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SHORT COMMUNICATION

The correlation between insulin and OCT-6 transcription factor in Schwann cells and sciatic nerve of diabetic rats

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Abstract Insulin signal is one of the vital signaling cascade required for Schwann cells to myelinate the axons of peripheral nervous system (PNS). Myelin formation of peripheral nerve is a complex molecular event controlled by different neurotrophic and transcription factors. The altered or failure in this signaling progression is one of the reasons behind the demyelination of peripheral neurons in diabetic peripheral neuropathy (DPN). The Schwann cell in PNS includes POU domain transcription factor OCT-6 expression. This factor is considered as crucial for the initiation and enhancement of myelination during nerve regeneration. To know the importance of OCT-6 gene, here we studied the long term expression of OCT-6 nuclear protein in sciatic nerve of normal and diabetic neuropathic rats. Also for the first time we elucidated the role of insulin in controlling the expression of OCT-6 in hyperglycemic Schwann cells and sciatic nerve of diabetic neuropathic rats. The results shows that, there will be long term OCT-6 expression in sciatic nerve of adult rats and also their significant decrease is observed in the diabetic condition. But, addition of Insulin for primary Schwann cells and diabetic rats shows the increased OCT-6 expression in both *in vivo* and *in vitro*. Together these results indicate the failure of OCT-6 support in neuropathy and also the importance of insulin signaling cascade in the expression of OCT-6 transcription factor.

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Introduction

The Myelin sheath is an insulating component of neurons in both Central and Peripheral nervous system. It is derived from the Schwann cells in the peripheral nervous system and plays a vital role in the potential transmission of nerve impulses.[1,2] Along with the myelination, Schwann cells also provide a trophic and metabolic support required to maintain the structural and functional integrity of the axons. Myelin structural formation is a complex and controlled event which involves the exchange of several complex molecular signals between Schwann cells and axons.[3] It is well known that a small alteration or failure in these signaling cascades can be responsible for the demyelination of neurons in the several neuropathic disease like Charcot–Marie–Tooth disease, multiple sclerosis and also in diabetic neuropathy.[2]

Insulin and insulin like receptors are the neurotrophic factors which plays a crucial role in myelin formation and maintenance. In the early studies, we have shown that insulin will regulate the expression of myelin proteins like P0 and MAG,[4,5] whereas the failure in insulin signaling propagation greatly decrease the myelin protein expression in diabetic neuropathic condition.[6]

Among the different class of transcription factors, OCT-6 appears to be a potent factor that shares a major primary role in Schwann cell differentiation and myelination.[7] OCT-6 is a class of nuclear protein and POU domain transcription factor required in transition of promyelinating to myelinating Schwann cells and also in regulating the transcription of certain genes like krox20 that is required throughout the myelin formation.[8,2] The alteration in the expression of OCT-6 factor was reported in Charcot–Marie–Tooth disease type 1A (CMT1A) and other peripheral neuropathies,[9,10] but the role and expression pattern of OCT-6 was yet to be understood in the diabetic neuropathy.

It is of greater importance to know the expression level of these factors in long term diabetic neuropathy. Therefore, we investigated the expression of OCT-6 in sciatic nerve of diabetic neuropathic rats and also in Schwann cells grown in hyperglycemic condition. In addition, we checked the role of long term insulin treatment in the expression of OCT-6 transcription factors in diabetic rats. Overall, this data extend our understanding of the molecular mechanism behind the failure of normal signaling cascade required for myelination in diabetic neuropathy.

Materials and methods

Materials

DMEM and FBS were purchased from Gibco (USA). Real time PCR reagents were procured from Invitrogen (USA). Antibodies were obtained from Abcam (UK). S-100 is obtained from Biogenex (USA), PVDF membrane is purchased from Millipore (India), Streptozotocin (purity: 98%), Dulbecco's phosphate buffered saline (DPBS) and skimmed milk powder were obtained from Himedia (India). All other chemicals were procured from Sigma (USA) unless mentioned.

Ethical statement

All animal experiments were confirmed to the guidelines of animal care and use. All efforts were made to minimize the number and the suffering of animals used.

Animals and grouping

Wistar rats were obtained from the Animal facility, SS Institute of Medical Sciences, Davangere, India. The rats were kept in an animal house facility and maintained under a 12 h light/dark cycle. Water and pelleted food were given *ad libitum*. Animals (male and female) 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.

