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Kallikrein 6 is a Novel Molecular Trigger of Reactive Astrogliosis

Isobel A. Scarisbrick[1,2,4,*], **Maja Radulovic**[1], **Joshua Burda**[1], **Nadya Larson**[2], **Sachiko I. Blaber**[3], **Caterina Giannini**[4], **Michael Blaber**[3], and **Alexander G. Vandell**[1]

[1]Neurobiology of Disease Program, Mayo Medical and Graduate School, Rochester MN 55905

[2]Department of Physical Medicine and Rehabilitation, Mayo Medical and Graduate School, Rochester MN 55905

[3]Department of Biomedical Sciences, Florida State University College of Medicine, Tallahassee, FL 32306-4300

[4]Department of Neurology, Mayo Medical and Graduate School, Rochester MN 55905

Abstract

Kallikrein-related peptidase 6 (KLK6) is a trypsin-like serine protease up regulated at sites of central nervous system (CNS) injury, including *DE NOVO* expression by reactive astrocytes, yet its physiological actions are largely undefined. Taken with recent evidence that KLK6 activates G-protein coupled protease activated receptors (PARs), we hypothesized that injury-induced elevations in KLK6 contribute to the development of astrogliosis and that this occurs in a PAR-dependent fashion. Using primary murine astrocytes and the Neu7 astrocyte cell line, we show that KLK6 causes astrocytes to transform from an epitheliod to a stellate morphology and to secrete interleukin 6 (IL-6). By contrast, KLK6 reduced expression of glial fibrillary acidic protein (GFAP). The stellation promoting activities of KLK6 were shown to be dependent on activation of the thrombin receptor, PAR1, as a PAR1 specific inhibitor, SCH79797, blocked KLK6-induced morphological changes. The ability of KLK6 to promote astrocyte stellation was also shown to be linked to activation of protein kinase C (PKC). These studies indicate that KLK6 is positioned to serve as a molecular trigger of select physiological processes involved in the development of astrogliosis and that this is likely to occur at least in part by activation of the G-protein coupled receptor, PAR1.

Keywords

Astrocyte; Protease Activated Receptor; Spinal Cord Injury; Multiple Sclerosis; Glioblastoma; GFAP; IL-6

Introduction

Kallikrein 6 (KLK6), also referred to as kallikrein-related peptidase 6, is a member of a family of 15 serine proteases aligned in tandem on human chromosome 19q13.3-4 (Borgono and Diamandis 2004). Of all kallikreins, KLK6 is the most abundant in the adult central nervous system (CNS) (Scarisbrick et al., 2006a). Within the CNS, KLK6 is expressed at the highest levels in the brain stem and spinal cord where expression is primarily associated with neurons and oligodendrocytes with a relative paucity of expression by astrocytes (Scarisbrick et al., 2000; Scarisbrick et al., 2001). In cases of CNS injury however, there is

[*]Correspondence to: Isobel A. Scarisbrick Ph.D, Molecular Neuroscience, 642B Guggenheim Building, Mayo Clinic Rochester, 200 First St., SW., Rochester, MN 55905, Tel: (507)-284-0124, Fax: (507)-266-4716.

an overall elevation in KLK6 levels within the lesion microenvironment, including an induction of expression by reactive astrocytes (Scarisbrick et al., 2002; Scarisbrick et al., 2006B). Given the important role that astrocytes play in the response of the CNS to injury and its ability to undergo repair (Bush et al., 1999; Faulkner et al., 2004; Ishida et al., 2006; Fitch and Silver 2008; Li et al., 2008; Rolls et al., 2009; Sofroniew 2009), we have in this study examined the potential activity of KLK6 in promoting astrogliosis.

Despite the abundance of KLK6 in both the normal and pathologic CNS (Scarisbrick et al., 1997; Scarisbrick et al., 2000; Scarisbrick et al., 2002; Terayama et al., 2004; Uchida et al., 2004; Scarisbrick et al., 2006B), very little is currently known regarding its physiologic and pathophysiologic roles. KLK6 has been shown to degrade extracellular matrix proteins, including laminin, fibronectin and heat denatured collagen, as well as myelin proteins such as myelin basic protein and myelin oligodendrocyte glycoprotein (Bernett et al., 2002; Blaber et al., 2002; Scarisbrick et al., 2002). In addition to degradative actions, we recently demonstrated that KLK6 cleaves thereby activating select members of a family of G-protein-linked receptors referred to as protease activated receptors (PARs) (Angelo et al., 2006; Oikonomopoulou et al., 2006a; Oikonomopoulou et al., 2006b; Vandell et al., 2008), positioning it to serve as an endogenous signaling molecule in the CNS. There are four known PARs, with PAR1, 2 and 4 activated by proteolytic cleavage of their N-terminus revealing a cryptic tethered ligand that binds intramolecularly to initiate intracellular signaling. PAR3 is also similarly cleaved by proteolysis but it remains controversial the extent to which it signals independently (Ostrowska and Reiser 2008; Seminario-Vidal et al., 2009), or rather acts as a regulator of thrombin signaling by serving as a co-factor for PAR4 (Nakanishi-Matsui et al., 2000) or by heterodimerzing with PAR1 (McLaughlin et al., 2007). In a recent study we showed that KLK6 activates PAR1 in neurons and PAR1 and PAR2 in astrocytes to elicit intracellular Ca^{2+} flux and regulation of the mitogen activated protein kinase (MAPK) signaling pathway (Vandell et al., 2008).

PARs have been widely implicated in disease, including CNS pathology (Adams et al., 2011). PAR1 is commonly referred to as the thrombin receptor and is of particular interest with regard to CNS function and disease since it is not only the most abundant PAR in the CNS (Junge et al., 2004; Vandell et al., 2008), but has also been implicated in ischemia related pathogenesis (Striggow et al., 2001), in traumatic spinal cord injury (Citron et al., 2000), and in HIV induced encephalitis (Boven et al., 2003). At sites of CNS pathology, PAR1 has been linked to direct neural injury (Turgeon et al., 1998; Festoff et al., 2000; Striggow et al., 2000), in N-methyl-D-aspartate receptor potentiation (Gingrich et al., 2000; Hamill et al., 2009; Han et al., 2011), and in astrogliosis (Wang et al., 2002; Boven et al., 2003; Sorensen et al., 2003; Nicole et al., 2005; Wang et al., 2007). Given the up regulation of KLK6 with CNS injury, including *DE NOVO* expression in reactive astrocytes (Scarisbrick et al., 2000; Scarisbrick et al., 2006b), in this study we tested the hypothesis that KLK6 participates in astrogliosis by activation of PAR1. To address this hypothesis we have examined the effects of KLK6 on hallmarks of reactive astrogliosis *IN VITRO*, including astrocyte stellation, proliferation, expression of glial fibrillary acidic protein (GFAP) and production of the cytokine interleukin 6 (IL-6).

