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Research Article

Salubrinal inhibits the expression of proteoglycans and favors neurite outgrowth from cortical neurons *in vitro*

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ABSTRACT

After CNS injury, astrocytes and mesenchymal cells attempt to restore the disrupted *glia limitans* by secreting proteoglycans and extracellular matrix proteins (ECMs), forming the so-called glial scar. Although the glial scar is important in sealing the lesion, it is also a physical and functional barrier that prevents axonal regeneration.

The synthesis of secretory proteins in the RER is under the control of the initiation factor of translation eIF2α. Inhibiting the synthesis of secretory proteins by increasing the phosphorylation of eIF2α, might be a pharmacologically efficient way of reducing proteoglycans and other profibrotic proteins present in the glial scar. Salubrinal, a neuroprotective drug, decreased the expression and secretion of proteoglycans and other profibrotic proteins induced by EGF or TGFβ, maintaining eIF2α phosphorylated. Besides, Salubrinal also reduced the transcription of proteoglycans and other profibrotic proteins, suggesting that it induced the degradation of non-translated mRNA.

In a model *in vitro* of the glial scar, cortical neurons grown on cocultures of astrocytes and fibroblasts with TGFβ treated with Salubrinal, showed increased neurite outgrowth compared to untreated cells. Our results suggest that Salubrinal may be considered of therapeutic value facilitating axonal regeneration, by reducing overproduction and secretion of proteoglycans and profibrotic protein inhibitors of axonal growth.

Abbreviations: BBB, blood–brain barrier; CSPGs, chondrotin sulfate proteoglycans; CTGF, connective tissue growth factor; DMEM, Dulbecco's modified Eagle's medium; ECMs, extracellular matrix proteins; EGF, epidermal growth factor; FBS, Fetal bovine serum; P/S, Penicillin/Streptomycin; P-eif2α, eIF2α phosphorylated; qPCR, quantitative real-time PCR; RER, rough endoplasmic reticulum; RPS29, 40S ribosomal protein S29; RT, room temperature; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrilamide gel electrophoresis; TBST, TBS with Tween 20; TGFβ, transforming growth factor β; XylT, Xylosyltransferases

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Introduction

Severe lesions of the CNS caused by cerebrovascular pathologies or mechanical contusions (*e.g.* spinal cord injury), disrupt the blood–brain barrier (BBB) that protects the CNS microenvironment from direct contact with blood substances and cells. Glial cells (mainly astrocytes) and mesenchymal cells (fibroblasts and pericytes) react to the lesion, secreting extracellular matrix proteins and inducing a new *glia limitans* called the glial scar [1]. Although it has some beneficial effects [2], the glial scar is one of the main obstacles to axonal regeneration after injury [3].

The major axon growth inhibitory components of the glial scar, that block successful regeneration [4], are the chondrotin sulfate proteoglycans (CSPGs). They consist of a large variety of core proteins covalently linked to chondroitin sulfate glycosaminoglycans [5]. The protein core of the CSPGs is synthesized in the rough endoplasmic reticulum (RER) [6] and CSPGs are glycosylated along their transport from the RER to the plasma membrane, through the Golgi apparatus [7]. Glycosylation of the core protein of CSPGs is initiated by xylosyltransferases (XylT) that add a xylose to core protein serine residues [8]. After xylose addition, complex saccharide chains are generated by the addition of β-GalNAc [9] and the polymerization catalyzed by the chondroitin synthase [10] and the protein chondroitin polymerizing factor [11].

Glial scar formation is regulated by various soluble mediators, including cytokines, and growth factors released from platelets, helped by blood cells and CNS endogenous cells (glia and neurons), that initially respond to the lesion and then to the subsequent inflammation. Growth factors such as EGF (epidermal growth factor), TGFβ (transforming growth factor β) and CTGF (connective tissue growth factor), and cytokines such as IL-6, IFNγ, TNFα and IL-1β regulate the expression and secretion of CSPGs in astrocytes [12]. Reactive astrocytes express and secrete most of the CSPGs in the scar, such as brevican, neurocan, versican and phosphacan [13], all of which have axon growth inhibitory properties [14]. Moreover, a large population of mesenchymal cells invade the lesion core, participating in the glial scar formation through extracellular protein deposition (*e.g.* fibronectin, collagen) and promoting astrocyte reactivity [15]. Although this population of profibrotic mesenchymal cells has been traditionally associated with invading meningeal fibroblasts [16], pericytes [17] and perivascular fibroblasts [18] have recently been postulated to contribute to glial scar formation.

