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Role of insulin in the expression of PMP22, a myelin protein during diabetic peripheral neuropathy

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Abstract: *One of the long-term complications of diabetes is diabetic peripheral neuropathy (DPN). DPN, which is characterized by the neuron demyelination often causes sensory loss.We have earlier reported the role of insulin in the expression of myelin proteins such as myelin basic protein (MBP) and myelin associated glycoprotein (MAG) during diabetic peripheral neuropathy condition. But the effect of insulin on the expression of Peripheral Myelin Protein 22 (PMP22) was not clear. Therefore, in this current study, we have analyzedthe effect of insulin on the expression of PMP22, both in vitro and in vivo using protein and mRNA expression studies. Results from both Western blotting and real time PCR confirmed that, expression of PMP22 concentration was decreased in diabetic condition and was recovered to normal upon treatment with insulin. These results corroborate the possibleneurotrophic role of insulin in enhancing the expression of PMP22 in the diabetic neuropathy condition.*

Keywords*: Diabetic peripheral neuropathy, Insulin, Schwann cells, Myelin protein, Sciatic nerve*

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I. Introduction

Myelin sheath(MS) is the extension of a specialized plasma membrane around the axons.Glial cells, such as the Schwann cells and Oligodendrocytes are responsible for the formationof myelin sheaths in Central and Peripheral Nervous System(PNS), respectively. Myelin facilitates the faster conduction of nerve impulses[1] and is composed of 70% of lipids and 30% of proteins of significant contribution. Major myelin proteins in the PNS are myelin basic protein (MBP), myelin-associated glycoprotein (MAG), P zero(P0) and peripheral myelin protein 22 (PMP22).Among these, peripheral myelin protein 22 (PMP22) is vital for the functioning of peripheral nerve, besides being the minor constituent of myelin sheath [2]. As confirmed by genetic evidence, this small intrinsic membrane protein possessesthe structural role in compact myelin, which involved in the initial steps of myelination process. Previous studies have shown that PMP22 might be involved in regulating the myelin thickness by controlling the expression of other myelin molecules [3].

It is well known that the inadequate production or disability to use insulin by the body leads to a chronic metabolic complication referred to as Diabetes Mellitus [4]. This disorder is characterized by increased blood glucose levels. Majorly, there are two types of diabetes: type 1 and type 2. Patients affected by either condition may develop microvascular complications like retinopathy, nephropathy,and neuropathy[5]. Out of these, diabeticperipheral neuropathy (DPN), characterized by demyelination of neurons that results in a sensory loss [6], is one of the most common and long-term complications of diabetes. An estimated prevalence of DPN, with respect to the degree and period of diabeteshas ranged between 13% to 58%[7,8]. The main reasons behind DPN in diabetes involves the poor glycemic control and reduced support for insulin [9]. Schwann cells (SCs) play a major role in maintaining the health of PNS by remyelinating the axons under demyelinated condition [10, 11].

The neurotrophic role of insulin was earlier reported in earlier studies, which confirmed that insulin induces the formation of neurite [12, 13]. Previous works from our laboratory have shown the presence of insulin receptors on Schwann cells[14]. Also, we have reported theneurotrophic role of insulin in enhancing the expression of myelin proteins such as MBP and MAG in the diabetic neuropathy condition, and the correlation between the expression of these myelin proteins and insulin receptor [15]. But, the role of PMP22 protein during the DPN was poorly understood. Therefore, in this current work, we studiedthe differential expression of the PMP22 protein in the Sciatic nerve of diabetic neuropathy rats with or without the insulin treatment. Further, the expression of PMP22 in Schwann cells at high glucose and insulin-treated conditions were also analyzed. Results confirmed that the PMP22 protein has a potent role in the myelination which was altered during diabetic peripheral neuropathy.