Results presented indicate that KLK6 is elevated in astrocytes at sites of pathology in the human brain and spinal cord and that KLK6 regulates select aspects of reactive astrogliosis *IN VITRO* in a PAR1-dependent fashion. Taken together, these findings support the hypothesis that KLK6 can serve as an endogenous regulator of astrocyte physiology and that elevations in KLK6 at sites of CNS pathology are positioned to participate in events which drive reactive astrogliosis in a graded fashion.

Results

KLK6 is Prominent in Hypertrophic Astrocytes in a Variety of Human Pathological Conditions

Cellular hypertrophy is one of the hallmarks of reactive astrogliosis and is a common characteristic of acute and chronic CNS lesions including those seen in spinal cord injury, multiple sclerosis, Alzheimer's, hydrocephalus, and glioma (Holley et al., 2003; Morcos et al., 2003; Rodriguez et al., 2009). In the uninjured CNS, KLK6-immunoreactivity is predominantly expressed by oligodendroglia (Figure 1A) (Scarisbrick et al., 2000; Scarisbrick et al., 2002). Hypertrophic astrocytes in cases of neuropathology are readily recognized by their large size (Morcos et al., 2003) and these were densely KLK6-immunoreactive in the spinal cord injury (Figure 1C) and multiple sclerosis (Figure 1E) cases examined. In glioblastoma multiforme, KLK6-immunoreactivity was also prominent in astrocytes and readily evident in those with a gemistocytic morphology as seen in Figure 1G. The prominence of KLK6 in reactive astrocytes across a range of CNS disorders, but not in astrocytes of uninjured brain (Scarisbrick et al., 2000; Scarisbrick et al., 2001; Scarisbrick et al., 2002; Scarisbrick et al., 2006b), underscores the need to determine whether KLK6 may be a significant contributor to the process of astrogliosis.

KLK6 Promotes Astrocyte Stellation

The potential impact of KLK6 on murine astrocyte morphology was determined by analysis of the arrangement of cytoskeletal actin in response to treatment with recombinant enzyme. Since serum can cause high basal levels of astrocyte stellation, and contains both proteases and protease inhibitors, all experiments were performed using defined serum free media. Under these conditions more than 90% of all primary astrocytes were non-stellate, that is did not have two or more processes at least one cell body in length (11.6 + 5.8% stellate, Figure 2). Treatment of primary astrocytes with 300 nM (10 μg/ml) of recombinant KLK6 resulted in a 7-fold increase in stellation over a period of 24 hr (74.1 + 5.1% stellate, Figure 2B). By contrast at parallel concentrations, another member of the kallikrein gene family, KLK1, which was expressed and activated under conditions identical to KLK6 (Laxmikanthan et al., 2005), did not alter astrocyte shape (Figure 2C). Subsequent experiments to determine the temporal features of the KLK6-stellation response indicated that significant changes in stellation could already be observed by 4 hr after KLK6 application (Figure 2H, $P<0.05$, SNK). Since we recently demonstrated that KLK6 can activate astrocyte PAR1 and PAR2 (Vandell et al., 2008), and activation of these receptors is known to promote a non-stellate morphology (Wang et al., 2002; Sorensen et al., 2003; Nicole et al., 2005; Park et al., 2006; Wang et al., 2007), we examined the effects of peptides that specifically activate each receptor (PAR1- and PAR2-APs). In agreement with prior studies, when treated with PAR1- (40 μM) or PAR2-APs (200 μM), primary astrocytes maintained an epitheliod, non-stellate appearance. The effects of KLK6 were also compared to effects of lipopolysaccharide (LPS) which other studies indicate promotes astrocyte activation (Brahmachari et al., 2006). Treatment of primary astrocytes with LPS (25 μg/ml) resulted in robust astrocyte stellation (73.2 + 1.9% stellate, Fig. 2F) paralleling the response observed with KLK6 treatment. KLK6-induced stellation in primary astrocytes was blocked by the pan-serine protease inhibitor aprotinin (Figure 2J).

While much remains to be learned regarding what would be considered physiological levels of KLK6 in the brain and the changes that occur with injury and disease, the level of KLK6 in human cerebrospinal fluid has been shown to range from 0.5 to over 30 μg/ml (Zarghooni et al., 2002; Borgono and Diamandis 2004; Shaw and Diamandis 2007). We therefore examined the effects of a range of KLK6 concentrations to determine the minimal dose sufficient to promote astrocyte stellation (Figure 2). There was a dose dependent increase in

KLK6-triggered stellation at both 30 nM (1 μg/ml, 38.1 + 2.1% stellate) and at 300 nM (10 μg/ml, 64.8 + 5.4% stellate), each promoting a significant increase relative to controls (11.6 + 5.8% stellate), while treatment with 3 nM (0.1 μg/ml) trended towards an increase but did not reach the level of statistical significance at the 24 hr time point ($P<0.05$). The stellation promoting effects of KLK6 *IN VITRO* therefore occur over a range of concentrations that would be considered physiological relative to normal human CSF and which are likely encompassed by the elevated levels known to occur in cases of CNS pathology (Scarisbrick et al., 1997; Scarisbrick et al., 2002; Uchida et al., 2004; Scarisbrick et al., 2006b).

The ability of KLK6 to promote astrocyte stellation was further evaluated using the Neu7 murine astrocyte cell line (Fok-Seang et al., 1995) that we have utilized in prior studies to dissect KLK6-receptor mediated signaling cascades (Vandell et al., 2008). Notably, Neu7 astrocytes have a high basal level of stellation under the defined media conditions used herein, permitting further comparisons between the stellation promoting/reversing effects of KLK6 relative to PAR-APs without the introduction of serum (Figure 3A-G). Neu7 astrocytes grown on charged cover glass were treated with concentrations of KLK6, KLK1 or PAR-APs identical to those used in experiments involving primary astrocytes. Under resting conditions, Neu 7 astrocytes exhibited a high basal level of stellation (74.5 ± 2.1% stellate). PAR1- and PAR2-APs caused a significant reversal of astrocyte stellation leaving only 26.7 ± 4% and 44.7 ± 4.1% of cells stellate, respectively ($P<0.001$). PAR-inactive 'scrambled' control peptides had no effect on stellation (not shown). These observations parallel those of prior studies which have demonstrated the ability of PAR1- and PAR2-APs to reverse astrocyte stellation in cultures with high basal stellation levels (Debeir et al., 1997; Pindon et al., 1998; Nicole et al., 2005; Park et al., 2006). Since we have shown KLK6 activates both PAR1 and PAR2 in Neu7 astrocytes, the expected result with KLK6 treatment would be a parallel reversal of stellation. Instead of reversing astrocyte stellation however, and mirroring the results seen in primary astrocytes, KLK6-induced a significant increase in the number of stellate astrocytes (85.2 ± 1.2% stellate, $P=0.002$). In addition to increasing the number of stellate cells present, KLK6 also increased astrocyte process length from an average of 29.1 ± 1.3 μm to 36.6 ± 0.8 μm ($P<0.001$, Figure 3H). As was the case for primary astrocytes, identical treatment with recombinant KLK1 did not alter the morphology of Neu7 astrocytes (79.1 ± 1.7% stellate).

