Biomaterials 82 (2016) 20–33

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Analgesic drug delivery via recombinant tissue plasminogen activator and microRNA-183-triggered opening of the blood-nerve barrier

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ARTICLE INFO

Article history:
Received 28 August 2015
Received in revised form
15 November 2015
Accepted 29 November 2015
Available online 17 December 2015

Keywords:
(4–6): drug delivery
microRNA
Peripheral nerve
Biocompatibility
In vivo test
In vitro test

ABSTRACT

The peripheral nerve contains three barriers which include the blood-nerve barrier consisting of endoneurial vessels and the perineurium as well as autotypic junctions in Schwann cells. The perineurium prevents diffusion of perineurally injected drugs that can be used for selective regional pain control. It is composed of a basal membrane and layers of perineurial cells sealed by tight junction proteins like claudin-1. Claudin-1 expression and barrier function are regulated via low-density lipoprotein receptor-related protein (LRP-1). Perisciatic application of recombinant tissue plasminogen activator (rtPA) or the catalytically inactive rtPAi – both agonists of LRP-1 – reduced claudin-1 mRNA and protein expression in the rat nerve. This facilitated an increase of nociceptive thresholds after local application of hydrophilic opioids or the voltage gated sodium channel blocker ($Na_V1.7$) ProToxin-II without apparent nerve toxicity. RtPA-induced barrier opening was mediated by LRP-1 and intracellularly by Erk phosphorylation. *In silico*, microRNA (miR)-rno-29b-2-5p and rno-miR-183-5p were identified as potential regulators of claudin-1 transcription in the rat. RtPA application increased miR-183-5p in the sciatic nerve. MiR-183-5p mimics functionally opened the perineurium and downregulated claudin-1 expression *in vivo*. *In vitro*, hsa-miR-183-3p mimics reduced claudin-1 expression in human HT-29/B6 cells. Overall, rtPA regulates perineurial barrier tightness via LRP-1, Erk phosphorylation and miR-183-5p/3p. This mechanism might serve as a new principle to facilitate drug delivery to peripheral nerves in humans.

1. Introduction

In peripheral nerves, three barriers are found: the blood-nerve barrier (BNB) consisting of the endothelium of endoneurial blood vessels, the perineurium surrounding nerve fascicles and autotypic junctions in Schwann cells. Barriers are sealed by tight junction proteins that can be divided into claudins, comprising 27 members in mammals, and tight junction-associated MARVEL proteins (TAMP), comprising occludin, tricellulin, and MarvelD3 [1]. The perineurium is composed of perineurial cells connected by the tight junction proteins occludin, claudin-1, claudin-3, claudin-19, tricellulin and the intracellular scaffolding protein ZO-1 [2,3]. Claudin-1 is essential for perineurial barrier sealing [4] as siRNA targeting claudin-1 opens the barrier for drug delivery [5,6]. Claudin-1 is also found in endoneurial cells, where claudin-5 is also present. In addition, claudin-1 is expressed in the membrane of mammalian myelinated Schwann cells (Schmidt-Lantermann incisures, outer mesoaxon, paranodal regions and periaxonal membranes) [7,8]. The perineurium limits paracellular drug

Abbreviations: BBB, blood–brain barrier; BNB, blood-nerve barrier; CLDN1, claudin-1 gene; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin; miR, microRNA; GSK-3, glycogen synthase kinase 3; IDV, integrated density value; LRP-1, low density lipoprotein (LDL) receptor-related protein-1; MMP, metalloproteinase; PBS, phosphate-buffered saline; ProTx-II, ProToxin-II; RAP, receptor-associated protein; rtPA, recombinant tissue plasminogen activator.

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http://dx.doi.org/10.1016/j.biomaterials.2015.11.053
0142-9612/

transport to the nerve when applied perineurally [6,9]. Indeed, hydrophilic opioids or blockers of voltage-gated sodium channels like $Na_V1.7$, which selectively target nociceptors, can only be used for regional analgesia if applied together with a barrier-opening enhancer [2].

Recently, we have shown that the perineurial barrier can be unsealed for several hours by perineurially injected hypertonic saline [5,6]. Hypertonic saline triggers the release of metalloproteinase-9 (MMP-9), which binds to low density lipoprotein (LDL) receptor-related protein-1 (LRP-1) via its hemopexin domain and leads to the downregulation of claudin-1 in the perineurium [5]. The LRP gene family is a group of related endocytic receptors. Members of this family are present on most cells and bind a diverse set of ligands. For example, LRP-1 binds tissue plasminogen activator (tPA) and MMP-9 [10]. LRP-1 activation in the peripheral nerve promotes Schwann cell survival and axonal regeneration [11]. Following LRP-1 activation, phosphorylation of Erk as well as of Akt and Rac is observed [11,12].

TPA is the protease known to generate plasmin from its inactive precursor, which in turn activates the fibrinolytic system. Previous studies have examined recombinant tPA (rtPA) at the blood brain barrier (BBB) because rtPA is used clinically to lyse emboli in acute stroke. At the BBB, rtPA directly increases vascular permeability in the early stages through receptor-mediated cell signaling of LRP-1 [13–15]. Yet, tPA-mediated BBB breakdown involves more than one pathway: In addition to LRP-1 activation rtPA is dependent on plasmin formation and potentiated in the presence of plasminogen. Indeed, application of a chemically inactive rtPA (rtPAi) does not open the BBB *in vitro*. Plasmin, generated on the cell surface selectively by tPA, modulates the astrocytic cytoskeleton, leading to an increase in BBB permeability [14].