II. Materials and Methods

2.1. Chemicals and reagents

All chemicals were procured from Sigma (USA) unless mentioned. Bovine Serum Albumin (BSA), Streptozotocin (STZ) and Dulbecco's phosphate buffered saline (DPBS) were obtained from Himedia (India). All real-time PCR reagents were from Invitrogen (USA). Antibodies were from Abcam (UK). DMEM and fetal bovine serum (FBS) were purchased from Gibco (USA).

2.2. Animals and grouping

Animal experiments were conducted in agreement with the animal ethical guidelines. Male and female Wistar rats were obtained from SS Institute of Medical Sciences and Research Centre, Davangere, India and maintained in an environment with 12 h light/dark period. Animals were fed with standard rat chow with water ad libitum. Total of 24 animals was grouped into three groups of 8 each. Group 1 is a control group (C) consists of non-diabetic normal animals, whereas group 2 includes streptozotocin-induced diabetic rats (STZ). Further, group 3 includes diabetic rats treated with insulin (STZ + I). Isolation of Schwann cell was performed by using 3-4 days old pups.

2.3. Induction of diabetes and insulin treatment

Diabetes was induced in adult male Wistar rats (200-250 g) by administering two doses of streptozotocin, 20 and 40 mg/kg body weight, respectively, with an interval of one week.Both the streptozotocin doses were given intraperitoneally, after overnight fasting. After 48 h of the second dose of streptozotocin, induction of diabetes was confirmed by measuring blood glucose. Rats with a mean plasma glucose level more than 250 mg/dl were considered as diabetic. Hot plate method was performed to validate the diabetic neuropathy [16]. Control group animals were injected with 0.1 M citrate buffer. 1 IU insulin was subcutaneously injected daily to group 3 rats. The sciatic nerves from all the three group animals were isolated each month for two months time period.

2.4. Isolation and culture of primary Schwann cells

Schwann cells (SCs) were isolated from sciatic nerve of 4-day old pups as per earlier reported method [17] with some modifications [14,15]. In brief, the sciatic nerve extracted from 4-day old pups were digested with collagenase (0.05%) and trypsin (0.25%). The digested nerve was then cultured on poly-l-lysine coated culture plates in DMEM with 10% FCS for 24 h, before treating the cells with 10 μM cytosine arabinoside for 12 h. Cells were further trypsinized and pure SCs were pelleted by centrifugation, washed in calcium and magnesium free DPBS and cultured in 1:1 DMEM and Ham's F12 media without FCS and containing gentamycin (40 mg/l), sodium selenite (30 nM), putrescine (100 μM), progesterone (20 nM), transferrin (5 mg/l) and 1% BSA. Approximately, 10×10^5 cells were seeded in $75cm^3$ tissue culture flasks and 90% confluent cells were used for the study.

2.5. Western blotting

Expression of PMP22 protein was studied as reported earlier [15]. Briefly, SCs were cultured in serum-free 1:1 DMEM and Ham's F12 medium containing 60 mM glucose with or without 10 nM insulin for 72 h. Protein lysates were prepared by sonicating the harvested cells in RIPA buffer at 4◦ C. Also, lysates were prepared similarly from the sciatic nerves of animals of all the three groups. The concentration of protein was estimated by performing Bradford's assay.

All the protein samples (40 μg) were resolved on SDS-polyacrylamide gel electrophoresis (15%) and transferred to PVDF membranes (Millipore, India). After transferring, membranes are blocked with skimmed milk solution and blotted with primary antibody of PMP22 overnight at 4◦C. Then, the blots were washed in tris-buffered saline with 0.1% Tween-20, probed with alkaline phosphatase linked secondary antibody and developed with NBT/BCIP substrate (Genei, India).