Diabetes induction and insulin treatment

Wistar rats weighing 200–250 g were induced diabetes by intraperitoneal injection of streptozotocin at 20 mg/kg body weight after overnight fasting. Then second dose of 40 mg/kg body weight was injected after one week. The STZ was dissolved in citrate buffer (0.1 M) and control rats were injected with vehicle buffer. The blood glucose measurements were performed to confirm the diabetes (Fig. 1). Rats showing fasting blood glucose level above 250 mg/dl (>15 mM) after 48 h of second dose of streptozotocin were considered as diabetic. DPN was confirmed by hot plate method as described earlier.[11] Group 3 rats were daily injected with insulin (1 IU)subcutaneously. The sciatic nerves from control, diabetic and insulin treated diabetic rats were dissected at 4–12 week time interval.

Schwann cell culture

For isolation of SC, sciatic nerve of 3–4 days pups were dissected and carried according to the method of Brockes[12] with slight modifications.[13] Neonatal rat sciatic nerves were subjected for enzymatic and mechanical dissociations as

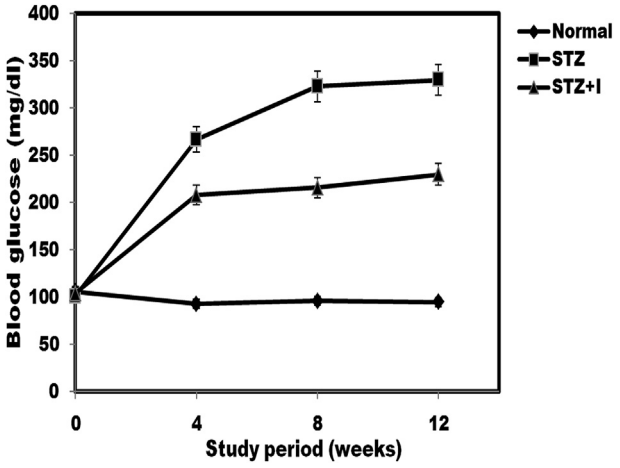

Figure 1 Alteration in the blood glucose levels of normal control, STZ and insulin treated STZ rats. Error bars represent ±SEM (n = 6).

previously described,[6] the obtained cell pellet was resuspended in DMEM supplemented with 10% Fetal bovine serum (FBS) and grown on laminin/poly-L-lysine coated Petri plates. After obtaining the pure culture the cells were 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 plated at a density of 4×10^4 cells/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. After attaining 90% confluence the cells were used for experiments. The purity of the SCs was confirmed with S-100 protein staining technique and morphological appearance.[4]

Immunofluorescence

SCs grown for 24 h and 48 h in 40 mM of glucose with and without 10 nM insulin were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.5% Saponin for 15 min. The cells were blocked with DPBS containing 10% FBS for 1 h at room temperature and incubated overnight with a 1:400 and 1:200 dilution of anti-pJNK and anti-pAKT DPBS containing 10% FBS. After the primary antibody incubations, 1:50 dilution of FITC-conjugated goat anti-rabbit were added for 1 h at room temperature. The coverslips were washed in DPBS and mounted on slides using permount. The fluorescence immunoreactivity was visualized by epifluorescence microscopy (Olympus, Japan) and photographed in the dark.

Western blotting

Sciatic nerve of control, diabetic and insulin (1U) treated rats at different time interval were lysed and incubated on ice for 1 h in RIPA lysis buffer containing 1% protease inhibitor cocktail and were sonicated for 1 min. Protein lysates were recovered by centrifuging the sample at 12000 rpm for 20 min at 4 °C. Similarly, protein lysates were prepared from SCs grown in DMEM and Ham's F12 medium with 40 mM Glucose, in the absence and presence of 10 nM insulin for 12 h and 24 h time interval. Protein concentration of the supernatant was estimated by Bradford assay and protein was separated on a 12%—15% SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked in 5% skimmed milk solution in TBS-T (Tris buffered saline with 0.1% Tween-20) and incubated overnight with primary antibody (2:1000) against OCT-6 at 4 °C. Further blots were washed in TBS-T and then incubated with secondary antibody (1:4000) conjugated with alkaline phosphatase, and developed with NBT/BCIP substrate (Genei, India).