Given the opposing effects of KLK6 and PAR1-AP on stellation of Neu7 astrocytes, we examined the effect of concomitant treatment (Figure 3I). In this series of experiments, Neu7 astrocytes treated with vehicle alone were 66.6 ± 4.9% stellate, while those treated with PAR1-AP showed the expected reversal of stellation (16.9 ± 4.6% stellate), and those treated with KLK6 showed enhanced stellation (78.2 ± 2% stellate). When Neu7 astrocytes were treated simultaneously with both PAR1-AP and KLK6, stellation reversal was still observed, but the magnitude was significantly reduced (35.8 ± 2.9% stellate, $P=0.005$). We note that the cleavage specificity of KLK6 is hydrolysis C-terminal to arginine and therefore we would not expect KLK6 to cleave and inactivate the PAR1-AP (TFLLR-amide) directly, although we did not directly test this possibility. The ability of KLK6 to oppose PAR1-induced stellation reversal was blocked by the pan-serine protease inhibitor 4-amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF, $P=0.004$, Figure 3).

PAR1 Activation is Necessary for KLK6-Induced Astrocyte Stellation

To evaluate the involvement of PAR1 in mediating KLK6-induced astrocyte stellation, primary or Neu7 astrocytes were treated with either vehicle alone or the PAR1 antagonist SCH79797 (35 nM) for 30 min, followed by application of KLK6 at 30 or 300 nM (1 or 10 μg/ml) for 24 hr (Figure 4A, B). Pre-treatment with the PAR1 antagonist alone did not alter astrocyte stellation under control conditions in primary or Neu7 astrocytes. The PAR1 inhibitor did however block the ability of 300 nM KLK6 to induce stellation in Neu7

astrocytes (63.5 ± 1.7% compared to 76.4 ± 2.2% for cells treated with KLK6 alone, P<0.001) and in primary astrocytes treated with 30 nM of KLK6 (5.2 ± 1.7 compared to 22.8 ± 3.5 for cells treated with KLK6 alone, P<0.001). At higher KLK6 concentrations (300 nM), which induced very high levels of stellation above baseline, reductions in primary astrocyte stellation seen with the PAR1 inhibitor did not reach the level of statistical significance.

KLK6 Induced Astrocyte Stellation is mediated by PKC

In order to begin to address the intracellular signaling pathways that mediate KLK6-induced stellation, primary or Neu7 astrocytes were pre-treated with vehicle alone or the protein kinase C (PKC) inhibitor Go6983 (60 nM) for 30 min prior to application of KLK6 (300 nM) and cells incubated for an additional 24 hr (Figure 5). The PKC inhibitor blocked KLK6-stellation in both primary and Neu7 astrocytes. In primary astrocytes, KLK6-treated cells were 53.7 + 4.0% stellate while this was reduced to 12.0 + 3.7% when PKC was blocked. In Neu7 astrocytes treatment with KLK6 resulted in 75.4 ± 2.8% stellate and pre-treatment with the PKC inhibitor reduced KLK6-mediated stellation to 56.7 ± 3.8%, (P<0.05) (Figure 5B).

Effects of KLK6 on IL-6 Secretion, GFAP Expression and Astrocyte Proliferation

The participation of KLK6 in astrogliosis was further evaluated by determining its ability to impact three additional hallmarks of reactive astrocytes; IL-6 secretion (Hariri et al., 1994), GFAP expression (Eng et al., 2000), and proliferation (Nicole et al., 2005; Wang et al., 2007). KLK6 treatment (300 nM, 24 hr) elicited a 2-fold increase in IL-6 secretion from primary astrocytes (166.9 + 5.1 pg/ml KLK6-treated compared to 79.8 + 2.1 pg/ml in the case of controls) (Figure 6A). By contrast, GFAP RNA expression was significantly decreased in the same cultures (Figure 6B). Quantification of the mean number of DAPI stained nuclei across experiments (Figure 2) indicated KLK6 does not cause a significant increase in astrocyte number (Figure 6C).

Discussion

Despite the fact that reactive astrogliosis is a ubiquitous component of CNS injury and disease, and essential to the wound healing process, the extracellular factors and intracellular mechanisms mediating this response are poorly understood. We have identified KLK6 as a novel serine protease with abundant expression in adult CNS and which is induced in astrocytes with neurological injury (Scarisbrick et al., 1997; Scarisbrick et al., 2000; Scarisbrick et al., 2001; Scarisbrick et al., 2002; Scarisbrick et al., 2006b). Given our recent findings demonstrating the ability of KLK6 to activate astrocyte PARs (Vandell et al., 2008), we hypothesize that elevations in KLK6 available from astrocyte and non-astrocyte sources at sites of CNS pathology promote astrogliosis. Studies herein demonstrate that KLK6 is capable of mediating discrete aspects of astrogliosis, including induction of a stellate morphology and IL-6 production. The ability of KLK6 to promote astrocyte stellation was further shown to be dependent, at least in part, on activation of PAR1 and the PKC intracellular signaling pathway. These studies therefore identify a novel molecular signaling pathway mediating astrogliosis *IN VITRO* that warrants further study as a target to modulate the development of astroglial scar tissue in cases of neurological injury and disease.

