Cyclic mechanical stress modulates neurotrophic and myelinating gene expression of Schwann cells

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Abstract

Objectives: This study aimed to investigate the response of Schwann cells to cyclic compressive and tensile stresses of different durations of stimulation.

Materials and methods: RSC96 cells were subjected to cyclic tensile stress or compressive stress; for either, cells in five groups were treated for 0, 1, 2, 24 and 48 h respectively. Enzyme-linked immunosorbent assay was conducted to detect secretion of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4 in the culture medium. Real-time PCR was conducted to quantify mRNA expression of neurotrophins including *NGF*, *BDNF*, *neurotrophin-3* and *neurotrophin-4*, and myelin-related genes including *Sox10*, *Krox20*, *neuregulin 1*, *NCAM*, *N-cadherin*, *P0*, *MAG* and *MBP*. Immunofluorescent staining was performed to visualize *Krox20* and F-actin in the tensile groups.

Results: Within 24 h, cells treated with cyclic tensile stress expressed and secreted significantly more BDNF, while cyclic compression down-regulated BDNF expression. Cells treated with both tensile and compressive stress down-regulated expression of *NRG1*, *NCAM*, *Krox20* and *Sox10* at all time points. Expression of *N-cadherin* was not affected by either stretch or compression. F-actin was down-regulated by tensile stress.

Conclusions: Both tensile and compressive loading down-regulated expression of several important

myelin-related Schwann cells genes and thus facilitated demyelination. Tensile stress meanwhile promoted secretion of BDNF by Schwann cells within 24 h, which may contribute to maintenance and repair of damaged axons. These effects of mechanical stress might have been mediated by the actin cytoskeleton.

Introduction

Schwann cells fabricate nerve cells' myelin sheaths in the peripheral neural system, and participate in development, functional maintenance and repair of peripheral nerves *via* their interaction with axons (1,2). When nerve bundles are traumatized, axons distal to the traumatic site undergo Wallerian degeneration whereas corresponding myelinating Schwann cells undergo demyelination which involves lysis of myelin proteins, clearance of axon debris (by demyelinated Schwann cells and macrophages), and accelerated proliferation of demyelinated Schwann cells (3–5). After completion of Wallerian degeneration and demyelination, Schwann cells involved align along the original path of the injured nerve to form the Bungner's band which subsequently guides extension of any residual axon by secreting neurotrophins, and remyelinate to form myelin of the regenerated nerve (4).

Mechanical stimulus has been widely reported to be involved in damage and repair of many tissues through a variety of cellular and molecular events (6–10). The role of mechanical factors in nerve damage and regeneration has drawn much attention to itself; excessive compressive and shearing stress have generally been considered to be detrimental to nervous tissue, whereas tensile stretch within an appropriate range of strength and frequency has been shown to favour nerve repair, though the underlying mechanisms remained under-investigated (11–13).

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Repair of damaged peripheral nerves involves sustained morphological and functional evolution of injured axons and related Schwann cells, and interaction between them. Recent studies on axons under mechanical stress suggest that tensile stretch of low magnitude stimulates axon elongation (14–16). However, published reports concerning effects of mechanical stimuli on Schwann cells, the other important player in nerve repair, are scarce. The work of Gupta *et al.* on models of compressive neuropathies has indicated that compression induced apoptosis as well as proliferation of Schwann cells. Also, shear stress down-regulates expression of myelin-associated glycoprotein (MAG) and myelin basic protein (MBP) by Schwann cells, implying that mechanical stimuli might favour demyelination of Schwann cells (17–19), but further evidence has still been lacking.

The present study was therefore proposed to specifically investigate myelinating and neurotrophic behaviour of Schwann cells under mechanical stress of varied forms, and stimulus duration, and thus to provide clues concerning the role of Schwann cells in responses of nerves to mechanical stimuli.

Materials and methods

Cell culture and immunofluorescence staining of Schwann cell marker S100

Rat Schwann cell line RSC96 (American Type Culture Collection, Manassas, VA, USA) was used in the present study. Cells were seeded in 25 cm² flasks and cultured in low-glucose DMEM supplemented with 10% foetal bovine serum. Confluent cells were trypsinized with 0.25% trypsin and EDTA then seeded in 24-well plates. After cells were completely attached and displayed typical spindle or tri-polar forms of cultured Schwann cells, they were washed in phosphate buffered saline (PBS) and fixed in paraformaldehyde at 4 °C. Fixed cells were then treated with 0.5% Triton X-100 for 10 min (to permeabilize plasma membranes), blocked in 1% bovine serum albumin at 37 °C for 30 min, incubated overnight with rabbit anti-S100 polyclonal antibody (1:200) (Abcam, Cambridge, UK), washed three times in PBS, and incubated with FITC-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) for 1 h. Lastly, nuclei were stained with DAPI before observation under a fluorescence microscope.