MicroRNAs (miRs) are small RNA molecules (~22 nt) that act as negative regulators of gene expression, either by blocking mRNA translation or through RNA degradation. A single miR is predicted to typically target hundreds of mRNAs. Deregulated expression of miRs might play a role in the development of several diseases including autoimmune disorders, malignancies and possibly pain [16]. MiRs are also involved in the control of barrier tightness [17]. In the gut, miR-29 is upregulated in irritable bowel syndrome with diarrhea in the colon, which is accompanied by a downregulation of claudin-1 [18]. MiR-29a/b KO mice are resistant to irritable bowel syndrome and maintain barrier sealing in the gut. Likewise, miR-155 KO mice have less BBB leakage in experimental autoimmune encephalomyelitis [19].

This study was designed to (i) explore rtPA as an enhancer to induce downregulation of claudin-1 expression and to increase perineurial barrier permeability, (ii) thereby facilitate antinociception by opioids or NaV1.7 blockers applied at the nerve, (iii) examine potential neurotoxic side effects of rtPA, (iv) decipher the molecular mode of action of rtPA *in vivo* and (v) identify miRs that posttranscriptionally regulate the tightness of the BNB after rtPA via claudin-1.

2. Materials and methods

2.1. Animals

Animal protocols were approved by the animal care committee of the regional government of Würzburg and are in accordance with the International Association for the Study of Pain [20]. Male Wistar rats weighing 180–220 g were always injected under brief isoflurane anesthesia as described below. Experiments were conducted at indicated time points.

2.2. Perineurial injection at the sciatic nerve and treatment schedules

Under brief isoflurane anesthesia, the right sciatic nerve was located using a 22 G atraumatic needle (to avoid intraneural injection) connected to a nerve stimulator (Stimuplex Dig RC; Braun, Melsungen, Germany) as previously described [5]. Rats received a perisciatic injection of 10 μg rtPA (Actilyse; Boehringer Ingelheim, Biberach, Germany) or rtPAi (non-enzymatic human tPA, HTPA-ALA; Molecular Innovations, Novi, MI USA) in 150 μl 0.9% saline. At indicated time points, mechanical nociceptive thresholds were obtained before and after application of DAMGO or tissue samples were taken. For the blockage of rtPA or rtPAi, 10 μg/150 μl RAP (30R-AR008, Fitzgerald Industries International, Acton, MA, USA) or 30 μg/150 μl PD98059 (#9900, Cell Signaling, USA) were injected into the vicinity of sciatic nerve 20 min before, then rtPA or rtPAi were applied.

2.3. Measurement of nociceptive thresholds

Mechanical nociceptive (pain) thresholds were determined using the paw pressure algesiometer (modified Randall–Selitto test; Ugo Basile, Comerio, Italy) as described before [5]. A blinded investigator determined the pressure required to elicit paw withdrawal, i.e. the paw pressure threshold. Three measurements per paw and per treatment were obtained. Averages were calculated subsequently. Pre-treatment measurements were performed before the perisciatic injection of rtPA. After indicated time points, baseline nociceptive thresholds were measured and 30 μg DAMGO or 5 or 25 nmol ProTx-II (both Sigma Aldrich Chemical, St. Louis, MA, USA) in 150 μl 0.9% saline were applied. The total injected volume was 300 μl maximum. Paw pressure thresholds were determined 10 min thereafter.

A decrease in paw pressure threshold was interpreted as hyperalgesia (pain) whereas a rise in paw pressure threshold was interpreted as antinociception (analgesia).

2.4. Western blotting

After indicated treatments, sciatic nerves were taken and homogenized in lysis buffer for Triton X-100 soluble proteins (25 mM Tris pH 7.6, 120 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 25 mM NaF, 1% (v/v) Triton X 100) containing protease inhibitors (Complete, Roche Applied Science) [5]. Cytosol fractions were obtained by homogenization with minipistil and sonification (3 × 5 s/3 s break), followed by a centrifugation at 4500× *g* for 10 min and subsequent centrifugation of the remaining supernatant at 40,000× *g* for 30 min. The Triton X-100-insoluble pellet, which contains the membrane fraction mainly of the plasma membrane and but also membranes of organelles, was resuspended in an equal volume of extraction buffer (25 mM Hepes pH 7.6, 2 mM EDTA, 25 mM NaF, 1% (w/v) SDS). Extracted protein was diluted in lysis buffer and incubated with BCA protein assay reagent (Pierce, Rockford, IL, USA) for quantification on a plate reader (Tecan, Grödig, Austria). Aliquots of protein were mixed with sodium dodecyl sulfate (SDS) containing buffer (Laemmli buffer), denatured at 95 °C for 5 min, fractionated on SDS polyacrylamide gels and subsequently blotted onto PVDF membranes (PerkinElmer, Boston, MA, USA). Proteins were detected using specific antibodies rabbit polyclonal anti-claudin-1 (# 51-9000, 1:1,000, Life Technologies, Darmstadt, Germany), pERK (# 9101, 1:400, Cell Signaling, Danvers, MA, USA), ERK (# 9102, 1:1000, Cell Signaling), rabbit anti-ZO-1 (# 61-7300, Thermo Fisher Scientific) and as protein loading control β-actin (#A3854, 1:20,000, Sigma Aldrich). Peroxidase conjugated goat anti-rabbit IgG, goat anti-mouse IgG and the