KLK6 Promotes Astrocyte Stellation

Astrocytes treated with KLK6 rapidly transform from an epitheliod polygonal shape to a stellate morphology. Astrocytes are known to be morphologically plastic and their shape plays a critical role in their functional properties (Eng et al., 2000; Liu et al., 2000; Messing

and Brenner 2003; Sofroniew 2009). The most exaggerated KLK6-stellation response was seen in primary astrocytes which exhibit a low resting level of stellation under defined culture conditions. Notably, KLK6 also promoted stellation of the Neu7 astrocyte cell line which had an overall higher basal level of stellation at rest. By contrast to the effects of KLK6, activation of either the thrombin receptor PAR1, or PAR2, or a combination of these receptors using synthetic peptide mimics (PAR-APs) promoted a reversal of astrocyte stellation when resting stellation levels were high, as seen in the Neu7 astrocyte cell line. These results parallel prior studies which show that thrombin, trypsin or PAR1-AP all promote reversal of astrocyte stellation (Cavanaugh et al., 1990; Debeir et al., 1997; Pindon et al., 1998; Park et al., 2006) and point to the unique capacity of KLK6 to drive astrocytes toward a stellate morphology. Interestingly, thrombin is also elevated at sites of CNS injury, largely by extravasation from blood (Gingrich and Traynelis 2000). Since thrombin and PAR1 activation promote a reversal of astrocyte stellation, and we show herein that KLK6 can partially oppose these effects, it is possible that elevations in KLK6 in CNS lesions serves in part to counterbalance the PAR1-mediated effects of thrombin, and that this continuum of proteolytic activity plays a unique role in regulating astroglial biology, including cellular morphology.

To begin to understand the functional significance of stellation to the process of astrogliosis, we compared the effects of KLK6 to those of LPS, a pro-inflammatory component of the cell wall of gram-negative bacteria, which is known to promote astrocyte activation *IN VITRO* (Brahmachari et al., 2006) and *IN VIVO* (Guerra et al., 2011). Notably, like KLK6, LPS promoted robust stellation of primary astrocytes. Taken together these data support the view that stellation is an important component of the physiological processes that take place during the astrocyte activation cascade and that KLK6 is a newly identified participant. Since our prior studies demonstrate that KLK6 is expressed *DE NOVO* in reactive astrocytes (Scarisbrick et al., 2006b), and current studies demonstrate expression in hypertrophic astrocytes in a range of human CNS pathologies, including spinal cord injury, multiple sclerosis and glioblastoma multiforme, we hypothesize that astrocyte-KLK6 acts in an autocrine or paracrine fashion to regulate specific aspects of the astrogliosis cascade, including astrocyte stellation. While the functional significance of stellation *IN VIVO* is not clear, one can envision that this morphological change may not only affect permeability of the blood brain barrier, but also play an important role in wound healing and the eventual development of glial scar tissue, each a common feature across CNS pathologies.

PAR1 Activation is Necessary for KLK6-Induced Astrocyte Stellation

While KLK6 promoted astrocyte stellation and activation of PAR1 promoted stellation reversal, it is interesting that blocking PAR1 activation alone, by pre-incubation of cells with the PAR1-specific inhibitor SCH79797 (Ma et al., 2005; Wang et al., 2006; Vandell et al., 2008), significantly reduced KLK6-astrocyte-stellation promoting effects. These results support the concept of biased agonism at PAR1, whereby activation of the same receptor by different agonists has divergent physiological effects. For example, there are several prior reports that activation of PAR1 can result in divergent phenotypes depending on the agonist involved. While both thrombin and activated protein C activate PAR1 on endothelial cells, thrombin induces changes in endothelial cells which promote breakdown of the blood brain barrier, while activated protein C promotes blood brain barrier integrity (Feistritzer and Riewald 2005). Notably, activated protein C retains its protective effects even in the presence of high concentrations of thrombin (Schuepbach et al., 2008), mirroring the ability of KLK6 to oppose PAR1-AP stellation reversal. Thrombin and activated protein C signaling also produce markedly different changes in gene expression in cytokine-stimulated cells (Riewald and Ruf 2005). Other studies indicate that divergent physiologic effects depending on the specific PAR1 agonist may reflect differences in receptor trafficking,

compartmentalization and/or transactivation events. Following activation of PAR1 by thrombin for example, PAR1 is rapidly internalized, while after activation by activated protein C, PAR1 remains on the cell surface (Schuepbach et al., 2008). Recent studies also suggest that compartmentalization of PAR1 in caveolae is critical for activated protein C-specific endothelial protective effects (Russo et al., 2009). While results in the present study demonstrate that PAR1 is a necessary component of the signaling pathway that results in KLK6-induced stellation, these results do not rule out the involvement of other receptors, including PAR2 which we have shown KLK6 activates in astrocytes (Vandell et al., 2008), and in other cell types (Oikonomopoulou et al., 2006a; Oikonomopoulou et al., 2006b). Interestingly, depending on the agonist, activation of PAR1 has also been associated with transactivation of the Epidermal Growth Factor receptor (Prenzel et al., 1999; Darmoul et al., 2004; Arora et al., 2008) and potentiation of N-methyl-D-aspartate receptor responses (Gingrich et al., 2000; Hamill et al., 2009; Han et al., 2011). Any differences in receptor localization, trafficking or transactivation between KLK6 and PAR1-AP-mediated activation of PAR1, and how this may affect intracellular signaling and astrocyte stellation will require further study. The results herein do support the concept that KLK6-signals at least in part through PAR1 to mediate astrocyte stellation and therefore PAR1 may serve as a target to modulate KLK6-induced changes in astrogliosis in cases of CNS injury.

PKC Signaling is Necessary for KLK6-Induced Astrocyte Stellation

The ability of KLK6 to promote astrocyte stellation depends on signaling through PKC since the stellation promoting effects of KLK6 were blocked by a PKC small molecule inhibitor. Prior studies have shown that activation of the PKC signaling cascade is essential to the regulation of astrocyte stellation with the effects observed dependent on the duration of activation. For example, a short 90 min exposure of astrocytes to the PKC activator PMA induced stellation reversal (Pindon et al., 1998; Park et al., 2006), while longer treatment periods, on the order of several hours, promoted stellation (Abe and Saito 2000). This is of particular interest with regard to the concept of biased agonism since it is possible that KLK6 and PAR1-AP engagement of the thrombin receptor differentially activate downstream signaling pathways such as PKC resulting in differential phenotypic effects, namely stellation or reversal of stellation. The PKC family contains at least 12 different isoforms and PAR1 has been shown to activate the α, β, δ and ε isoforms across a range of cell types (Pindon et al., 1998; Fang et al., 2004; Chiu et al., 2008; Fang et al., 2008). Of these, only the β-1 isoform has been linked to PAR1 mediated stellation reversal (Pindon et al., 1998). No specific PKC isoform has yet been linked to the promotion of astrocyte stellation, but the PKC inhibitor Go6983, shown to block KLK6-induced stellation herein, is known to inhibit the α, β, γ, δ, and ς isoforms, suggesting that one or more of these may be involved.