Grouping and mechanical loading of cells

Cells were assigned to two categories, one was subjected to cyclic compressive stress and the other to

cyclic tensile stress. Each category had five groups, one as control and the other four treated by compressive or tensile stress for 1, 2, 24 or 48 h respectively. Cells were seeded on the plastic loading plate of our loading system (described below), and 24 h later, after all cells were fully attached to the plate, cyclic loading was initiated. Strain magnitude was 2000 ϵ and frequency was 0.5 Hz. Strength and frequency of cyclic loading were determined by a pilot study that involved gradients of parameters. With higher forces or frequencies, significant amounts of cells became detached from the loading plate. Current parameters were the highest among those that caused no vast detachment of cells.

A four-point bending device for mechanical loading of cultured cells was adopted in our study (Fig. 1). As we previously reported (20), the working components of the device were composed of a box with two wedges on the lid and two wedges on the base. A rectangular plastic

Figure 1. The loading device. Magnitude of strain exerted by this device calculated according to the formula $\epsilon = td/a (L - 1.33a)$, where ϵ refers to strain applied on the cells, t to thickness of the plate, d to vertical displacement of the lid, L to the length of the plate and a to horizontal distances between wedges on the lid and base, unilaterally.

plate with cells seeded on the middle one third of its upper surface, was placed between the four wedges. When the lid was depressed by a step motor, the plate became bent by the four wedges. When the distance between wedges of the lid was larger than that of the bottom, the plate curved upward, and cells on its middle portion of the upper surface would be stretched. Similarly, when distance between the wedges on the lid was smaller than that of the base, the plate curved downward, and cells on the plate became compressed. Magnitude of the strain was calculated according to the formula:

$$\varepsilon = td/a(L - 1.33a)$$

where ε refers to strain applied to the cells, t refers to thickness of the plate, d refers to vertical displacement of the lid, L refers to length of the plate and a refers to horizontal distances between wedges on the lid and base, unilaterally.

Enzyme-linked immunosorbent assay (ELISA) for secreted neurotrophins

At the end of the last loading cycle, samples of culture medium were collected from each group. Secretion of four major types of neurotrophin, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) in the sample medium was quantified by rat NGF ELISA kit, rat BDNF ELISA kit, rat NT-3 ELISA kit and rat NT-4 ELISA kit.

Real-time polymerase chain reaction of neurotrophin and myelin-related gene mRNAs

Total RNA was extracted from cells of each group using Simply P Total RNA Extraction kit (Bioflux,

Zhejiang, China) immediately after all loading cycles were finished, and cDNA of each sample was obtained by reverse transcription with PrimeScript™ RT reagent Kit (Takara, Shiga, Japan). Real-time PCR was subsequently performed using SYBR Premix Ex Taq™ (Takara) on ABI 7300 Real-time PCR System (Applied Biosystems, Waltham, MA, USA) to detect mRNA expression of neurotrophins *NGF*, *BDNF*, *NT-3* and *NT-4*, and myelin-related genes including *Sox10* [master regulator of Schwann cell phenotype and myelin maintenance (21,22)], *Krox20* [immediate early gene essential for Schwann cells to enter the myelinating stage (21,23)], *NRG1* [potent inducer of myelination produced by both the axons and Schwann cells (4,24)], *NCAM* and *N-cadherin* [adhesion molecules mediating interaction between axons and glial cells and early initiation of myelination (25,26)], and marker genes of myelin *MAG*, *MBP* and myelin protein zero (*P0*) (27), with *GAPDH* the reference gene. Relative expression of each gene was normalized to *GAPDH* and calculated with the formula $2^{-\Delta\Delta Ct}$. Primers of each gene are shown in Table 1.

Immunofluorescent staining of *Krox20* and *F-actin*

Based on the results of above mentioned tests, cells treated with tensile stress for 24 and 48 h, were further subjected to immunofluorescence staining of *Krox20* and *F-actin*. Briefly, cells were washed in PBS three times and fixed in paraformaldehyde. After fixation, they were treated with 0.5% Triton X-100 and 1% bovine serum albumin, then incubated in rabbit anti-*Krox20* polyclonal antibody (1:200) (Abcam) overnight. FITC-conjugated secondary antibody was used to visualize the immune complex, rhodamine-conjugated phalloidin was used to stain *F-actin*, and DAPI was used to stain nuclei.

