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Research article

Electrophysiological characterization of Müller cells from the ischemic retina of mice deficient in the leukemia inhibitory factor

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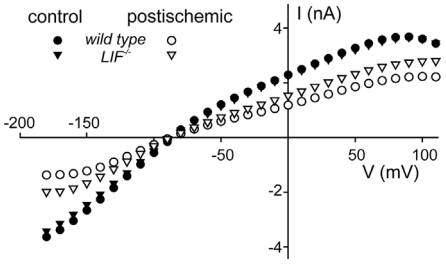
GRAPHICAL ABSTRACT



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ABSTRACT

Leukemia inhibitory factor (LIF) is a cytokine that exerts different effects in the nervous system. It is involved in neuronal injuries and diseases and is assumed to be neuroprotective and to regulate reactive gliosis. In LIF-deficient ($LIF^{-/-}$) mice, expression of glial fibrillary acidic protein in retinal Müller glial cells as a hallmark of reactive gliosis is suppressed during retinal degenerations. Here, we detected expression of LIF and its receptors in Müller cells of the murine retina. Moreover, electrophysiological alterations of Müller cells 7 days after transient retinal ischemia were studied by the patch-clamp technique. The amplitude of inward currents in Müller cells from the postischemic retina was reduced to 51% in wild type and to 70% in $LIF^{-/-}$ mice. This demonstrates that decrease of inward currents takes place in reactive Müller cells even in the absence of LIF.

1. Introduction

Leukemia inhibitory factor (LIF) is a cytokine from the interleukin-6 (IL-6) family [1]. The effects of LIF are mediated by binding to two types of receptors: gp130, the common receptor of the IL-6 family, and LIF receptor (LIFR) [2]. In the nervous system, LIF is involved in development and differentiation as well as in pathology [2]. Glial cells respond to injuries and diseases of the nervous tissue with the process of reactive gliosis. An increased expression of glial fibrillary acidic protein (GFAP) is a hallmark of reactive gliosis [3]. It has been found that LIF regulates the expression of GFAP in glial cells [4]. Therefore, LIF might be one factor that regulates reactive gliosis. Moreover, LIF expression itself is upregulated in glial cells after injuries and neurological disorders and LIF has been shown to be a mediator in inflammatory processes [5,6]. Within the retina, LIF and its receptors are important for retinal differentiation during development [7]. Moreover, LIF plays a neuroprotective role in the degenerating retina [8].

The dominant macroglia cell type of the retina is the Müller glial cell. Müller cells are obviously involved in all types of retinal diseases and degenerations [9]. Whereas Müller cells in the healthy retina of many species (e.g., mouse and rat) do not display GFAP immunoreactivity, expression of GFAP is strongly upregulated during

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reactive gliosis [10]. It has been shown that lack of LIF in LIF-deficient ($LIF^{-/-}$) mice suppressed upregulation of GFAP in Müller cells in cases of retinal degenerations [11,12]. Specific alterations of Müller cell physiology have been observed to be induced by reactive gliosis [9,13]. For example, a decrease of currents mediated by inwardly rectifying K^+ (Kir) channels has been recorded in some but not all cases of Müller cell gliosis. Because of this known variability of Müller cell reactions, we investigated electrophysiological properties of Müller cells from $LIF^{-/-}$ mice after transient retinal ischemia to see whether LIF is involved in the downregulation of Kir currents.

2. Materials and methods

2.1. Animals

All experiments were done in accordance with the European Communities Council Directive 86/609/EEC and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the local authorities. Mice were maintained with free access to water and food in an air-conditioned room on a 12-h light-dark cycle. For RNA sequencing, C57Bl/6J mice at the age of 3–5 months were used. $LIF^{-/-}$ mice were backcrossed on a C57Bl/6 background. Because $LIF^{-/-}$ females are infertile, heterozygous $LIF^{+/-}$ animals were interbred to generate wild type and $LIF^{-/-}$ mice. Animals were used at an age of 2–6 months, wild type littermates were used as control. The following primer combination was used to genotype the animals: wt-band (192 bp) (forward: cgcctaacatgacagact tcccat, reverse: aggcccctcatgacgtctatagta) and knockout-band (514 bp) (forward: ccagctcttcagcaatatcacggg, reverse: cctgtccggtgccctgaatgaact).