KLK6 Stimulates IL-6 Secretion but Suppresses GFAP mRNA Expression

In addition to promoting robust astrocyte stellation, treatment of astrocyte cultures with KLK6 promoted secretion of IL-6. IL-6 is a cytokine known to be up regulated in reactive astrocytes and to be an important component of astroglial pathophysiology (Hariri et al., 1994). Interestingly, blockade of IL-6 has been shown to reduce astrogliosis, inflammation and connective tissue scarring and to improve behavioral outcomes in murine models of traumatic spinal cord injury (Okada et al., 2004). IL-6 is also known to play an important role in the development and malignant progression of astrocytomas (Weissenberger et al., 2004). Therefore, elevations in KLK6 at sites of CNS pathology (Scarisbrick et al., 2002; Blaber et al., 2004; Terayama et al., 2004; Uchida et al., 2004), including *DE NOVO* expression by astrocytes (Scarisbrick et al., 2006b), are in a position to drive IL-6 mediated effects in an autocrine or paracrine fashion. Targeting KLK6 therefore may represent a

novel strategy to regulate IL-6 and the pleiotropic actions of this cytokine in a range of CNS pathological conditions.

While KLK6 promoted robust astrocyte stellation, and IL-6 secretion, it significantly reduced the expression of another cardinal feature of reactive astrogliosis, namely, expression of GFAP. GFAP is the major intermediate filament protein present at low levels in resting astrocytes and among those proteins known to be up regulated in the injured CNS(Sofroniew 2009). Interestingly, prior studies examining the effects of PAR1-AP on GFAP levels also point to suppressive effects at a protein level (Nicole et al., 2005).

Underscoring the likely unique roles played by KLK6 in the process of reactive astrogliosis, KLK6 was not seen to promote astrocyte proliferation. By contrast, trypsin, thrombin and PAR1-AP have each been identified as potent astrocyte mitogens (Perraud et al., 1987; Wang et al., 2002; Nicole et al., 2005), and proliferation is considered another hallmark of astrogliosis. Taken together, the selective effects of KLK6 in mediating astrocyte stellation and IL-6 secretion, while suppressing GFAP production, support the concept that KLK6 plays unique physiological roles in the spectrum of events that characterize astrogliosis. Further studies will be necessary to determine whether the astrocyte-specific effects triggered by KLK6 can be modulated to facilitate repair in cases of neurological injury or disease.

Materials and Methods

KLK6 in Human CNS Lesions

All human materials studied were formalin-fixed paraffin-embedded archival material obtained at autopsy. All cases of chronic spinal cord injury examined were defined as greater than one month post-injury (n=10, mean time post injury 1844 + 846 days), and were confirmed as traumatic contusion or transection injury cases by chart review. The cause of injury reflected that in the general population with 68% a result of motor vehicle accident, 21% falls and 11% sports-related injury. Chronic active multiple sclerosis lesions studied (n=5) were clinically and pathologically confirmed cases (Poser et al., 1983). Glioblastoma multiforme cases examined (n=32) were arrayed as tumor core samples in triplicate on glass microscope slides. All glioblastoma multiforme cases were histologically proven as World Health Organization (WHO) Grade IV. Five-μm deparaffinized sections were stained for KLK6 using a KLK6-specific monoclonal antibody (MSP-3-3, 20 μg/ml) (Scarisbrick et al., 2001; Scarisbrick et al., 2002) and standard immunoperoxidase techniques. Prior to staining, endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide, sections treated with citrate buffer (pH 7.0, BioGenex, San Ramon, CA) and non-specific binding blocked with 20% normal goat serum. Following incubation with primary and biotinylated species appropriate secondary antibodies (Jackson ImmunoResearch, West Grove, PA), KLK6 immunoreactivity was visualized with 3′, 3′-diaminobenzadine tetrahydrochloride as the substrate. Tissue sections processed in the absence of primary antibody served as a control for potential non-specific binding of secondary antibodies. Immunostained sections were counterstained with Gills Hematoxylin and adjacent sections examined for pathology in the case of multiple sclerosis and spinal cord injury lesions using Luxol fast blue/periodic acid Schiff (LFB/PAS). The use of human materials in this study was approved by the Mayo Clinic Institutional Review Board and all clinical follow-up was obtained by review of medical records.

Cell Culture

Primary astrocytes were isolated from postnatal day 1 C57/BL6J mice (Jackson Laboratories, Bar Harbor ME) essentially as described previously (Stieg et al., 1980).

Following isolation, astrocytes were expanded in Dulbecco's Modified Eagle Medium with 10% fetal calf serum, 0.45% glucose, 2 mM Glutamax, 1 mM sodium pyruvate, and 50 U/mL penicillin-streptomycin for 10 d on poly-D-lysine coated cell culture plastic (Sigma, St. Louis, MO). Prior to subculturing astrocytes in defined serum free media, oligodendroglia were removed from the cultures by overnight shaking at 200 rpm. Cultures of primary astrocytes were greater than 95% pure based on immunoreactivity for GFAP. The Neu7 astrocyte cell line was generated by retroviral immortalization of murine cortical astrocytes (Fok-Seang et al., 1995) and was kindly provided by Dr. Herb Geller (National Institutes of Health Bethesda, MD). Neu7 astrocytes express GFAP (Heck et al., 2003) and mirror properties of an astroglial scar in that they produce an extracellular matrix inhibitory to neurite outgrowth (Fok-Seang et al., 1995). All experiments were performed on cells cultured in defined serum free media which consisted of Neurobasal A media supplemented with 2% B27, 1% N2 (Invitrogen, Carlsbad CA), 50 U/mL penicillin-streptomycin, 0.45% glucose, 2 mM Glutamax, 1 mM sodium pyruvate and 50 μM beta-mercaptoethanol. In all cases cells were grown on surfaces coated with 10 μg/mL poly-D-lysine. All cells were maintained at 37°C in 95% air and 5% CO_2. All animal studies were performed in adherence to NIH Guidelines for animal care and safety and were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Recombinant KLK1 and KLK6 Protein Production