Table 1. Primer sequences for real-time PCR

Genes	Forward (5'–3')	Reverse (5'–3')
<i>NGF</i>	CTGGGCGAGGTGAACATTAACA	CAGCCTGTTTGTCTCTGTTGTC
<i>BDNF</i>	TCTACGAGACCAAGTGAATCCCA	CTTATGAACCGCCAGCCAAT
<i>NT-3</i>	AGTGTGTGACAGTGAGAGCCTGTGG	GAGAGTTGCCGGTTTTGATCTCTCC
<i>NT-4</i>	CCCTGCGTCAGTACTTCTTCGAGAC	CTGGACGTCAGGCACGGCCTGTTC
<i>Krox20</i>	CCTACAATCCGCACCACCTG	GAACCTCCTGTCGCAACCCT
<i>Sox10</i>	TGCCAAAGCCCAGGTGAAGA	AGACTGAGGGAGGTGTAGGCGAT
<i>NRG1</i>	CCTGTCAAACCCGTCAAGATACT	CTCCGCTTCCATAAATTCAATCC
<i>NCAM</i>	TTCAGTGACGACAGTTCCGGAGC	TGCGAAGACCTTGAGGTGGAT
<i>N-cadherin</i>	CAATGGAGACTGCACCGACG	TTGGCCTGGCGCTCTTTATC
<i>P0</i>	GTGGTTTACACGGACAGGGAAGT	GGTTGACCCTTGCCATAGTGG
<i>MAG</i>	TGGAAGCCCACAGTGAAT	CTTGAAGATGGTGAGAATAGGG
<i>MBP</i>	GAAGTCGCAGAGGACCCAAGA	CTGCCTCCGTAGCCAAATCC
<i>GAPDH</i>	CCGTATCGGACGCCTGGTTA	CCGTGGGTAGAGTCATACTGGAAC

Statistical analysis

All data were analysed by independent sample *t*-test with the statistics software spss 16.0 (IBM, Armonk, NY, USA). *P*-values lower than 0.05 were regarded statistically significant.

Results

Identification of cells

Immunofluorescence staining showed that all cells were S100 positive and spindle or tri-polar shaped, typical for the Schwann cell phenotype (Fig. 2).

Neurotrophin expression and secretion

Cyclic compressive and tensile stresses had contrary effects on BDNF expression and secretion, while the other three neurotrophins, NGF, NT-3 and NT-4, were detected neither by ELISA of secreted proteins nor by real-time mRNA PCR. Specifically, cyclic tensile stress promoted BDNF expression, mRNA peaking at 1 h and secreted protein peaking by 24 h, whereas cyclic compressive stress down-regulated BDNF expression; though the difference was not statistically significant in terms of secreted protein, the general trend of mRNA and protein coincided with each other (Fig. 3).

Figure 2. Immunofluorescent staining of Schwann cell marker S100 in RSC96 cells. All cells were stained homogeneously by green fluorescent dye conjugated with antibody. (a) Nuclei stained with DAPI. (b) S100 protein stained green. (c) Merged image. Scale bar = 50 μ m.

Figure 3. Brain-derived neurotrophic factor (BDNF) expression and secretion by RSC96 cells under compressive and tensile stresses for different durations. Compressive stress down-regulated expression and secretion of BDNF in a time-dependent manner with a rebound at 24 h (a, c), while tensile stress promoted BDNF expression and secretion with mRNA peaking at 1 h, and secreted protein peaking at 24 h (b, d). **P*-value lower than 0.05.

Expression of myelin regulators and myelin marker genes

Sox10, master regulator of Schwann cell phenotype and myelin maintenance, was massively down-regulated after treatments at all time points, both in compression and

tension groups (Fig. 4a,b). A similar temporal pattern applied to expression of *Krox20* after mechanical treatment (Fig. 4c,d), namely, expression of both genes rapidly reduced within 1 h, rebounded slightly by 2 h, and continued to be down-regulated afterwards, regardless of form of mechanical stimulus. Expression of *NRG1* by

Figure 4. Expression of myelin-related genes *Sox10*, *Krox20* and *NRG1* by RSC96 cells under compressive and tensile stresses, for different durations. Both compressive and tensile stresses significantly down-regulated *Sox10*, *Krox20* and *NRG1* at all time points. **P*-value lower than 0.05.

Figure 5. Expression of neural adhesion molecules *NCAM* and *N-cadherin* by RSC96 cells under compressive and tensile stresses, for different durations. Compressive and tensile stresses significantly down-regulated *NCAM* at all time points, while expression of *N-cadherin* was not affected by either type of mechanical stress. **P*-value lower than 0.05.

Schwann cells was also dramatically down-regulated by both compressive and tensile stress. The only difference from *Sox10* and *Krox20* was absence of rebound by 2 h (Fig. 4e,f).

