Short communication

Myelin-related gene silencing mediated by LPA₁ – Rho/ROCK signaling is correlated to acetylation of NFκB in S16 Schwann cells

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Abstract

Lysophosphatidic acid (LPA) initiates demyelination following peripheral nerve injury, which causes neuropathic pain. Our previous in vivo and ex vivo studies using mice have demonstrated that LPA-induced demyelination of spinal dorsal roots is attributed by the LPA₁-type receptor-mediated down-regulation of myelin-related molecules, such as MBP and MPZ. In this study using S16 mature-type Schwann cells, we found that LPA-induced down-regulation of myelin-related genes is attributed by the activation of LPA₁ receptor, Rho kinase, and p300, leading to an acetylation of NFκB, which down-regulates the transcription of Sox10, MBP and MPZ genes.

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Acute pain such as nociceptive and inflammatory pain plays important roles as an alarm, and this type of pain is sensitive to nonsteroidal anti-inflammatory drugs and opioids. Chronic pain including neuropathic pain, on the other hand, is refractory to these medical treatments. Neuropathic pain is often characterized by abnormally hyper-responsive sensory perception, called hyperalgesia or allodynia, in which innocuous (tactile) stimuli cause intense pain. In a series of our studies, we have proposed that lysophosphatidic acid (LPA)-mediated demyelination of sensory neurons is one of neurobiological mechanisms underlying neuropathic allodynia (1). As the cluster of biochemical events involved in demyelination are being reconstructed, the unique role of LPA in initiation of neuropathic pain through demyelination is becoming clearer (1–3). We have revealed that LPA functions as an initiator of neuropathic pain, and moreover, LPA itself amplifies LPA biosynthesis through an activation of LPA₁ receptor and microglia (4,5). This amplified LPA acts on dorsal root as a retrograde signal, which in turn results in demyelination through LPA₁ receptor. We found that LPA-mediated demyelination occurs in in vivo and ex vivo systems and this signaling is mediated by Rho/Rho kinase (ROCK) pathway (1,2), while detailed cell-based mechanisms remain elusive. In this study, we report that LPA initiates down-regulation of myelin-related gene expression via LPA₁ in S16 cells, a mature-type of Schwann cell line, and this signaling is associated with Rho/ROCK pathway and unique acetylation of NFκB.

S16 cell line was purchased from ATCC (Manassas, VA) and maintained with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS (Gibco, Grand Island, NY) supplemented with 100 IU/ml penicillin G and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere.

For quantitative PCR, S16 cells were plated onto 6-well plates at 4.0 × 10⁴ cells per well in DMEM containing 10% FBS. After 48 h, the cells were serum-starved in DMEM containing 0.1% BSA for 3 h, and then pre-treated with 1 μM of the following compounds: Ki16425 (a general gift from KIRIN Brewery Co., Ltd, Gunma, Japan), Y27632 (Sigma–Aldrich, St Louis, MO), or C646 (Sigma–Aldrich) for 30 min. The cells were then treated with vehicle or LPA (1-oleoyl-2-hydroxy-sn-3-glycerol-3-phosphate, Cayman Chemical, Ann Arbor, MI) for 3 h. Total RNA was isolated using TRIzol (Life technologies, Gaithersburg, MD) according to the manufacturer’s instructions. cDNA was synthesized with PrimeScript RT reagent kit (Takara Bio, Inc., Japan). Quantitative PCR analysis was performed using GeneAce SYBE qPCR Mix (Nippon Gene, Japan) on LightCycler 480II Real-Time PCR system (Roche, Indianapolis, IN). The PCR conditions were as follows: 95 °C for 30 s and 60 °C for 60 s with initial activation of enzyme at 95 °C for 10 min for a total of 40 cycles. The following primer sets were used: Mbp forward: 5’-CCCTCCAAGGCACAGACAC-3’ and reverse: 5’-AGGGAGCCGTTAGTTGATG-3’, Mpz forward: 5’-GGATTTGCTGATGGC-3’.