Quantitative real time PCR

Total RNA was isolated using total RNA isolation kit by following the manufacturer's instructions. The concentration and purity of RNA was calculated using a spectrophotometer (Analytik Jena, Germany). Isolated RNA was then reverse transcribed to cDNA using pure link RNA mini kit and 2.0 μg of cDNA and power SYBR green master mix (Applied Biosystems, USA) was used to perform real-time PCR amplification with a StepOne plus real-time PCR system (Applied Biosystems, USA). The relative expressions of genes were analyzed by StepOne™ software v2.2.2. The fold changes were calculated by $2^{(-\Delta\Delta Ct)}$ method by normalizing with RPL19 house keeping gene expression, and relative gene expression is represented.

All reactions were run in triplicate. The primer sequences used were as follows:

OCT-6 forward: 5′-CGAGAGCCGCATCTGTGTAT-3′.
OCT-6 reverse: 5′-CGTTCTTGAAGGAGGAGAGGG-3′.
RPL19 forward: 5′-CGTCCTCCGCTGTGGTAAA-3′.
RPL19 reverse: 5′-AGTACCCTTCCTCTTCCCTAT-3′.

Statistical analysis

All the assays were carried out in triplicates. Immunofluorescence and western blott data were analyzed using Multi Gauge software. Statistical analyses of the experimental data were carried out by an unpaired student's t-test. All data were presented as the mean ± SEM. Values of $p < 0.05$ were considered significant.

Results

Developmental regulation of OCT-6 expression in sciatic nerve of rats

Western blotting technique was carried out to study the developmental expression of OCT-6 in sciatic nerve of rats. Proteins isolated from the sciatic nerve of 4, 8 and 12 weeks rats were separated using 15% SDS-PAGE and subjected for western blotting analysis. Results showed that OCT-6 is expressed along with peripheral nerve development. The level of expression is varied among 4, 8 and 12 week sciatic nerve sample. Compared to 4 week increased expression is observed in 8 and 12 week sample [Fig. 2A (Lane C)]. However the level of OCT-6 is not constantly increased along with the time, but the expression is observed throughout all the time points.

Insulin elevates the deteriorated OCT-6 expression in sciatic nerve of diabetic rats

Real time PCR and Western blotting studies were used to know the significance of insulin in expression of OCT-6 transcription factor. Western blotting results showed that OCT-6 expression in diabetic rats was decreased by 0.31, 0.60, and 0.43 folds in 4, 8 and 12 week sample respectively when compared to the sciatic nerve sample of control. Whereas in the insulin administrated rats a significant elevation of 0.16, 0.60 and 0.26 fold compared to diabetic rats of 4, 8 and 12 week was observed (Fig. 2A and B).

Further, mRNA of sciatic nerve sample was subjected for quantitative PCR studies to know the OCT-6 gene expression in diabetic and insulin treated rats. In diabetic rats decreased fold changes of 8 and 12 weeks were 0.4 folds. In contrast, the OCT-6 gene expression was significantly