2.6. RNA isolation and real-time PCR

Total RNA was extracted from both Schwann cells and sciatic nerve tissues with or without insulin treatment, using total RNA isolation kit as per the manufacturer's protocol. mRNA expression was analyzed using a StepOne plus real time PCR (Applied Biosystems, USA) [15]. The primer sets used were: PMP22 (forward 5'-CCATCCCTGGCTCTCGATTG-3'; reverse 5'-CAAGGCGGATGTGGTACAGT-3'), and RPL19 (forward 5'- CGTCCTCCGCTGTGGTAAA-3'; reverse 5'-AGTACCCTTCCTCTTCCCTAT-3'). Fold change in the expression of mRNA was estimated through delta-delta Ct method ($2^{-\Delta\Delta Ct}$) using StepOneTM software v2.2.2 by considering RPL19 as housekeeping gene.

2.7. Statistical analysis

Assays were performed in triplicate and data were expressed as mean±SEM. Statistical analysis of the data was performed by using Student's t-test. Significance was established upon a $p < 0.05$.

III. Results

Western blotting was performed to study the expression of PMP22 (Fig. 1) at two different time point in sciatic nerve samples isolated from control, STZ induced diabetic rats and 1U insulin-treated diabetic rats. 40 µg of protein sample extracted from these tissues were separated on 15% SDS-PAGE and the bands were obtained by western blotting using anti-PMP22 primary antibody. Reduction in the expression of PMP22 was observed in diabetic rat samples, compared to control. Interestingly, the PMP22 concentration was recovered to normal upon treatment with insulin (Fig. 1A).

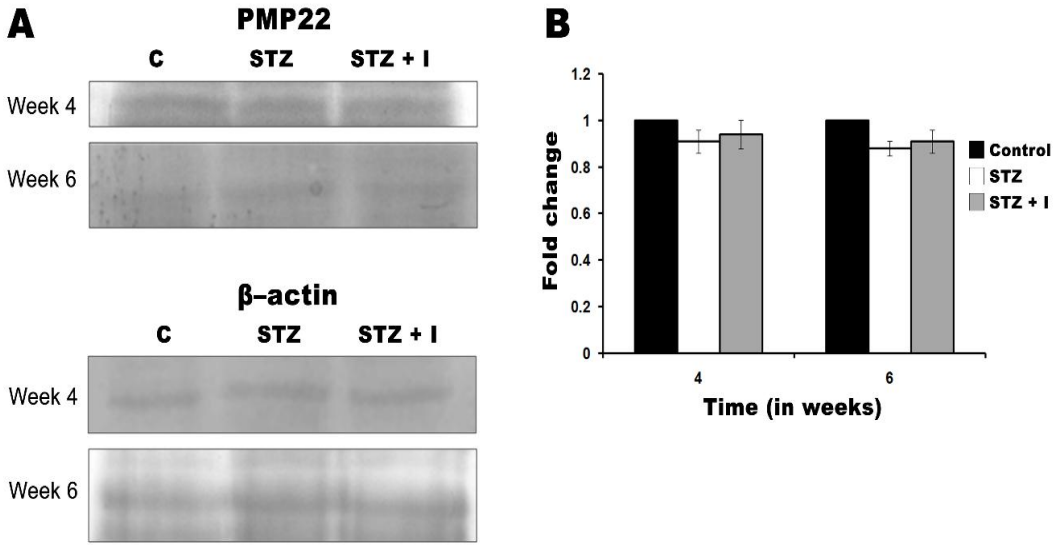

Figure 1: A. Evaluation of PMP22 protein expression in sciatic nerve of control (C), diabetic (STZ) and insulin treated diabetic (STZ+I) rats by western blotting. β-actin was used as loading control. **B.** Western blotting data presented as a bar chart.

A similar pattern of results was also obtained in the real-time PCR experiments (Fig. 2). The fold change was calculated between control, STZ induced diabetic rats and 1U insulin-treated diabetic rat samples. We observed decreased fold change in the STZ samples, compared to control, which further improved upon insulin treatment.