Protein translation in the ER is regulated by the phosphorylation status of the translational initiator eIF2α. Increasing the phosphorylation of eIF2α by different kinases attenuates the translation of secretory and transmembrane proteins that are synthesized in the ER. Four kinases induce the phosphorylation of translational initiator eIF2α: GCN2 (activated by amino acid starvation), HRI (activated by heme deprivation, as well as by osmotic and heat shocks), PKR (activated by viral infections; and some cytokines and growth factors) and PERK (activated by ER stress and hypoxia). Conversely, reducing the phosphorylation of eIF2α increases the translation of secretory and transmembrane proteins. GADD34 or CReP protein form a complex with PP1α phosphatase that dephosphorylates eIF2α [19].

Salubrinal was discovered while screening for small molecules that protected PC12 cells from death induced by ER stress [20]. Salubrinal had a neuroprotective effect in mice against injury caused by intracereventricular injection of kainic acid [21]. It also reduced glutamate toxicity for primary cultures of cortical neurons and for the hippocampal cell line HT22 [22]. Salubrinal inhibited eIF2α dephosphorylation through the inhibition of phosphatases containing GADD34 or CReP, maintaining eIF2α highly phosphorylated and reducing the translation of secretory proteins in the ER [20].

Here, we show that Salubrinal reduced the expression and secretion of proteoglycans and other profibrotic proteins such as CTGF. Additionally we show for the first time that Salubrinal reduced the mRNAs for CSPGs and CTGF, suggesting that it induced the degradation of non-translated ER-targeted protein mRNAs. Salubrinal favored neurite outgrowth from cortical neurons in a glial scar model *in vitro*.

Materials and methods

Reagents

Sal003, eIF-2α Inhibitor II (Salubrinal) was purchased from Calbiochem (La Jolla, CA, USA). Recombinant human TGF-β2 (TGFβ) and Recombinant human EGF (EGF) were purchased from Peprotech (Rocky Hill, NJ, USA); Dulbecco's modified Eagle's medium (DMEM) was purchased from Lonza (Barcelona, Spain); Penicillin/Streptomycin mix (P/S) and Poly-L-lysine were purchased from Sigma-Aldrich (St Louis, MO, USA). Neurobasal® Medium, B27® Supplements, GlutaMAX™ Supplement and Fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD).

Cell culture

Primary cultures of astrocytes were obtained from P0-P2 C57BL/6 mouse cortices [23]. The tissue homogenate was filtered through a 40 μm mesh (BD Falcon; Franklin Lakes, NJ, USA) and centrifuged at 168 × *g* for 10 min. The pellet was plated and grown in DMEM medium supplemented with 10% heat-inactivated FBS and 1% P/S (DMEM 10:1) in 75-cm^2 flasks, precoated with poly-L-lysine (10 μg/ml). The Medium was changed every 3–4 days and after reaching confluency (10 days of culture), the cells were shaken at 280 rpm at 37 °C in a shaker (Infors Minitron Botmingen; Switzerland). Detached cells were washed off with PBS and the attached astrocyte monolayer was trypsinized and centrifuged at 168 × *g* for 10 min. The cell pellet was resuspended in warm DMEM 10:1 and plated in multiwell plates.

Primary cultures of fibroblasts, obtained from P0-P2 C57BL/6 mouse meninges, were subjected to enzymatic digestion with 0.25% trypsin in HBSS (Sigma-Aldrich) for 20 min, at 37 °C, followed by mechanical homogenization. The tissue homogenate was centrifuged at 168 × *g* for 10 min and the cells in the pellet were cultured in complete DMEM medium in 75-cm^2 flasks, precoated with poly-L-lysine. After the cells reached confluency (10 days of culture), they were washed off with PBS, trypsinized and centrifuged at 168 × *g* for 10 min. Cell pellets were resuspended in warm DMEM, 10:1 and plated in multiwell plates.

Primary cultures of neurons, obtained from E17 – E18 C57BL/6 mouse cortices, were subjected to mechanical and enzymatic digestion with trypsin and DNase (20 mg/ml; Roche; Indianapolis, IN, USA) in HBSS for 15 min, at 37 °C, followed by homogenization. The tissue homogenate was centrifuged at 168 × *g* for 10 min and the pelleted cells were suspended in complete DMEM medium and