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Salmon-derived thrombin inhibits development of chronic pain through an endothelial barrier protective mechanism dependent on APC

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ABSTRACT

Many neurological disorders are initiated by blood–brain barrier breakdown, which potentiates spinal neuroinflammation and neurodegeneration. Peripheral neuropathic injuries are known to disrupt the blood-spinal cord barrier (BSCB) and to potentiate inflammation. But, it is not known whether BSCB breakdown facilitates pain development. In this study, a neural compression model in the rat was used to evaluate relationships among BSCB permeability, inflammation and pain-related behaviors. BSCB permeability increases transiently only after injury that induces mechanical hyperalgesia, which correlates with serum concentrations of pro-inflammatory cytokines, IL-7, IL-12, IL-1α and TNF-α. Mammalian thrombin dually regulates vascular permeability through PAR1 and activated protein C (APC). Since thrombin protects vascular integrity through APC, directing its affinity towards protein C, while still promoting coagulation, might be an ideal treatment for BSCB-disrupting disorders. Salmon thrombin, which prevents the development of mechanical allodynia, also prevents BSCB breakdown after neural injury and actively inhibits TNF-α-induced endothelial permeability in vitro, which is not evident the case for human thrombin. Salmon thrombin's production of APC faster than human thrombin is confirmed using a fluorogenic assay and APC is shown to inhibit BSCB breakdown and pain-related behaviors similar to salmon thrombin. Together, these studies highlight the impact of BSCB on pain and establish salmon thrombin as an effective blocker of BSCB, and resulting nociception, through its preferential affinity for protein C.

1. Introduction

Blood-brain barrier (BBB) disruption is characteristic of many neurological disorders, including stroke, Parkinson's disease and ALS, and contributes to the associated neuroinflammation and neurodegeneration in those diseases [1–4]. The neurovasculature of the BBB is comprised of endothelial cells that are trophically coupled to nearby neurons via glial cells, which together, make up the 'neurovascular unit' and interact closely to maintain inflammatory dysfunction in disease states [5–7]. The healthy BBB endothelium is bound together by tight junctions, which inhibit the transmission of serum components and blood-borne cells into the central nervous system (CNS) [6,8]. BBB disruption permits the entrance of neurotoxic factors into the CNS that impair normal neuronal function and exacerbate inflammation [3,9]. Peripheral nerve injuries also increase the permeability of the BBB, or more specifically the blood-spinal cord barrier (BSCB), where the injured afferents synapse [10–12]. Those injuries also induce chronic neuropathic pain, which is maintained by spinal neuroinflammation [11,13,14]. However, it is unclear whether BSCB disruption itself contributes to pain and should be investigated as a potential therapeutic target for chronic pain prevention. We hypothesize that a nerve root injury that initiates chronic pain also induces BSCB breakdown at times corresponding to either the development or maintenance of pain.

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Systemic inflammation contributes to spinal neuroinflammation both directly and indirectly. Pro-inflammatory factors stimulate peripheral neurons that can then become hyperexcitable at their synapses in the CNS [15] and indirectly contribute to inflammation within the CNS. Circulating pro-inflammatory cytokines, such as TNF-α and IL-1β, themselves can also increase vascular permeability promoting their own entrance into the CNS across a compromised BBB [11,16–19]. Once in the CNS, these inflammatory molecules directly stimulate neurons and glia, maintaining pain signaling [20,21]. Further supporting the role of pro-inflammatory molecules and the development of pain, blocking the actions of TNF-α and IL-1β centrally significantly attenuates that pain that can develop after compressive nerve root injury [22]. Systemic and central inflammation play important roles in pain and are integrated through BBB permeability [7,8,11]; yet, it is not known whether there is a relationship between nerve root-induced pain and levels of pro- or anti-inflammatory cytokines or chemokines at times of BSCB breakdown.

The protease thrombin, most notably recognized for its role in the coagulation cascade, also regulates a variety of endothelial processes via its enzymatic activation of cell-bound receptors [23]. Thrombin initiates distinct signaling cascades depending on the cofactor it binds and the substrate it cleaves [23–26]. In its unbound state, mammalian thrombin increases vascular permeability by directly activating the protease-activated receptor-1 (PAR1) on the endothelial surface [23,27]. In contrast, when bound to endothelial thrombomodulin, thrombin activates endothelial-bound protein C into activated protein C (APC), which stabilizes vascular integrity [25,28,29]. Clinical trials have tested APC for its enhancement of endothelial barriers in sepsis, stroke and traumatic brain injury [30–32], but its strong anticoagulant effects hamper its clinical safety [30]. For this reason, a major effort in thrombin mutagenesis and protein engineering has identified domains in thrombin's structure that control protein C activation in order to increase thrombin's innate affinity for protein C instead of PAR1 by manipulating protein structure [32–35]. We hypothesize that by activating the APC pathway, with either APC or thrombin with a higher affinity for protein C, will fortify the BSCB and prevent pain.