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and reverse: 5'-TTGGTGCTCGGCTGTGTC-3'; Sox10 forward: 5'-GCTATCCAGCTCCTACAAGAT-3' and reverse: 5'-CTCTGCTTTGGGGTTGTTG-3'; GAPDH forward: 5'-CCACGGCAAGGGGCGGAGATGAT-3' and reverse 5'-CACCGGAAGGGGCGGAGATGAT-3'. The data were analyzed using the delta cycle threshold method and the expression level of each gene was normalized to GAPDH.

For phalloidin staining, cells were seeded onto a lab-Tek-8-chamber slide (NUNC, Rochester, NY) at 2.0 \times 10^4 cells per well. The next day, after the serum-deprivation culture for 3 h, the cells were pre-treated with Ki16425, Y27632, or C646, followed by stimulated with vehicle or LPA as mentioned above. The cells were washed with PBS and fixed with 4% PFA for 1 h at room temperature. After fixing, rhodamine-phalloidin (0.05 U/mL, Invitrogen, Carlsbad, CA) was applied and incubated for 1 h. After PBS wash, the slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). The stained cells were placed on a Zeiss LSM 710 inverted laser scan confocal to capture the images.

As for western blotting, S16 cells were plated onto 6-well plates at 4.0 \times 10^4 cells per well. After 48 h, the cells were serum-starved and pre-treated with Ki16425, Y27632, or C646 followed by treated with LPA as described above. The cells were collected and subjected to western blot analysis to detect NF\(\kappa\)B and acetylated NF\(\kappa\)B (ac-NF\(\kappa\)B) (anti-NF\(\kappa\)B, Santa Cruz Biotech, sc-3034 and #3045, respectively from Cell Signaling Technology; 1:1000 dilution), or anti-\(\beta\)-actin (sc-47778, Santa Cruz Biotech; 1:1000 dilution) and developed using the SuperSignal chemiluminescence reagent (Thermo Scientific, Rockford, IL).

For statistical analysis, one-way ANOVA was applied followed by Dunnett’s test for multiple comparisons (GraphPad Prism6). Results were shown as means ± SEM.

We first determined the expression level of LPA receptors in mouse dorsal root (DR) and matured Schwann cell line, S16 cells. As shown in Fig. 1A and B, quantitative PCR analysis revealed that LPA1 receptor was dominantly expressed in both preparations. Mouse DR fibers most abundantly express LPA1, however, the expression of LPA4-6 was very low, while LPA2 and LPA3 expression was negligible. In S16 cells, LPA1 is also the most abundant, followed by LPA4, while other subtypes were negligible. The addition of LPA at 0.3–3 \(\mu\)M to S16 cells caused significant decreases in gene expressions of myelin basic protein (MBP) and myelin protein zero (MPZ), and their upstream transacting factor, Sox10 by 20–30% of control (Fig. 1C–E). The degree of decrease is equivalent to those found in nerve injury- or LPA-induced in vivo models (1), or ex vivo model using dorsal root fibers (2). It should be noted that LPA1 gene expression did not change after LPA induction in S16 cells (data not shown).