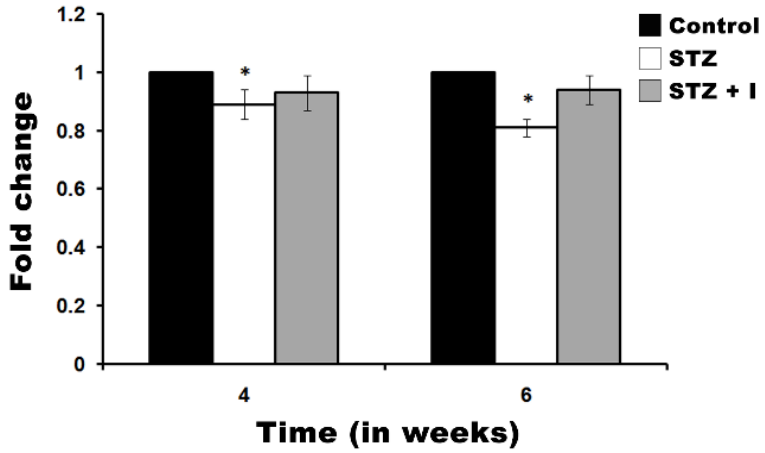

Figure 2: Quantitative mRNA expression of PMP22 in sciatic nerves of control (C), diabetic (STZ) and insulin treated diabetic (STZ+I) rats by qPCR analysis. RPL 19 gene was used as a housekeeping gene. * indicates $p< 0.05$.

In vitro studies were carried out using cultured primary Schwann cells (Fig. 3) grown at control, hyperglycemic and insulin-treated hyperglycemic conditions. After the stipulated time of treatment, cells were processed for protein isolation. An equal quantity of proteins from all samples was subjected to Western blotting for PMP22 protein. The bands obtained indicated the reduced PMP22 expression in SCs grown on high glucose condition, which further elevated slightly to normal level after supplemented with 10 nM insulin (Fig. 4). Real-time PCR results (Fig. 5) of SC samples were in agreement with that of western blotting. All these results collaboratively suggest the role of insulin in enhancing the expression of PMP22 in the diabetic neuropathy condition, thus acting as a neurotrophic factor.

