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Cholinergic, dopaminergic and insulin receptors gene expression in the cerebellum of streptozotocin-induced diabetic rats: Functional regulation with Vitamin D_3 supplementation

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ABSTRACT

The study was to find out the effect of Vitamin D_3 supplementation on preventing the altered gene expression of cholinergic, dopaminergic, insulin receptors and GLUT3 gene expression in cerebellum of diabetic rats. Radioreceptor binding assays and gene expression were done in the cerebellum of male Wistar rats. Rota rod has been used to evaluate motor coordination. Our results showed a significantly increased gene expression of dopamine D2, muscarinic M1, M3, α7 nicotinic acetylcholine, insulin receptors, acetylcholine esterase, GLUT3 and Vitamin D receptor in the cerebellum of diabetic rats. There was a down-regulation of dopamine D1 receptor. Total dopamine receptor showed a decreased and total muscarinic, muscarinic M1 and M3 receptors showed an increased binding parameter, B_{max}. Rota rod experiment showed a significant decrease in the retention time on the rotating rod in diabetic while treatment improved retention time near to control. Vitamin D_3 and insulin treatment markedly recovered the altered gene expression and binding parameters to near control. Our study showed Vitamin D_3 functional regulation through dopaminergic, cholinergic and insulin receptors and glucose transport mechanism through GLUT3 in the cerebellum of diabetic rats which play a major role in neuroprotection in diabetes which has clinical application.

1. Introduction

Vitamin D_3 is traditionally recognized as a potent regulator of calcium and phosphorus metabolism. Vitamin D_3 is either synthesised in the epidermis from 7-dehydrocholesterol by the absorption of ultraviolet light, or obtained from the diet in a limited number of foods such as eggs, fish oils, and fortified milk (DeLuca, 1993). Hyperglycaemia during uncontrolled diabetes is known to cause oxidative stress, which has been implicated in various secondary complications of diabetes. Diabetes mellitus has been reported to be accompanied by a number of behavioral and hormonal abnormalities, including hyperphagia, reduced motor activity (Marchall et al., 1976; Marchall, 1978). The biological actions of Vitamin D_3 are mediated through binding to the Vitamin D receptor (VDR), a member of the nuclear steroid hormone receptor family (Strugnell and DeLuca, 1997). An increased prevalence of diabetes has been described in Vitamin D-deficient individuals (Boucher et al., 1995; Isaia et al., 2001; Chiu et al., 2004). Insulin synthesis and secretion has been shown to be impaired in β cells in Vitamin D-deficient animals.

In the cerebellum, nicotinic acetylcholine receptors mediate the release of glutamate (Reno et al., 2004), GABA (De Filippi et al., 2001; Rossi et al., 2003) and norepinephrine (O'Leary and Leslie, 2003). These receptors significantly influence the activity within the cerebellar circuitry, and any deregulation of this activity contributes to functional disorders involving the cerebellum. Diabetes is also found to be associated with changes in somatic sensations which involve the cerebellum, cerebral cortex and thalamus. Symptoms, like loss of pain, impaired touch perception and decreased position sense, have been commonly documented in a diabetic patient (Waxman and Sabin, 1981). Atrophy of the cerebellum has been reported in diabetic patients, and this is not associated with the duration of the disease or glycaemic control (Lunetta et al., 1994).

Dopamine in the central nervous system is involved in the control of both motor and emotional behavior (Vallone et al., 2000) and peripherally modulates insulin secretion in the pancreatic islets (Nogueira et al., 1994). Nafadotride, a preferential antagonist of dopamine D3 receptors administered at low doses directly into the cerebellum, has been shown to activate locomotor activity (Barik and de Baurepaire, 1996).

Acetylcholine is a major neurotransmitter of the peripheral parasympathetic nervous system. It helps to facilitate the release of insulin in a glucose-dependent mode. Hence this activity has been

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shown to be mediated by the activation of muscarinic acetylcholine receptors located on the pancreatic β cells (Ahren, 2000; Gilon and Henquin, 2001). The brain glucose uptake is ultimately dependent on facilitative glucose transporters, the modulation of brain glucose transporters intrinsic activity. GLUT3 is the main neuronal glucose transporter (Maher et al., 1993) abundant in the brain.

Immunohistochemistry showed the presence of VDR in pituitary cells and mRNA and protein VDR expression in human pituitary gland (Perez-Fernandez et al., 1997), suggesting a possible role of Vitamin D in regulation of the brain endocrine system. A putative receptor for 1,25(OH)2D has been detected in chick brain (Jia and Nemere, 1999), allowing speculation that 1,25(OH)2D could act like other neuroactive hormones in modulating neuronal activity and neurotransmitter receptors (Rupprecht and Holsboer, 1999; Zakon, 1998). It is of particular importance that VDR and catalytic enzymes are colocalized in the brain (Baulieu, 1998), supporting an autocrine/paracrine function for Vitamin D. These findings support a functional role for Vitamin D in the human brain (McGrath et al., 2001).

The role of Vitamin D_3 in regulating the cholinergic and dopaminergic receptors function in the cerebellum has not been studied. In the present study we examine the effect of Vitamin D_3 in modulating the cholinergic, dopaminergic and insulin receptors and GLUT3 in the cerebellum of STZ-induced diabetic rats for understanding the therapeutic role of Vitamin D_3 in diabetes associated functional disorders involving the cerebellum. Our present study on the anti-diabetic property of Vitamin D_3 in cerebellum mediated through cholinergic and dopaminergic receptors will definitely enlighten novel therapeutic possibilities for diabetes.