Interestingly, fish differ significantly from mammals in their inflammatory responses and have naturally evolved enzymes with distinct capabilities from mammalian counterparts [36]. Specifically, thrombin derived from salmon exhibits nearly indistinguishable clotting capabilities, but initiates different cell signaling cascades compared to human thrombin [36–39]. Salmon thrombin induces lower levels of platelet aggregation and astrocytic transcription of pro-inflammatory cytokines compared to human thrombin at the same concentration [37,38]. We have shown that salmon thrombin uniquely inhibits the development of neuropathic pain after a nerve root compression injury and activates PAR1 to a lower degree than human thrombin [38,39]. We hypothesize that salmon thrombin may prevent neural pain by exhibiting a higher affinity for protein C than mammalian thrombin promoting its ability to provide vascular protection.

In this study we use a nerve root compression model in the rat to define BSCB breakdown after painful and non-painful nerve root injuries and to determine whether the resulting pain intensity correlates with serum inflammatory cytokines levels. We further investigate the effects of salmon thrombin on mediating nerve root-induced BSCB breakdown, and use in vitro studies to define its effects on inflammation-induced endothelial permeability and protein C activation. In order to establish whether directly blocking BSCB inhibits the development of pain similar to salmon thrombin, we administer intravenous APC in studies with painful nerve root injury. These three complementary studies integrate in vivo and in vitro assays and are augmented by an in silico study using protein modeling of fish and human thrombin to identify mechanistic differences that may explain the experimental observations.

2. Materials & methods

2.1. Study design & objectives

Three experimental studies were performed to characterize BSCB breakdown, to investigate the potential for thrombin to attenuate/prevent BSCB breakdown, and to compare thrombin to known APC actions. Adult male Holtzman rats were used in all in vivo studies, and all experiments were approved by the Institutional Animal Care and Use Committee. The first set of studies characterizes the time course of BSCB breakdown following painful and non-painful nerve root compression in the rat and investigate potential relationships between peripheral inflammation and pain at times of BCSB breakdown. Two durations of nerve root compression were administered in the rat: 15 min because it has been shown to induce sustained nociception and 3 min to serve as a loading control in which the nerve root undergoes injury, but pain-related behaviors do not develop [40,41]. BSCB permeability was investigated by immunolabeling the spinal cord for IgG, a blood protein that is not present in the CNS under normal conditions, at day 1 and day 7. Those time points were selected since they correspond to the development and maintenance of pain in this injury model [13,38–41]. To investigate the relationship between inflammation, pain and BSCB breakdown, serum levels of a panel of inflammatory cytokines were assayed at day 1, which corresponds to maximal BSCB breakdown. Of the cytokines correlating to mechanical hyperalgesia, TNF-α was the most robust and so was also immunolabeled in the spinal cord to assess whether this inflammatory mediator enters the CNS to exacerbate neuroinflammation.

The second study with salmon thrombin evaluated the effectiveness of salmon thrombin in preventing BSCB breakdown and nociception after neural injury and determines whether it acts through protein C to protect endothelial barriers. This study integrated in vivo and in vitro methods. For the in vivo portion of this study, rats undergoing painful nerve root compression were treated with salmon thrombin, human thrombin for comparison, or neurobasal media as a vehicle control. Since findings from the characterization study identified day 1 as the time point of maximal BSCB breakdown, spinal IgG was labeled and quantified on day 1 after injury with the different treatments. To test whether salmon thrombin directly protects endothelial barriers, an in vitro microchannel setup was used to measure the relative effects of salmon and human thrombin on inflammation-induced vascular permeability. Those experiments were performed in the presence of serum to expose the endothelial surface to protein C, as well as under serum-free conditions in order to examine if thrombin acts through surface receptors or serum components to modulate permeability. The production rate of APC also was compared between salmon and human thrombin using a fluorogenic peptide assay to determine if the two species exhibit different protein C activation rates.

Lastly, APC was administered after painful nerve root compression in vivo to identify whether that protein can prevent BSCB breakdown and the development of pain-related behaviors similar to salmon thrombin. In that study spinal IgG and mechanical hyperalgesia were measured on day 1 after injury. Moreover, based on the findings of these three experimental studies, we also performed protein modeling to compare the protein structures between fish and human thrombin in order to better understand how their structures influence their mechanisms of action.