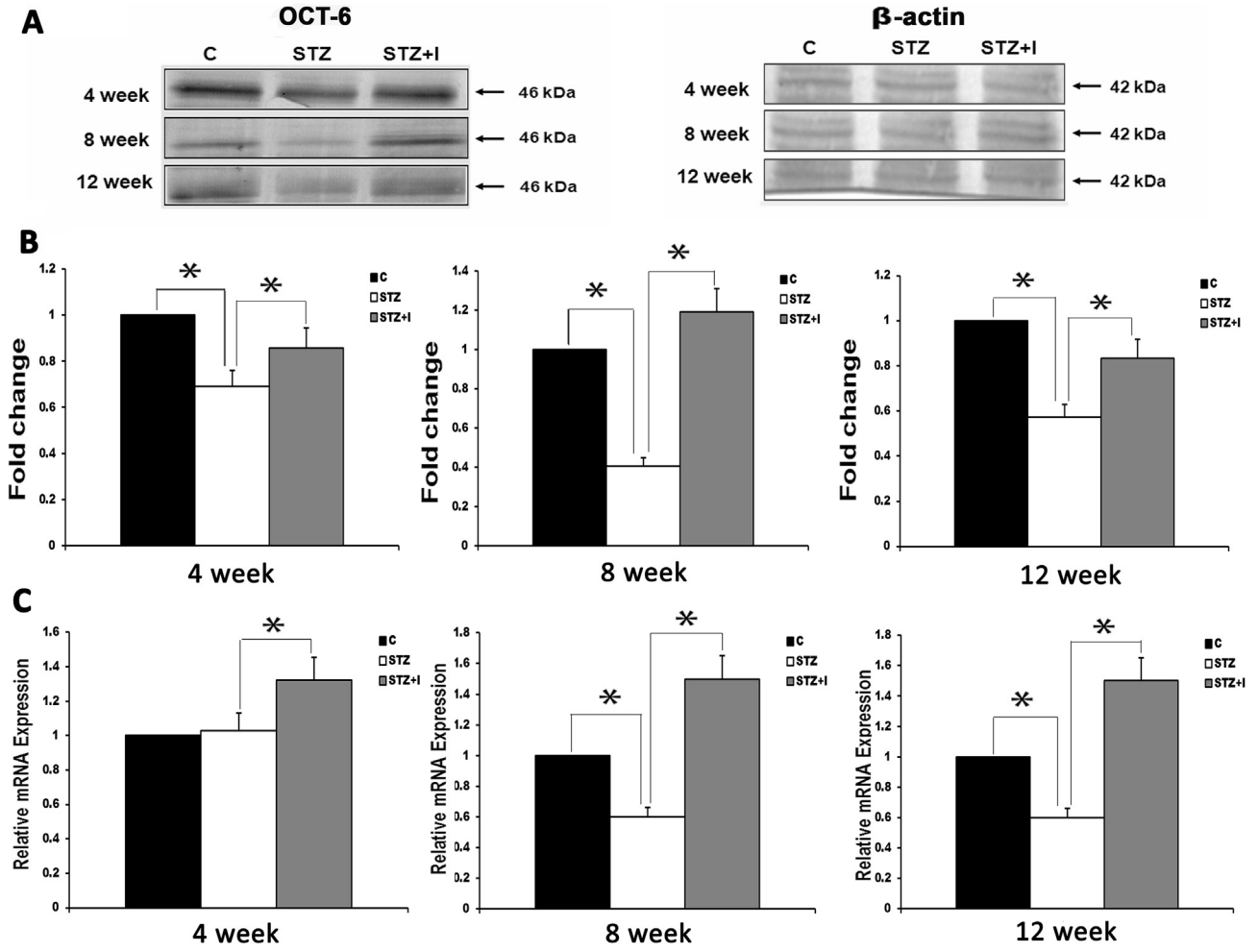

Figure 2 Differential expression of OCT-6 protein and OCT-6 mRNA level at 4, 8 and 12 week sciatic nerve samples correlated between control (C), diabetic (STZ) and insulin treated diabetic rats (STZ+I). (A) Western blot analysis for OCT-6 protein expression of sciatic nerve in STZ and STZ+I compared to Control. β actin was used for normalization. (B) Western blotting data were presented as a bar chart. (C) Real time PCR studies showing change in relative mRNA expression of OCT-6 among STZ and STZ+I of sciatic nerve. RPL19 was used as house keeping gene.*indicates $p < 0.05$.

increased by 0.2, 0.6 and 0.9 folds in insulin treated compared to 4, 8 and 12 week diabetic nerve sample (Fig. 2C).

Insulin alters the OCT-6 expression in Schwann cells grown in hyperglycemic condition

In the *in vitro* studies, OCT-6 expression of Schwann cells grown in hyperglycemic condition was assessed by Western blotting and quantitative PCR. The OCT-6 expression in protein isolated from Schwann cells grown in high glucose with and without insulin condition for 12 h remains unchanged, where Schwann cells grown in high glucose medium for 24 h sample showed 0.2 fold decreased compare to control cells, But insulin treated cells showed the significant increase of 0.9 folds compared to non treated cells (Fig. 3A and B). Further, quantification of OCT-6 mRNA showed the similar result as of OCT-6 protein expression, there is no significant mRNA expression in the cells grown for 12 h in all three different conditions. In 24 h high glucose condition 0.3 fold decreased expression was observed compare to control and 0.6 fold increased expression in insulin treated cells compared to high glucose condition (Fig. 3C).

