in islets maintained for 1 day at 20 mM glucose than in islets maintained at 1 mM glucose. In addition to insulin regulation, an increase in affinity and number of 5-HT_{2A} receptors have a role in pathogenesis of major depression during diabetes [31, 32].

Thus, from our study we conclude that STZ induced diabetes causes an increase in affinity of cerebral cortex 5-HT_{2A} receptors without any change in the number of receptors. The brain stem 5-HT_{2A} receptors are upregulated accompanied by the appearance of a low affinity site which was reversed to control by insulin treatment. The enhanced 5-HT_{2A} receptor binding observed in brain regions can mediate an increased sympathetic nerve discharge in a similar way as central 5-HT_{1A} receptors leading to inhibition of insulin release from pancreas and can also mediate diabetes induced depression.

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