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Research article

Insulin influenced expression of myelin proteins in diabetic peripheral neuropathy

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HIGHLIGHTS

- Down regulation of myelin specific proteins, MBP and MAG in DPN.
- IR fluctuation in experimental model of DPN and Schwann cells.
- Improved expression myelin proteins upon insulin treatment.
- Correlation between expression of IR and myelin specific proteins.

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ABSTRACT

Diabetic peripheral neuropathy (DPN) is one of the downstream complications of diabetes. This complication is caused by the deficiency of insulin action and subsequent hyperglycemia, but the details of their pathogenesis remain unclear. Hence, it is of critical importance to understand how such hormonal variation affects the expression of myelin proteins such as myelin basic protein (MBP) and myelin associated glycoprotein (MAG) in the peripheral nerve. An earlier report from our lab has demonstrated the expression of insulin receptors (IR) in Schwann cells (SCs) of sciatic nerve. To assess the neurotrophic role of insulin in diabetic neuropathy, we studied the expression of these myelin proteins under control, DPN and insulin treated DPN subjects at developmental stages. Further, the expression of these myelin proteins was correlated with the expression of insulin receptor. Expression of myelin proteins was significantly reduced in the diabetic model compared to normal, and upregulated in insulin treated diabetic rats. Similarly, an *in vitro* study was also carried out in SCs grown at high glucose and insulin treated conditions. The expression pattern of myelin proteins in SCs was comparable to that of *in vivo* samples. In addition, quantitative study of myelin genes by real time PCR has also showed the significant expression pattern change in the insulin treated and non-treated DPN subjects. Taken together, these results corroborate the critical importance of insulin as a neurotrophic factor in demyelinized neurons in diabetic neuropathy.

1. Introduction

Diabetic peripheral neuropathy (DPN) is a microvascular complication of diabetes mellitus, characterized by demyelination of neurons accompanied by sensory loss. It is the result of abnormalities in pathways and molecules related to neuronal stress, and abnormal insulin signaling [1]. Poor glycemic control and reduced support of trophic factor like insulin are the prominent reasons of neuropathy in diabetic mellitus [2]. DPN encompasses functional deficits in motor, sensory, and sympathetic neurons, causing majorly pain in the distal lower extremities and hands [3,4]. Schwann cells (SCs) ensheathing the axons provide structural and functional support to the nerves during development and injury [5]. These SCs are integral parts of peripheral nervous system (PNS) for saltatory conduction of nerve impulse by forming a thick myelin around axons, and also creates a microenvironment to provide neurotrophic factors for the regeneration of neurons. During DPN, the function of SCs is altered due to hyperglycemia, oxidative stress and mitochondrial dysfunction leading to its decreased support to neurons [6].

Insulin is one of the growth factors utilized by various cells for its growth and differentiation [7]. It promotes neurite outgrowth and regeneration of nerves in both central and peripheral nervous

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system. Altered insulin support may contribute to DPN in PNS [8]. Recently, our laboratory reported the localization of insulin receptors (IRs) on SCs, suggesting the role for insulin in PNS [9]. A large number of studies support the prominent role of insulin and its receptor in the nerve development [10–13]. Among the many functions of insulin, gene expression and protein synthesis via MEK and PI3K pathways of insulin receptor signaling plays a major role in the promotion of cell growth [14]. A less is known about the mechanism of insulin action on the nervous system, in turn its role in DPN [8].

To study the molecular mechanism of insulin on diabetic models, we investigated the role of insulin on PNS myelin proteins like myelin basic protein (MBP) and myelin associated glycoprotein (MAG). Correlation studies with IR expression were also performed. Though myelin proteins comprise 20–30% of total myelin, it plays a major role in the maintenance of myelin. MBP and MAG are the two major proteins of myelin synthesized by SCs, which play a key role in the early stages of myelination, and maintenance of stable axon-myelin interaction [15]. Decreased expression of these myelin proteins was observed in sciatic nerve of diabetic rats [16]. Upon treatment of diabetic rats with insulin, improvement in the expression of myelin proteins in the sciatic nerve was observed. Similarly, these protein expressions were studied in SCs cultured at high glucose condition in the absence and presence of insulin. These results further supported the *in vivo* studies. Our observations indicate the neurotrophic role of insulin in diabetic peripheral neuropathy.

2. Materials and methods

2.1. Chemicals and reagents

Streptozotocin, Dulbecco's phosphate buffered saline (DPBS) and BSA were purchased from Himedia (India). All antibodies were procured from Abcam (UK). DMEM and FCS were from Gibco (USA). All real time PCR reagents were obtained from Invitrogen (USA). All other chemicals were purchased from sigma (USA) unless mentioned.