Further to know the significance of insulin in OCT-6 expression, immunofluorescence studies were carried using OCT-6 specific primary antibody and FITC conjugated secondary antibody. The expression of OCT-6 and Schwann cell number was increased by 0.3 and 0.6 folds along with 10 nM insulin treatment [Fig. 4A (e and f)] compared to the cells grown in 40 mM glucose medium [Fig. 4A (c and d)]. And about 0.6 and 0.8 fold decreased fluorescence expression and SC number were observed in the cells grown in 40 mM glucose medium compared with control [Fig. 4A (a and b)] grown for 12 and 24 h respectively. This result supports the role and significance of insulin in OCT-6 expression.

Discussion

Insulin is a class of peptide hormone, which is considered as one of the potent neurotrophic factor required to maintain the homeostatic environment of neuronal tissues both in

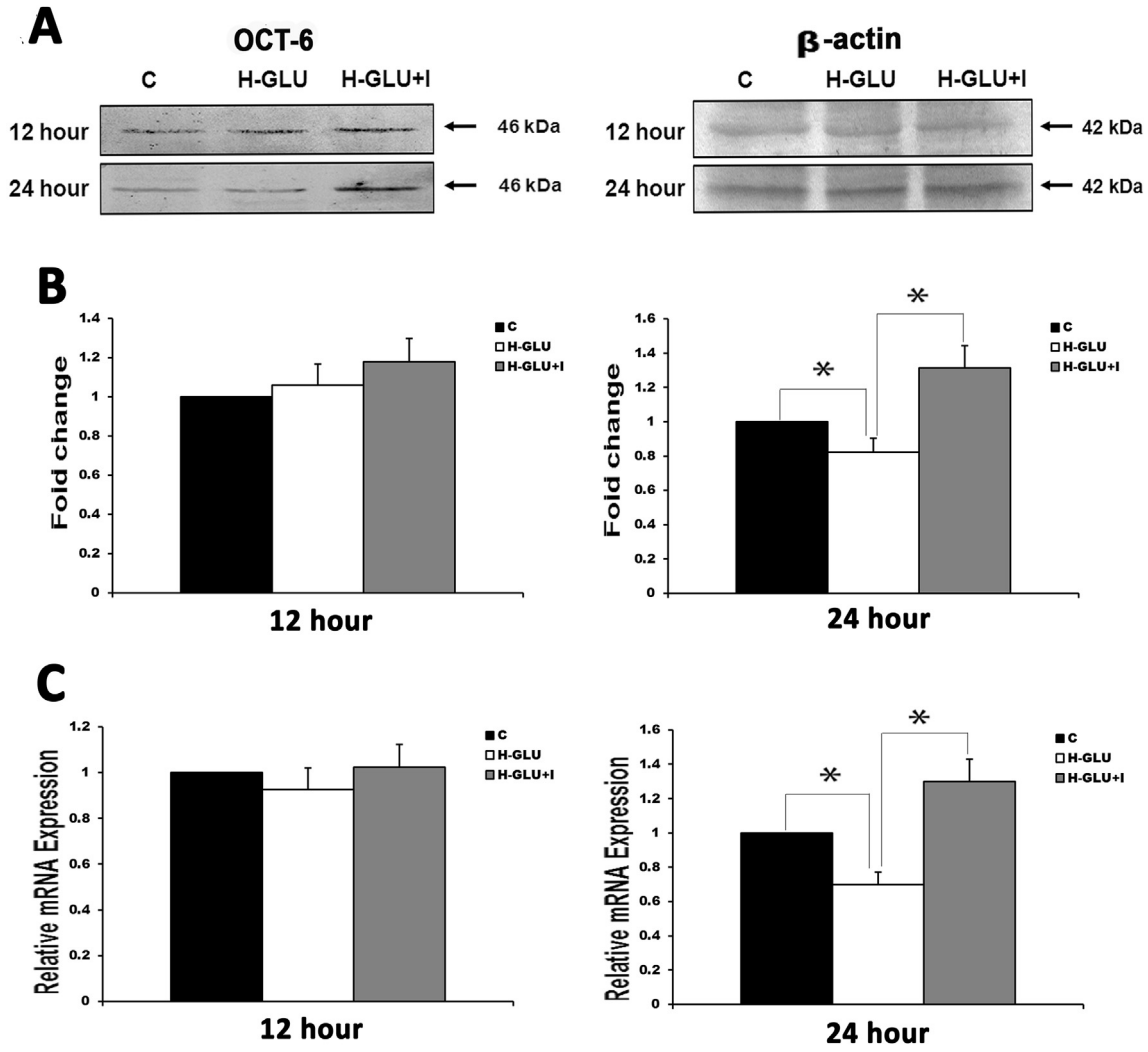

Figure 3 Differential expression of OCT-6 protein and OCT-6 mRNA level at 12 h and 24 h of the SCs grown in normal medium (C), 40 mM glucose (H-GLU) and 10 nM insulin added to 40 mM glucose medium (H-GLU+I). (A) Western blot analysis for OCT-6 protein expression of SCs in H-GLU and H-GLU+I compared to Control. (B) Western blotting data were presented as a bar chart. (C) Real time PCR studies showing change in relative mRNA expression of OCT-6 among H-GLU and H-GLU+I of SCs. RPL19 was used as house keeping gene. *indicates $p < 0.05$.

the central and peripheral nervous system. In the central nervous system insulin induces the neurite out growth, it controls the neuronal growth via phosphatidylinositol 3-kinase (PI3K) pathway and also they have role in the formation of neuronal dendritic spine in hippocampus.