Recombinant mature KLK1 and KLK6 protein was produced from insect cell heterologous expression as previously described (Bernett et al., 2002; Blaber et al., 2002; Laxmikanthan et al., 2005). Briefly, an amino terminal 6x-His tag and enterokinase (Asp_4-Lys) pro-sequence was substituted for the KLK1 and KLK6 pro-sequence to enable efficient purification and subsequent controlled activation by enterokinase (EK). EK activation was performed under acidic pH conditions to limit any subsequent KLK6 autolytic inactivation (in contrast, KLK1 does not undergo internal autolysis). EK, mature KLK and released pro-peptide have substantially different molecular masses and were effectively and rapidly separated by size exclusion chromatography. The final purified mature KLK1 and KLK6 protein were characterized by N-teminal sequence analysis to quantify the extent of activation (and potential autolysis), and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE; combined with Coomassie brilliant blue staining and scanning densitometry) to quantify overall purity as well as extent of any autolysis. Mature purified KLK1 and KLK6 N-terminal sequencing showed the correct mature N-terminus, in both cases, with no evidence of the 6x-His uncut pro-peptide. Scanning densitometry of the SDS PAGE showed an overall purity of >95% for both KLK1 and KLK6 proteins. MALDI-TOFF mass spectrometry analysis showed a principle mass of 28, 780 Da for purified KLK1 and 25, 866 Da for purified KLK6, indicating the presence of 13 N-acetylglucosamine moieties at the single site of glycosylation at residue Asn95 in KLK1 and six N-acetylglucosamine moieties at the single site of glycosylation at residue Asn132 in KLK6. Mass spectrometry showed minor heterogeneous glycosylation for both proteins and involving 1–6 additional 180 Da hexose units (resulting in increased mass heterogeneity of 0.7–4.2 %); thus, 28.8 kDa and 25.9 kDa were utilized as the molecular mass of the purified KLK1 and KLK6 proteins, respectively; and an extinction coefficient of $E_{280\ 1cm}$ (1%) = 18.0 AU and12.8 AU, respectively, were utilized for routine determination of protein concentration. Purified KLK1 and KLK6 proteins were subjected to spectrofluorometric active site titration using 4-methylumbelliferyl 4-guanidinobenzoate (MUGB). The ester complex with these kallikrein-related peptidases is not as stable as with trypsin, however burst titration can be effectively extrapolated to the zero time point to determine active site concentration. This analysis indicated approximately 90% and 85% active protein for KLK1 and KLK6 proteins, respectively, based upon their individual extinction coefficients, and therefore in good agreement with the overall purity and integrity of the mature forms as

determined by the N-terminal sequencing and SDS PAGE scanning densitometry. Thus, active site titration indicates essentially all the mature KLK1 and KLK6 protein is correctly folded and active, in good agreement with the X-ray structure determination of both proteins produced and purified by these same methods (Bernett et al., 2002; Laxmikanthan et al., 2005).

Astrocyte Stellation Assay

The ability of KLK6 to alter astrocyte stellation and the possible involvement of PAR1 in this process was examined using a combination of recombinant kallikrein, PAR-activating peptides, and a PAR1 specific inhibitor, SCH79797 (Wang et al., 2006; Strande et al., 2007; Grisaru-Granovsky et al., 2009). First, the effects of treating primary astrocytes or the Neu7 astrocyte cell line for 24 hr with recombinant KLK1 or KLK6, each at 300 nM (10 μg/ml), or with PAR1- (TFLLR-amide, 40 μM) or PAR2-APs (SLIGRL-amide, 200 μM) (Peptides International, Louisville KY) were compared. PAR-APs mimic the tethered ligand of their respective receptors and therefore mediate receptor-specific activation. The concentrations of PAR-APs and KLK6 used were those we have demonstrated are sufficient to evoke Ca^{2+} flux and MAPK signaling in Neu7 astrocytes (Vandell et al., 2008). Negative control peptides for PAR1- (RLLFT-NH2) and PAR2- (VKGILS-NH2) were also examined at parallel concentrations. The minimal dose of KLK6 required to promote stellation of primary astrocytes was established by quantifying the effects of 3, 30 or 300 nM (0.1, 1 or 10 μg/ml) at the 24 hr time point. The time course of the stellation response was also determined by examination of cells at 30 or 90 min as well as 4, 8 or 24 hr post-KLK6 treatment. To determine whether KLK6-specific effects on astrocyte stellation were mediated by its enzymatic properties, stellation experiments were repeated using KLK6 that had been pre-incubated for 30 min with a pan-serine protease inhibitor, either APMSF (50 μM) or aprotinin (5 μM, Sigma). The involvement of PAR1 in KLK6-mediated changes in astrocyte stellation was examined using a PAR1-specific inhibitor, SCH79797 (35 nM, Tocris). SCH79797 is a selective non-peptide antagonist of PAR1 that has demonstrated specificity *IN VITRO* in a wide variety of cell types including, mouse kidney cells (Hocherl et al., 2011), a mouse motoneuron cell line (Vandell et al., 2008), rat primary astrocytes (Wang et al., 2006) and gingival fibroblasts (Ohuchi et al., 2011), as well as human melanoma (Silini et al., 2011) and cytotrophoblast cells (Grisaru-Granovsky et al., 2009). The role of PKC signaling was examined using a PKC specific inhibitor (Go6983, 60 nM, Tocris). SCH79797 or Go6983 were applied to cells 30 min prior to the application of recombinant KLK6.

To examine astrocyte stellation, astrocytes were plated at a density of 12, 500 cells per cm^2 on poly-D-lysine coated cover slips. Following treatment, cells were fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, stained with 1 U rhodamine-conjugated phalloidin (Invitrogen) to visualize actin. Phalloidin stained cells were cover slipped using 4′, 6-diamidino-2-phenylindole (DAPI) containing Vectashield mounting media enabling enumeration of all cells in each case (Vector Laboratories, Burlingame CA). Five digital images were collected at 20X magnification per cover slip in predetermined fields (at the center and four poles) using an Olympus AX70 microscope equipped with SPOT software (Olympus, Center Valley PA). Astrocytes were scored as stellate if they possessed two or more processes at least one cell body in length. Astrocyte process length was measured using Interactive Pathology Laboratory software (BD Biosciences, Franklin Lakes NJ). Cell number was determined by enumeration of the mean number of DAPI labeled nuclei in each microscopic field. In each case, cells were treated in triplicate and experiments repeated at least twice. At least 300 cells were scored without knowledge of the treatment conditions in each experiment and stellation in treated and untreated conditions compared using unpaired Students t-tests.

Quantitative PCR and ELISA

In addition to determining the effect of KLK6 on astrocyte stellation, its ability to alter other facets of astrogliosis, namely IL-6 secretion and GFAP expression were also examined. To determine the potential effect of KLK6 on IL-6 secretion and GFAP expression, primary cortical astrocytes were grown in 6 well plates and treated with either vehicle alone or KLK6 (300 nM) for 24 hr. Cell culture supernatants were collected and snap frozen at minus;70 °C for measurement of IL-6 protein. IL-6 secretion was measured in cell culture supernatants using the eBioscience Mouse IL-6 ELISA kit according to the manufacturer instructions (eBioscience, San Diego CA).