2. Materials and methods

Bio chemicals used in the present study were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents of analytical grade were purchased locally. Quinuclidinyl benzilate, L-[Benzilic-4,4′-3H], ([^{3}H] QNB)(Sp. Activity 42 Ci/mmol), 4-DAMP, [N-methyl-3H] (Sp. Activity 83 Ci/mmol), [^{3}H] dopamine were purchased from NEN Life Sciences Products Inc., Boston, U.S.A. Pirenzepine, 4-DAMP mustard, dopamine and cholecalciferol were from Sigma Chemical Co., USA. Tri-reagent kit was purchased from MRC, USA. Real Time PCR Taqman probe assays on demand were from Applied Biosystems, Foster City, CA, USA.

Male adult Wistar rats of 180–240 g body weight were used for all experiments. The animals were allowed to acclimatise for 2 weeks before the experiment. They were housed individually in separate cages under 12 h light and 12 h dark periods. Rats had free access to standard food and water ad libitum. All animal care and procedures were done in accordance with the Institutional and National Institute of Health guidelines. All efforts were made to minimize the number of animals used and their suffering. Diabetes was induced in rats by single intra femoral vein injection of STZ freshly dissolved in 0.1 M citrate buffer, pH 4.5, under anaesthesia (Junod et al., 1969). STZ was given at a dose of 55 mg/kg body weight (Hohenegger and Rudas, 1971; Arison et al., 1967). Animals were divided into the following groups: (i) Control, (ii) diabetic, (iii) insulin-treated diabetic and (iv) Vitamin D_3-treated diabetic rats. Each group consisted of 6–8 animals. The insulin-treated diabetic group received subcutaneous injections (1 U/kg body weight) of Lente and Plain insulin (Boots India) daily during the entire period of the experiment. The last injection was given 24 h before sacrificing the rats. Vitamin D_3-treated groups received 12 μg/kg Vitamin D_3 dissolved in 0.3 ml of coconut oil. The supplementation was administrated *via* gavage for a period of 2 weeks (De Souza Santos and Marques Vianna, 2005) for the entire period of the experiment. Rats were sacrificed on 15th day by decapitation. The cerebellum was dissected out quickly over ice according to the procedure of Glowinski and Iversen (1966), and the tissues collected were stored at −80 °C until assayed.

2.1. Estimation of blood glucose

Blood glucose was estimated by the spectrophotometer method using glucose oxidase–peroxidase reactions. Blood samples were collected from the tail vein at 0 h (Before the start of the experiment), 3rd, 6th, 10th and 14th day and the glucose levels were estimated subsequently. Along with this blood samples were collected 3 h after the administration of morning dose of insulin and Vitamin D_3. The results were expressed in terms of milligram per decilitre of blood.

2.2. Rota rod test

Rota rod has been used to evaluate motor coordination by testing the ability of rats to remain on revolving rod (Dunham and Miya, 1957). The apparatus has a horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70 cm long rod was divided into four sections by wooden partitions. The rod was placed at a height of 50 cm to discourage the animals to jump from the rotating rod. The rate of rotation was adjusted in such a manner that it allowed the normal rats to stay on it for 5 min. Each rat was given five trials before the actual reading was taken. The readings were taken at 10, 15 and 25 rpm after 15 days of treatment in all groups of rats.

2.2.1. Total muscarinic, muscarinic M1 and M3 receptor binding studies in the cerebellum

Binding assay in cerebellum was done according to the modified procedure of Yamamura and Synder (1981), Cerebellum was homogenised in a polytron homogeniser with 20 volumes of cold 50 mM Tris–HCl buffer, pH 7.4 containing 1 mM EDTA. The supernatant was then centrifuged at 30,000×*g* for 30 min and the pellets were resuspended in appropriate volume of Tris–HCl-EDTA buffer pH 7.4.

Total muscarinic, and muscarinic M1 receptor binding parameter assays were done using [^{3}H]QNB (0.1–2.5 nM) and M3 receptor using [^{3}H]DAMP (0.01–5 nM). The non-specific binding was determined using 100 μM atropine for Total muscarinic, pirenzepine for muscarinic M1 and 4-DAMP for M3 receptor. Total incubation volume of 250 μl contains 200–250 μg protein concentrations. Tubes were incubated at 22 °C for 60 min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50 mM Tris–HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 10% in all our experiments.

2.2.2. Total dopamine receptor binding studies in the cerebellum

DA receptor assay was done using [^{3}H]DA according to Madras et al. (1988). Cerebellum was homogenised in a polytron homogeniser with 20 volumes of cold 50 mM Tris–HCl buffer, along with 1 mM EDTA, 0.01%ascorbic acid, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, pH 7.4 and centrifuged at 38,000×*g* for 30 min at 4 °C. The pellet was washed twice by rehomogenization and centrifuged twice at 38,000×*g* for 30 min at 4 °C. This was resuspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.25 nM–1.5 nM of [^{3}H]DA in 50 mM Tris–HCl buffer, along with 1 mM EDTA, 0.01% ascorbic acid, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, pH 7.4 in a total incubation volume of 250 μl containing 200–300 μg of proteins. Specific binding was determined using 100 μM unlabelled dopamine.

Tubes were incubated at 25 °C for 60 min. and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50 mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 10% in all our experiments.