For the purpose of pharmacological characterization of Ki16425, a selective inhibitor of LPA1 and LPA3 without affinity to LPA4-6 (6), and Y27632, a specific ROCK inhibitor, the effects of these inhibitors on LPA-induced stress-fiber formation were evaluated by use of phalloidin-staining in S16 cells. As shown in Fig. 2A and B, the addition of LPA caused an intense stress-fiber formation. The co-treatment with Ki16425 or Y27632 completely abolished the LPA-induced stress fiber formation, while these inhibitors alone had no significant changes (Fig. 2C–F). As shown in Fig. 2G, the LPA-induced down-regulation of Mbp, Mpz, and Sox10 gene expression was completely inhibited by Ki16425, suggesting that LPA1 receptor is responsible for these mechanisms, since LPA4 receptor is not expressed in S16 cells. In addition, Ki16425-treatment did not induce down-regulation of Mbp, Mpz, and Sox10 by 20–30% of control (Fig. 1C–E). The degree of decrease is equivalent to those found in nerve injury- or LPA-induced in vivo models (1), or ex vivo model using dorsal root fibers (2). It should be noted that LPA1 gene expression did not change after LPA induction in S16 cells (data not shown).
affect the LPA1 gene expression (data not shown). The complete blockade of down-regulation was also observed by the treatment with Y27632 (Fig. 2H), though either inhibitor alone had no significant effects on the expression of myelin-related genes. These findings confirmed our previous in vivo studies that the LPA1-receptor-mediated demyelination is mediated by its downstream Rho/ROCK signaling (1).

Chen and others reported that deacetylation of NFκB by HDAC1/2 are critical for myelination in Schwann cells (7,8). On the other hand, p300 and ac-NFκB complex induces chromatin modifications, such as recruitment of histone H3K4 or K9 methyltransferases. Moreover, the ac-NFκB/p300 complex represses expression of Sox10, a transcriptional factor and regulates the myelin-related genes (7). In addition, there are some reports that ROCK phosphorylates p300 and activates its acetyltransferase activity (9). However, the link between the extracellular signal and the subsequent intracellular signaling upstream of NFκB-acetylation remains unclear. Here we examined the effects of C646, an inhibitor of p300 (10), which is one of downstream factors of ROCK and a transcriptional co-activator with acetyltransferase activity (11). As shown in Fig. 2I, C646 significantly attenuated the down-regulation of gene expression of Mbp and Mpz as well as Sox10.

In the western blot analysis, on the other hand, LPA at 1 and 10 μM increased the levels of ac-NFκB in S16 cells, while no change was observed in the levels of non-ac-NFκB (Fig. 3A). The pretreatment with 1 μM Ki16425 inhibited the acetylation of NFκB by LPA, while Ki16425 alone showed no effect. Quantitative analysis demonstrated that LPA-induced increase in the ratio of ac-NFκB to non-ac-NFκB and its blockade by Ki14625 were significant (Fig. 3B). Quite similar findings were observed with the effects of Y27632 and C646 on LPA-induced ac-NFκB levels, while each inhibitor alone showed no significant effect (Fig. 3C–F).

The most important issue in this study is the downstream of Rho/ROCK signaling activated through LPA1 leads to gene silencing of myelin-related molecules. This is because most of studies regarding Rho/ROCK signaling are related to morphological functions, and gene regulation of myelin-related molecules is often reported in terms of transactivation systems (12,13). Furthermore, the role of ac-NFκB in the gene repression is also unique, since NFκB itself is known to activate the transcription of inflammation-related genes (14). In our preliminary study using another type of Schwann cell line, IMS32 (15), we failed to detect the LPA-mediated gene repression of myelin-related molecules. So the use of S16 cells could be beneficial for the study of LPA1-mediated cellular signaling of down-regulation of myelin-related gene, and for future drug discovery strategy to suppress demyelination-related neuropathic pain.
**Fig. 3.** LPA elicits acetylation of NF-κB through LPA₁-Rho/ROCK-p300 pathway. Western blotting analysis after exposure of 1 and 10 μM LPA for 3 h after pretreatment with either 0.1% BSA (vehicle), 1 μM Ki16425 (A and B), 1 μM Y27632 (C and D) or 1 μM C646 (E and F) for 30 min in S16 cells. β-actin was used as a loading control. A representative blot is shown. The intensity of the bands was analyzed using ImageJ software. Statistical analyses were performed using one-way ANOVA (mean ± SEM, n = 3). *p < 0.05 and **p < 0.01 versus vehicle control. #p < 0.05 and ##p < 0.01 versus 1 μM LPA. N.S. represents no significance.

**Conflict of interest**

All authors declare no conflict of interest.

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**References**