2.2. RNA sequencing

Müller cells were enriched as described [14]. Briefly, retinae were incubated in papain (0.2 mg/ml; Roche, Mannheim, Germany)-containing PBS (Ca^{2+}-/Mg^{2+}-free phosphate-buffered saline) for 30 min at 37 °C, followed by washing with PBS. After short incubation in PBS with DNase I (200 U/ml; Sigma–Aldrich, Taufkirchen, Germany; if not otherwise stated, all substances were from Sigma-Aldrich). The tissue pieces were triturated by a 1-ml pipette tip in extracellular solution (ECS) containing (mM): 135 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 Na_2HPO_4, 10 HEPES, and 11 glucose, adjusted to pH 7.4 with Tris-base. After centrifugation and for enrichment of microglia and vascular cells, the retinal cell suspension was incubated prior to Müller cell selection with CD11b- and CD31 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in two subsequent steps each for 15 min at 4 °C and subsequent positive-selection via LS-columns (Miltenyi Biotec). Thereafter, the cell suspension was incubated in ECS containing biotinylated hamster anti-CD29 (clone Ha2/5, 0.1 mg/ml, BD Biosciences, Heidelberg, Germany) for 15 min at 4 °C. Cells were washed in ECS, spun down, resuspended in the presence of anti-biotin MicroBeads (1:5; Miltenyi Biotec) and incubated for 10 min at 4 °C. After washing, $CD29^+$ Müller cells were separated using large cell columns according to the manufacturer's instructions (Miltenyi Biotec). Total RNA was isolated from all samples using the PureLink® RNA Micro Scale Kit (Thermo Fisher Scientific, Schwerte, Germany). Upon DNase-treatment to remove genomic DNA, first-strand cDNAs were synthesized from 50 ng of total RNA (RevertAid H Minus First-Strand cDNA Synthesis Kit, Fermentas by Thermo Fisher Scientific). After library preparation (Nextera XT, Clontech) samples were sequenced on an Illumina platform (NextSeq 500 High Output Kit v2; 150 cycles; Kompetenzzentrum Fluoreszente Bioanalytik, University of Regensburg) reaching a mean coverage of at least 20 million reads per sample. Fastq-data were filtered, reads were mapped to the mouse RefSeq protein-coding genes and analyzed for gene regulation at transcript resolution via Cufflinks.

2.3. Retinal ischemia

Transient retinal ischemia was induced in one eye by the HIOP (high intraocular pressure) method. The other eye remained untreated as internal control. Anesthesia was induced with ketamine (100 mg/kg body weight, intraperitoneal (ip); Ratiopharm, Ulm, Germany), xylazine (5 mg/kg, ip; Bayer Vital, Leverkusen, Germany), and atropine sulfate (100 mg/kg, ip; Braun, Melsungen, Germany). The anterior chamber of the test eye was cannulated from the pars plana with a 30-gauge infusion needle, connected to a saline bottle. The intraocular pressure was increased to 160 mmHg for 90 min by elevating the bottle. After removing the needle, the animals survived for 7 days and, subsequently, were sacrificed with carbon dioxide.

2.4. Histology

Isolated retinae were fixed in 4% paraformaldehyde (in PBS) for 2 h. After several washing steps in PBS, the tissues were embedded in PBS containing 3% agarose (w/v), and 45-µm thick slices were cut by using a vibrating microtome. The slices were incubated in 5% normal goat serum plus 3% Triton X-100 (Triton) plus 1% dimethylsulfoxide (DMSO) in PBS for one hour at 37 °C and, subsequently, in the primary antibodies overnight at 4 °C. After washing in PBS plus 3% Triton and 1% DMSO, the secondary antibodies were applied overnight at room temperature in PBS plus 0.3% Triton and 1% DMSO in the dark.

Cell nuclei were labeled with Hoechst 33258 (1:1000; Molecular Probes, Eugene, OR, USA). Control slices were incubated with secondary antibodies alone without primary antibodies; no unspecific labeling was observed (not shown). Images were taken with the laser scanning microscope LSM 510 Meta (Zeiss, Oberkochen, Germany). The following primary antibodies were used: mouse anti-GFAP (1:500; G-A-5 clone, Sigma-Aldrich), rabbit anti-GFAP (1:500; Dako, Glostrup, Denmark), mouse anti-glutamine synthetase (GS) (1:500; Merck-Millipore, Darmstadt, Germany), and rabbit anti-cellular retinaldehyde binding protein (CRALBP) (1:500; Santa Cruz Biotechnology, Heidelberg, Germany). The following secondary antibodies were used: Cy3-conjugated donkey anti-rabbit, Cy2-conjugated donkey anti-mouse, Cy3-conjugated goat anti-rabbit, and Cy2-conjugated goat anti-mouse. All secondary antibodies were applied in a 1:200 dilution and were obtained from Dianova (Hamburg, Germany).

2.5. Electrophysiology

For whole-cell patch-clamp experiments, cells were isolated as described above. The cells were stored at 4 °C in serum-free minimum essential medium until use within 4 h after cell isolation. Müller cells were identified according to their characteristic morphology. The currents were recorded at room temperature using the Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA) and the ISO-2 software (MFK, Niedernhausen, Germany). The signals were low-pass filtered at 1 or 6 kHz (eight-pole Bessel filter) and digitized at 5 or 30 kHz, respectively, using a 12-bit A/D converter. Patch pipettes were pulled from borosilicate glass (Science Products, Hofheim, Germany) and had resistances between 4 and 6 MΩ when filled with a solution containing (mM): 10 NaCl, 130 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 EGTA, and 10 HEPES, adjusted to pH 7.1 with Tris. The recording chamber was continuously perfused with ECS. To evoke membrane currents, de- and hyperpolarizing voltage steps of 250 ms duration, with increments of 10 mV, were applied from a holding potential of −80 mV. The amplitude of the steady-state inward currents was measured at the end of the 250-ms voltage step from −80 to −140 mV. The membrane capacitance of the cells was measured by the integral of the uncompensated capacitive artifact (filtered at 6 kHz) evoked by a 10-mV voltage step in the presence of extracellular $BaCl_2$ (1 mM). Current densities were calculated by dividing inward current amplitudes evoked by 60 mV hyperpolarization by the membrane capacitance. The resting