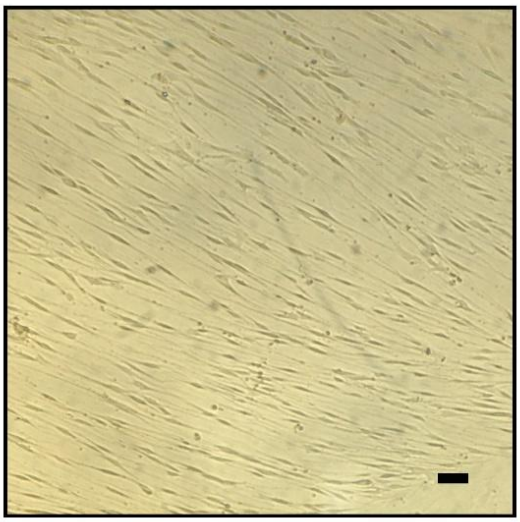

Figure 3: Cultured primary Schwann cells. Scale bar, 50 µm

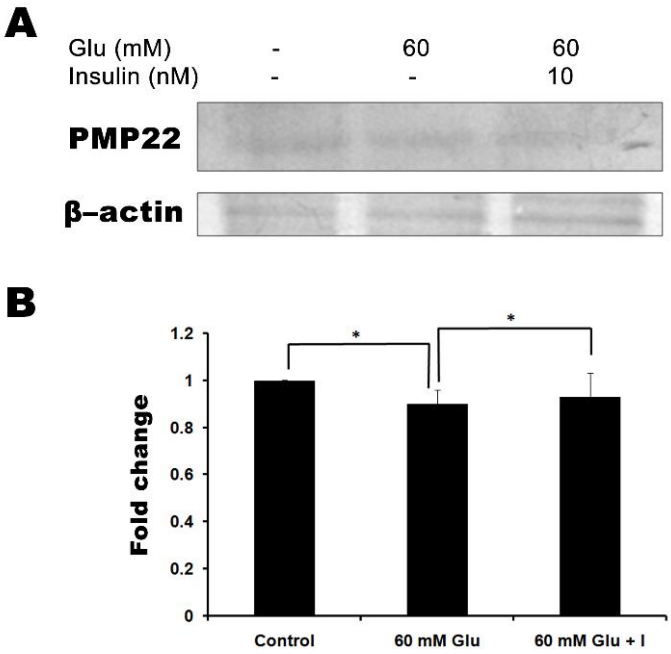

Figure 4: A. Expression of PMP22 in primary Schwann cells by Western blotting analysis. β-actin was used as loading control. **B.** Western blotting data were presented as a bar chart. * indicates $p<$ 0.05.

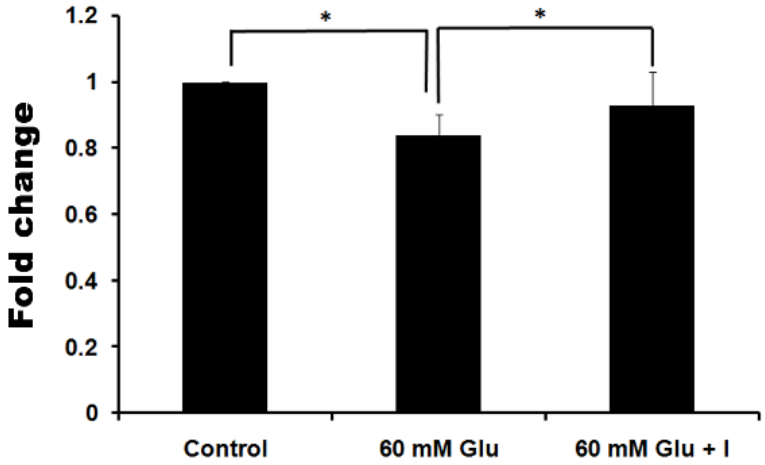

Figure 5: Quantitative mRNA expression of PMP22 in primary Schwann cells with high glucose in the absence and presence of 10 nM insulin by qPCR analysis. RPL 19 gene served as a housekeeping gene. * indicates $p< 0.05$.

IV. Discussion

Despite several attempts, till now no successful management of diabetic neuropathy was achieved. Therefore, the treatment of diabetic neuropathy is of higher importance in medicine. Demyelination being the major cause, needs to be addressed to treat the DPN.Demyelination is characterized by the degeneration of axons and retardation in the nerve conduction[18,19]. Earlier studies have reported the neuroprotective effect of steroid molecules,whichnot only inhibits peripheral nerve damage but also improvesthe expression of myelin proteins, P0 and PMP22 in diabetic neuropathy condition [20-22]. Schwann cells support the PNS during DPN by regenerating the myelin sheath. SCsare of critical importance, as they synthesize the proteins required for the myelination.

Glycoprotein zero (P0) and peripheral myelin protein 22 (PMP22) are involved in the maintenance of myelin structure of PNS [23,24]. Among these, PMP22 is an integral protein of myelin consisting of both extracellular and transmembrane domains [25].Early stages of myelination require PMP22, deficient of which results in retardation of myelin formation [3]. The reduced PMP22 expression was observed in the STZ induced diabetic rats[26], thus, could be one of the reasons for demyelination.Previous work from our laboratory, has shown the presence of insulin receptors on Schwann cells [14], signifying the role of insulin in PNS. On this basis, we studied and confirmed the neurotrophic role of insulin by elevating the level of MBP and MAG which are also an integral part of myelin [15]. In the current study, we demonstrated the role of insulin in enhancing the expression of PMP22 in diabetic neuropathy samples. Both *in vivo* study on sciatic nerve samples and *in vitro* study on Schwann cells confirmedthat theinsulin functions as a neurotrophic factor.

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Competing interests

The authors declare that they have no competing interests.

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