None of myelin marker genes *P0*, *MAG* and *MBP* was detected in mRNA samples of the control group, which conformed to the gene expression profile of RSC96 cells (28). Also, neither cyclic compressive nor tensile stress was able to restore expression of these genes by RSC96. Thus, these data in have not appeared in our column charts.

Expression of neural adhesion molecules

NCAM was down-regulated by both cyclic compression and tension, with a rebound in the compression groups at 48 h and in the tension groups at 24 h. In the tension groups at 48 h, *NCAM* was no longer detected (Fig. 5a,b). Expression of *N-cadherin* however, was not affected by either type of mechanical stress (Fig. 5c,d).

Immunofluorescent staining of *Krox20* and F-actin

As shown in Fig. 6, morphology of cells before and after tensile stress treatment showed no major difference,

Figure 6. Immunofluorescent staining of *Krox20* and F-actin. Tensile stretch at 24 and 48 h down-regulated expression of *Krox20* and F-actin. Distribution of F-actin was less concentrated in peri-nuclear regions after tensile stretch treatment.

implying that changes in gene expression was not caused by apoptosis. Also, protein expression of *Krox20* conformed to its mRNA expression, in that its fluorescence staining was much stronger in the control group than 24 h-tension and 48 h-tension groups. Meanwhile, expression and distribution of F-actin was also changed after tensile loading. Fluorescence staining was not as strong and concentrated in peri-nuclear regions as in the control group.

Discussion

In the present study, we examined the effect of two types of mechanical stimulus, cyclic compressive and tensile stresses, on gene expression profile of Schwann cells, in terms of their neurotrophic and myelinating behaviour. BDNF, the only detected neurotrophin among the four we examined, was promoted by cyclic tensile stress but down-regulated by cyclic compressive stress. Positive regulators of myelination *Sox10*, *Krox20*, *NRG1* and *NCAM* were all significantly down-regulated by both compression and tension. However, it should be noted that expression of myelin markers *MAG*, *MBP* and *P0* were not detected in the samples, as RSC96 cells do not form myelin when cultured *in vitro*. However, we did detect changes in expression of myelination regulators, implying that myelination by Schwann cells is likely to be influenced by cyclic mechanical stimuli.

Effects of various mechanical stimuli on nerves have been well documented previously. Compressive force has been identified to be an unfavourable factor of nerve maintenance and regeneration as it may induce demyelination, plasma membrane damage or reactive gliosis, as revealed by *in vivo* studies and clinical observations (12,13,29–32). Tensile stress, however, tends to facilitate regeneration of damaged nerves (11,33,34), partly due to its anabolic effect on neurite regrowth, independent of Schwann cells (16). Our study has indicated that in addition to the direct influence of tensile stress on axons, promoted secretion of neurotrophins from Schwann cells *in situ* might also contribute to accelerated axon elongation.

As previously reported, mechanical stimuli, specially compression, causes demyelination of neural tissues *in vivo* (12,13); Gupta *et al.* revealed that shear stress reduced *MAG* and *MBP* expression by Schwann cells *in vitro* (19). Our results agreed with these observations and suggested that down-regulated positive inducers of myelination could be one of the mechanisms through which mechanical stimuli induce demyelination, regardless of forms of stress. However, as mentioned above, it has long been noted that compressive and tensile stresses

affect nerve function in different, or even opposite, ways. Our study has implied that underlying mechanisms that cause the difference might not involve myelination by Schwann cells, but rather different effects of compressive and tensile stresses on axon remodelling. This has been documented previously with neurotrophin secretion from Schwann cells as our study has suggested.

As tensile stress caused more favourable effects on Schwann cells, we further examined changes in F-actin expression and distribution after tensile loading. The results agreed with previous studies reporting that F-actin is rapidly down-regulated by mechanical stretch in many cell types (35–37). The actin cytoskeleton has been described as being an important sensor of mechanical force, which interacts with many downstream molecules (38,39). Expression of *Krox* genes has been reported to be regulated by assembly and rearrangement of the actin cytoskeleton, through its effect on activity of serum response factor (40–42). It is thus reasonable to speculate that changes in Schwann cells after mechanical loading might be mediated by the actin cytoskeleton, although further investigation needs to be conducted to testify to the assumption, and to discover more specific signalling pathways.

As a whole, these data imply that after peripheral nerve damage, mechanical stress specially tensile stress of mild magnitude, may promote regeneration of nerves via first accelerating demyelination of Schwann cells distal to the injury site. Secondly, by increasing secretion of neurotrophins from these Schwann cells, and thirdly, facilitating axon elongation. The demyelinating effect of mechanical stress could be mediated by the actin cytoskeleton. For the very same reason that it favours demyelination, mechanical stress of any form should be avoided at later stages of nerve regeneration when re-myelination of Schwann cells becomes the primary objective.

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