GFAP expression was quantified in RNA isolated using RNA STAT-60 (Tel-Test Inc. Friendswood, TX) from KLK6-treated or untreated cultures. The level of mRNA expression in each case was determined in 0.25 μg of RNA using Light Cycler RNA Amplification SYBR Green I (Roche, Basel Switzerland) and an iCycler iQ5 (BioRad, Hercules CA). GFAP mRNA copy number was normalized to the level of the constitutively expressed gene, glyceraldehyde phosphate 3-dehydrogenase (GAPDH), amplified in the same sample. In each case copy number was determined by parallel amplification of cDNA clones diluted to known copy number as previously described (Scarisbrick et al., 2006a). Forward and reverse primers used for GFAP were 5′-GCAGATGAAGCCACCCTGG-3′ and 5′-GAGGTCTGGCTTGGCCAC-3′, and for GAPDH were 5′-ACCACCATGGAGAAGGC-3′ and 5′-GGCATGGACTGTGGTCATGA-3′, respectively.

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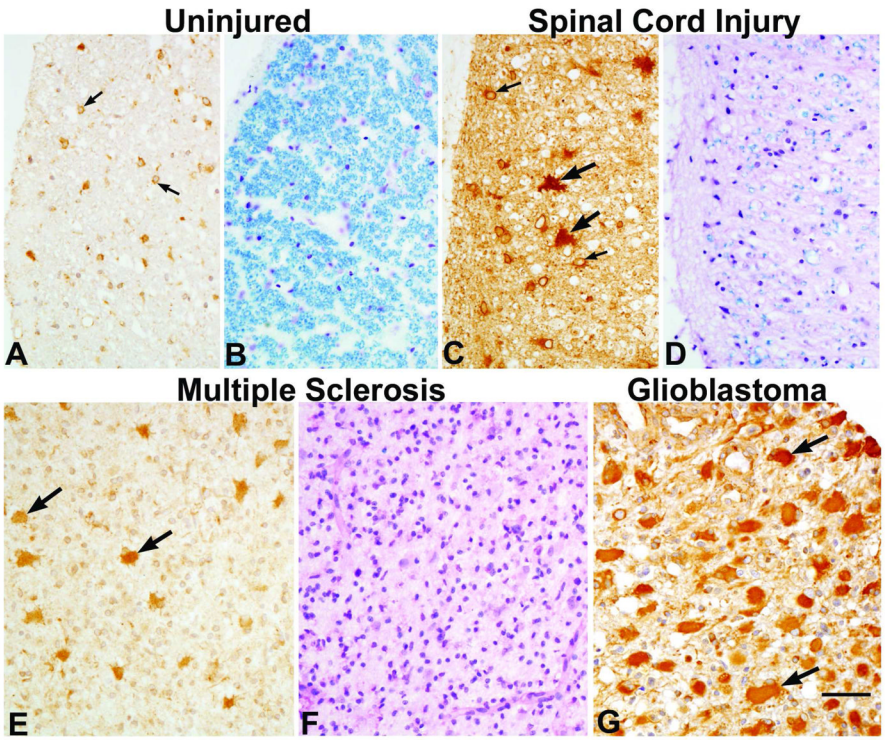

Figure 1. KLK6 is dense in hypertrophic astrocytes in spinal cord injury, multiple sclerosis and glioblastoma multiforme

In normal appearing spinal cord white matter (A, B), KLK6 is prominent in oligodendrocytes which bear round nuclei and a thin rim of cytoplasm (arrows, A), while astrocytes are not prominently stained(Scarisbrick et al., 2000; Scarisbrick et al., 2001) (B, shows adjacent LFB/PAS). In cases of traumatic spinal cord injury (C) and in multiple sclerosis lesions (E), KLK6 immunoreactivity is dense in hypertrophic astrocytes (D and F show Luxol fast blue/periodic acid Schiff stains in near adjacent sections demonstrating myelin loss; small arrows A and C indicate KLK6-immunoreactive oligodendrocytes, large arrows C and E indicate hypertrophic astrocytes). KLK6 is also dense in astrocytes with a gemistocytic morphology seen in glioblastoma multiforme tumor samples (G, large arrows) (Scale Bar = 50 μm in A–G).