[14–16] In the PNS insulin activates the AKT signaling pathway in dorsal root ganglion which is essential for survival and maintenance of peripheral neurons.[17] Therefore failure or alteration in insulin signaling pathway may leads to several molecular complications like Alzheimer's and Diabetic Neuropathy.[18–20] In the PNS, Schwann cells forms the myelin structure around the axons with the involvement of several growth factors and transcription factors in signaling cascade, so it is of greater interest to distinguish their role in failure of this pathway. It is reported that OCT-6 is absolute requirement for transition of Schwann cells from non myelinating to the myelinating stages. Earlier findings emphasize that acute axonal degeneration breakdown of myelin leads Schwann cells to up regulate OCT-6. During nerve regeneration OCT-6 translocated to the nucleus and promote the transcription of myelin protein.[9] In this study we have demonstrated the expression pattern of POU domain transcription factor OCT-6 in long term DPN and also the role of insulin signaling in overcoming the deprived expression of OCT-6 in DPN. But the mechanism of neurotrophic factors controlling the myelination events still remains unknown.

Demyelination of peripheral neurons is one of the complications of DPN. In earlier reports we have shown that insulin alters the myelin proteins expression (P0, MAG) in the Schwann cells and sciatic nerve of diabetic rats.[4,5] We also recently reported that increased phosphorylation of serine 731 of Insulin receptor substrate-2 (IRS2) and decreased growth factor receptor-bound protein-2 (GRB2) adaptor protein is one of the reasons behind the failure in propagation of insulin signaling pathway in DPN.[6] Insulin can regulates the expression of forkhead transcription factors (FKHR, Hnf-3β/Foxa-2) by Akt-mediated and