2.2. Animals and grouping

Animal care and procedures for animal experiments were conducted in conformity to the guidelines of animal care and use. Wistar rats (male and female) were obtained from the Animal facility, SS Institute of Medical Sciences and Research Centre, Davangere, India. The rats were maintained under a 12 h light/dark cycle and fed with standard rat chow, and had access to water *ad libitum*. 3–4 days old pups were used for the Schwann cell culture preparation. All efforts were made to minimize the number of animals used and their suffering. Animals were grouped into three groups [Group 1, Control (C); Group 2, streptozotocin induced diabetic rats (STZ); Group 3, Insulin treated diabetic rats (STZ + I)] of 12 animals each.

2.3. Diabetes induction and insulin treatment

Adult male Wistar rats weighing 200–250 g were injected with 20 mg/kg body weight streptozotocin after overnight fasting. After one week, a second dose of 40 mg/kg body weight streptozotocin was injected. Age matched male rats injected intraperitoneally with citrate buffer (0.1 M) were used as control. Diabetes was confirmed by estimating blood glucose after 48 h of second dose of streptozotocin. Rats which showed a mean plasma glucose level above 250 mg/dl were considered as diabetic. Diabetic neuropathy was confirmed by hot plate method as described earlier [28]. Group 3 rats were daily injected subcutaneously with insulin (1 IU). The sciatic nerves from control, diabetic and insulin treated diabetic rats were dissected at 1–4 month time interval.

2.4. Isolation and culture of primary Schwann cells

Primary culture of SCs was prepared from the sciatic nerve of 4 day old pups as previously described by Brockes [17] with slight modifications [7,9]. Briefly, the sciatic nerve from postnatal day 4 was dissected and digested enzymatically with collagenase (0.05%) and trypsin (0.25%), and plated on poly-L-lysine coated culture dishes in DMEM with 10% FCS. After 24 h, cells were treated with 10 μM cytosine arabinoside for 12 h, before being trypsinized and centrifuged to get pure SCs. The pellet was washed in calcium and magnesium free DPBS to remove the serum containing media. Cells were then suspended in serum free 1:1 DMEM and Ham's F12 media containing 40 mg/l gentamicin, 30 nM sodium selenite, 100 μM putrescine, 20 nM progesterone, 5 mg/l transferrin and 1% bovine serum albumin. The cells were then seeded at a density of 30,000–40,000 cells per 18 mm coverslip pre coated with poly-L-lysine in a 6-well plate for immunocytochemical studies. For protein and mRNA expression, cells were plated at a density of 10×10^5 cells/75 cm^2 tissue culture flask. Cultures were used for the experiment, after they attain approximately 90% confluency.

2.5. Western blotting

The protein expression study was carried out as described previously [18]. Briefly, SCs were cultured in serum free 1:1 DMEM and Ham's F12 medium with 60 mM glucose in the absence and presence of 10 nM insulin for 72 h. Cells were harvested and protein lysates were prepared in RIPA buffer at 4 °C by brief sonication. Similarly, protein lysates were prepared from sciatic nerves of the control, diabetic and insulin (1U) treated diabetic rats at different intervals of time. Protein concentrations were determined using the Bradford's method.

40 μg of protein from each sample was loaded and resolved on 10–18% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electrotransferred onto PVDF membranes (Millipore, India), blocked with skimmed milk solution, and blotted with each specific primary antibody overnight at 4 °C. Blots were then washed in wash buffer (Tris buffered saline with 0.1% Tween-20) and treated with secondary antibody conjugated with alkaline phosphatase and developed with NBT/BCIP substrate (Genei, India).

2.6. RNA isolation and real time PCR

After the time course of treatment with insulin, total RNA was isolated from cells and tissues using total RNA isolation kit according to the manufacturer's protocol. mRNA expression studies were carried out as prescribed previously [7] by using StepOne plus real time PCR (Applied Biosystems, USA). The primer sets used were MBP (forward 5′-CTTCCTCCCAAGGCACAGAG-3′; reverse 5′-AAATCTGCTGAGGGACAGGC-3′), MAG (forward 5′-TCAGGGAGACTGAGGTGAGG-3′; reverse 5′- CGAACTACTAGGTGGTGTTGT-3′), IR (forward 5′-CTGAAGGAGCTGGAGGAGTC-3′; reverse 5′-GATTTCATGGGTCACAGGGC-3′) and RPL19 (forward 5′-CGTCCTCCGCTGTGGTAAA-3′; reverse 5′-AGTACCCTTCCTCTTCCCTAT-3′). Analysis was done using StepOne™ software v2.2.2. The fold change was calculated for the mRNA expression by using delta–delta Ct method ($2^{-\Delta\Delta Ct}$) using RPL19 as housekeeping gene.