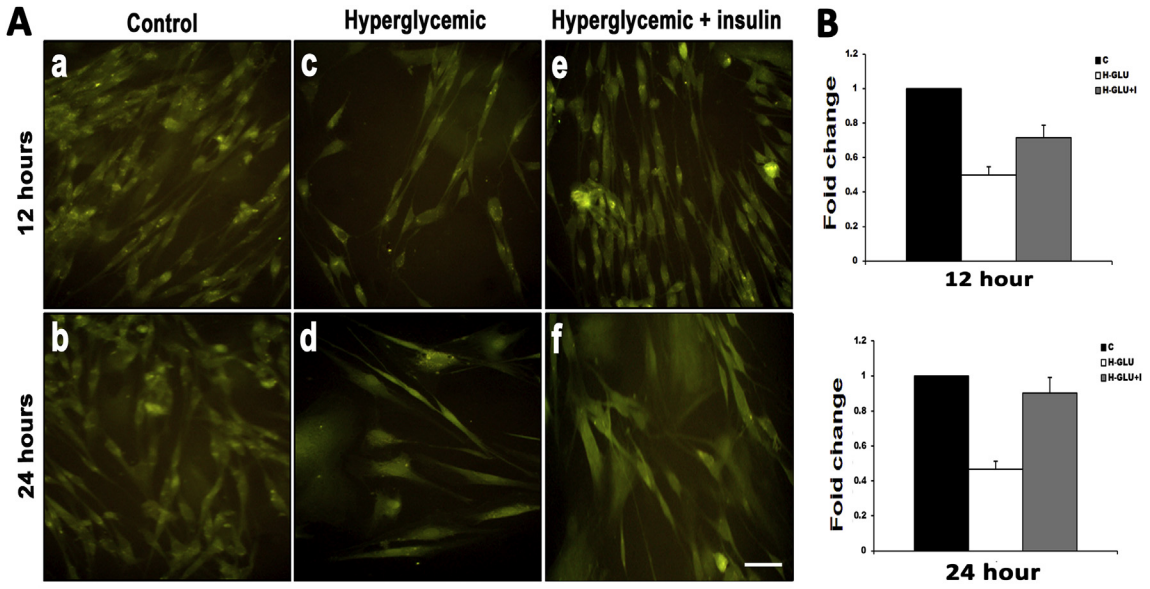

Figure 4 Immunofluorescence staining for evaluation of OCT-6 protein expression. (A) SCs grown for 12 h and 24 h in normal medium [(control) a and b], high glucose medium [(40 mM glucose) c and d] and insulin added high glucose medium [(40 mM glucose + 10 nM insulin) e and f]. (B) Relative fold change in the fluorescence intensity of OCT-6 protein expression of SCs in H-GLU and H-GLU+I compared to Control. Scale bar: 50 μm.

Wortmannin-sensitive signaling pathway.[21,22] Therefore, we deliberate to know the expression of POU domain transcription factor OCT-6 in response to insulin. Since Schwann cells require OCT-6 as a key transcription factor for myelination, neuronal development and regeneration,[23,10] their expression should have key role in long term DPN. Western blotting studies for 4, 8 and 12 weeks in normal rat sciatic nerve sample revealed the importance of OCT-6 in adult neurons.

In response to the above findings, we checked the expression of OCT-6 in neuropathic condition. It is well know that OCT-6 is one of the activator of zinc finger protein Krox-20 which is essential throughout the myelination, where myelination cannot occur in Krox-20 null mutant.[8,24] To carry the *in vivo* experiments we used normal, neuropathic and insulin injected neuropathic rats. The OCT-6 protein and gene expression studies showed the decreased expression in sciatic nerve of diabetic neuropathic rats, whereas the significant increase in the insulin treated rats were also observed. These findings suggests that, the neuropathic rats fails to expresses the OCT-6 which is expressed in sciatic nerve of normal rats, but the insulin administration for neuropathic rats activates insulin signaling cascade along with significantly restores the OCT-6 expression which is the sign of remyelination of neurons in demyelinated neuropathic rats. Addition to these studies, for *in vitro* experiments the SCs were grown in high glucose medium (40 mM) to imitate the diabetic condition. In the initial time point the expression of OCT-6 in SCs grown in hyperglycemic condition with and without insulin remains unaltered. Meanwhile in the hyperglycemic SCs without insulin showed the deprived expression than of 10 nM insulin supplemented cells. These results support our previous findings behind the failure of insulin signaling propagation in DPN.

In conclusion, our results strongly provide information that helps to understand the possibilities behind the demyelination of peripheral neurons in DPN. We found the importance of POU transcription factor OCT-6 in long term myelination and their decreased support in neuropathic condition. But insulin signaling cascade appears to be pivotal factor for regeneration of demyelinated neurons. Down regulated OCT-6 activation can be one of the odds connected to failure of insulin signaling cascade in long term DPN. However, the factors that link the insulin signaling pathway to OCT-6 transcription factor is yet to be elucidated. The findings on signaling molecules may help to understand more about the reasons behind neuronal degeneration in diabetic neuropathy and provides a platform for new drug discovery research.

Conflict of interest

All the authors disclose that there is no conflict of interest.

Acknowledgment

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References

1. Raine CS. Morphology of myelin and myelination. In: Morell P, ed. *Myelin*. 2nd ed. New York: Plenum; 1984:1—41.

2. Herbert AL, Monk KR. Advances in myelinating glial cell development. *Curr Opin Neurobiol*. 2017;42:53—60.
3. Bhatheja K, Field J. Schwann cells: origins and role in axonal maintenance and regeneration. *Int J Biochem Cell Biol*. 2006; 38:1995—1999.
4. Shettar A, Muttagi G. Developmental regulation of insulin receptor gene in sciatic nerves and role of insulin on glycoprotein P0 in the Schwann cells. *Peptides*. 2012;36:46—53.
5. Rachana KS, Manu MS, Advirao GM. Insulin influenced expression of myelin proteins in diabetic peripheral neuropathy. *Neurosci Lett*. 2016;629:110—115.
6. Manu MS, Rachana KS, Advirao GM. Altered expression of IRS2 and GRB2 in demyelination of peripheral neurons: Implications in diabetic neuropathy. *Neuropeptides*. 2016;62:71—79.
7. Jaegle M, Meijer D. Role of Oct-6 in Schwann cell differentiation. *Microsc Res Tech*. 1988;41(5):372—378.
8. Ghislain J, Charnay P. Control of myelination in Schwann cells: a Krox20 cis-regulatory element integrates Oct6, Brn2 and Sox10 activities. *EMBO Rep*. 2006;7(1):52—58.
9. Kawasaki T, Oka N, Tachibana H, Akiguchi I, Shibasaki H. Oct6 a transcription factor controlling myelination, is a marker for active nerve regeneration in peripheral neuropathies. *Acta Neuropathol*. 2003;105(3):203—208.
10. Jaegle M, Ghazvini M, Mandemakers W, et al. The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development. *Gene Dev*. 2003;17(11):1380—1391.
11. Malik ZA, Tabassum N, Sharma PL. Attenuation of experimentally induced diabetic neuropathy in association with reduced oxidative-nitrosative stress by chronic administration of *Momordica charantia*. *Adv Biosci Biotechnol*. 2013;4:356—363.
12. Brockes JP, Fields K, Raff MC. Studies on cultured rat Schwann cells. I. Establishment of purified population from cultures of peripheral nerves. *Brain Res*. 1979;165:105—118.
13. Shetter AR, Muttagi G, Sagar CBK. Expression and localization of insulin receptors in dissociated primary cultures of rat Schwann cells. *Cell Biol Int*. 2011;35:299—304.
14. Patel RA, Kurian P, Raizada MK, Crews FT. Insulin stimulates phosphatidylinositol 3-kinase activity in rat neuronal primary cultures. *J Neurochem*. 1993;61(1):360—363.
15. Lee CC, Huang CC, Wu MY, Hsu KS. Insulin stimulates postsynaptic density-95 protein translation via the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway. *J Biol Chem*. 2005;280(18):18543—18550.
16. Lee CC, Huang CC, Hsu KS. Insulin promotes dendritic spine and synapse formation by the PI3K/Akt/mTOR and Rac1 signaling pathways. *Neuropharmacology*. 2011;61(4):867—879.
17. Grote CW, Morris JK, Ryals JM, Geiger PC, Wright DE. Insulin receptors substrate 2 expression and involvement in neuronal insulin resistance in diabetic neuropathy. *Exp Diabetes Res*. 2011;2011:21—28.
18. Duarte AI, Moreira PI, Oliveira CR. Insulin in central nervous system: more than just a peripheral hormone. *J Aging Res*. 2012;2012, 384017.
19. Grote CW, Ryals JM, Wright DE. In vivo peripheral nervous system insulin signalling. *J Peripher Nerv Syst*. 2013;18(3): 209—219.
20. Blázquez E, Velázquez E, Hurtado-Carneiro V, Ruiz-Albusac JM. Insulin in the brain: its pathophysiological implications for States related with central insulin resistance, type 2 diabetes and Alzheimer's disease. *Front Endocrinol (Lausanne)*. 2014;5: 161.
21. Wolfrum C, Besser D, Luca E, Stoffel M. Insulin regulates the activity of forkhead transcription factor Hnf-3β/Foxa-2 by Akt-mediated phosphorylation and nuclear/cytosolic localization. *Proc Natl Acad Sci U S A*. 2003;100(20):11624—11629.
22. Nakae J, Park Byung-Chul, Accili D. Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. *J Biol Chem*. 1999;274:15982—15985.
23. Ghazvini M, Mandemakers W, Jaegle M, et al. A cell type-specific allele of the POU gene Oct-6 reveals Schwann cell autonomous function in nerve development and regeneration. *EMBO J*. 2002;21(17):4612—4620.
24. Blanchard AD, Sinanan A, Parmantier E, et al. Oct-6 (SCIP/Tst-1) is expressed in Schwann cell precursors, embryonic Schwann cells, and postnatal myelinating Schwann cells: comparison with Oct-1, Krox-20, and Pax-3. *J Neurosci Res*. 1996;46(5): 46630—46640.