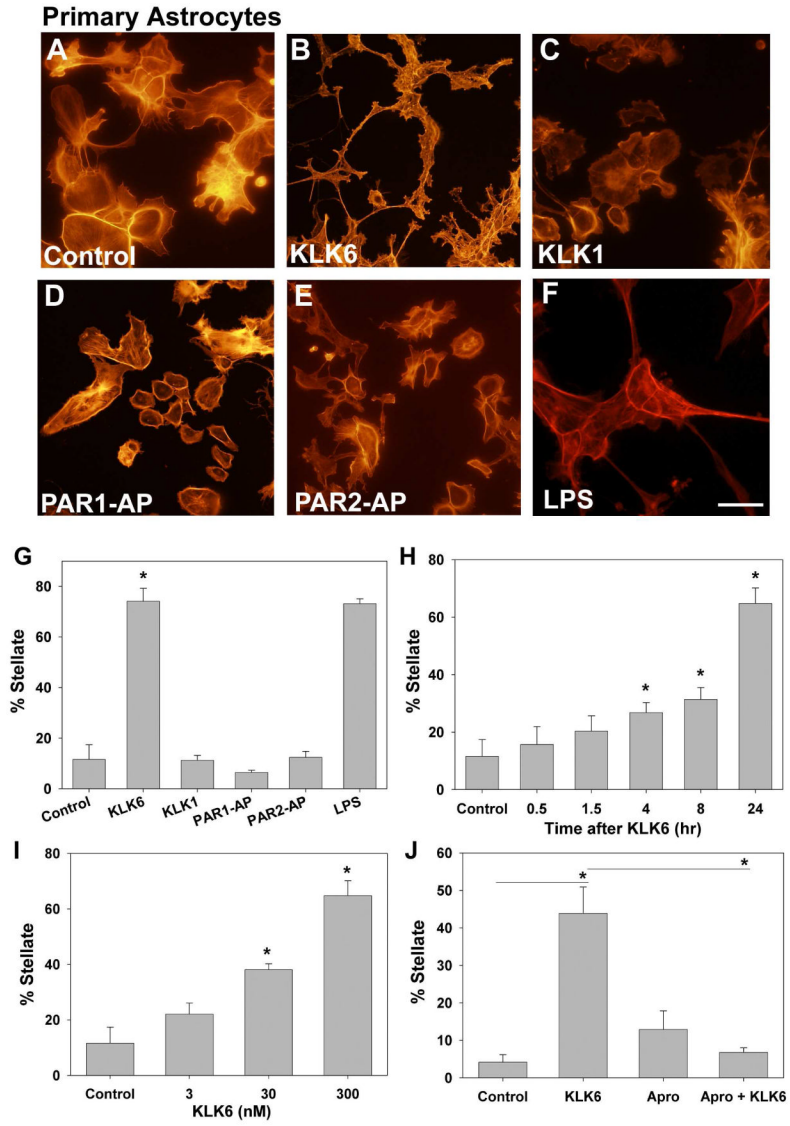

Figure 2. KLK6 promotes stellation of primary murine cortical astrocytes

Primary astrocytes were treated with (A) vehicle alone, (B) KLK6 (300 nM (10 μg/ml)), (C) KLK1 (300 nM (10 μg/ml)), (D) PAR1-AP (40 μM), (E) PAR2-AP (200 μM); or (F) LPS (25 μg/ml) for 24 hr and stained with rhodamine-conjugated Phalloidin to visualize actin. (G) Histogram shows percent stellation induced by each agonist. While KLK6-triggered robust stellation, neither KLK1, PAR1- or PAR2-APs increased stellation above baseline levels. LPS was used as a positive control for astrocyte activation and promoted stellation. (H) KLK6-induced stellation (300 nM) was observed as early as 4 hr after KLK6 treatment. (I) Significant KLK6-mediated stellation effects were seen at both 30 and 300 nM (1 and 10 μg/ml). (J) Aprotinin (Apro) blocked KLK6-induced astrocyte stellation (Asterisks $p<0.05$, SNK). (Scale Bar = 50 μm in A–F)

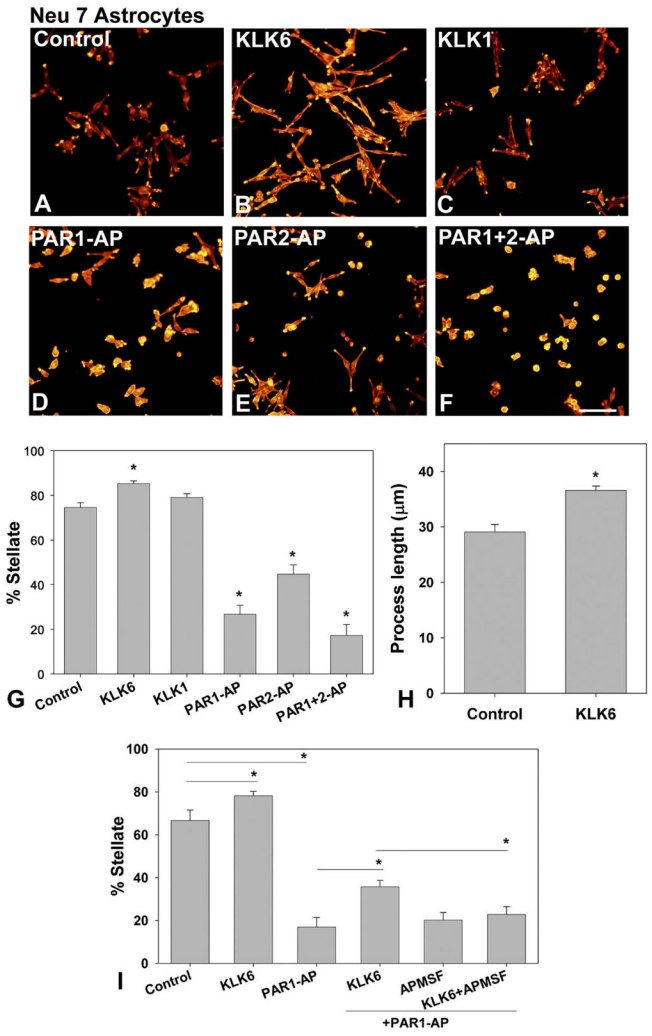

Figure 3. KLK6 promotes stellation of Neu7 astrocytes

The Neu7 astrocyte cell line was treated with (A) vehicle alone, (B) KLK6 or (C) KLK1 each at 300 nM (10 μg/ml), (D) PAR1-AP (40 μM), (E) PAR2-AP (200 μM), or (F) PAR1- in addition to PAR2-APs and stained with rhodamine-conjugated Phalloidin to visualize actin. (G) KLK6 significantly increased stellation above baseline, while treatment with PAR1-, PAR2-, or combined PAR1- and PAR2-APs, caused significant stellation reversal. KLK1 did not significantly alter stellation. (H) KLK6-triggered stellation was associated with a significant increase in astrocyte process length. (I) Less PAR1-AP mediated stellation reversal was observed when cells were concomitantly treated with KLK6. The ability of KLK6 to decrease PAR1-mediated stellation reversal was blocked by the serine protease inhibitor APMSF. (Asterisks $p<0.05$, SNK). (Scale Bar = 100 μm in A-F).

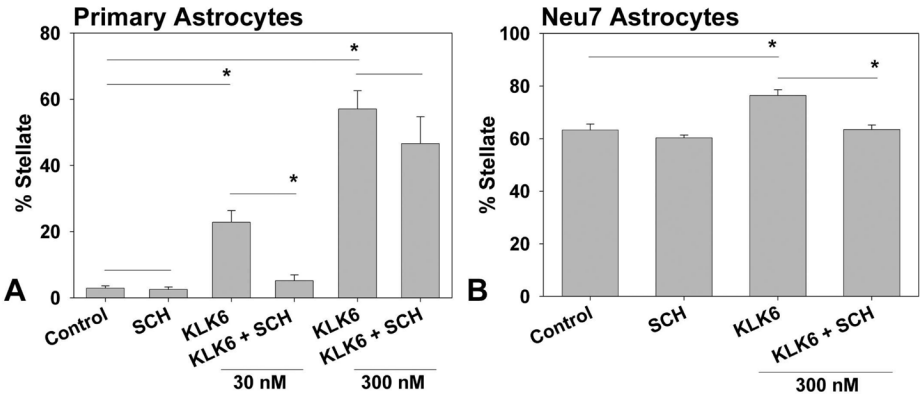

Figure 4. KLK6-induced astrocyte stellation is dependent on PAR1

Histograms show the PAR1 inhibitor SCH79797 (35 nM) blocks astrocyte stellation induced by (A) 30 nM (1 μg/ml) KLK6 in primary astrocytes and (B) 330 nM (10 μg/ml) KLK6 in the Neu7 astrocyte cell line. PAR1-inhibitor mediated reductions in KLK6-stellation seen at higher concentrations of KLK6 in primary astrocytes did not reach the level of statistical significance. (Asterisks $p<0.05$, SNK).

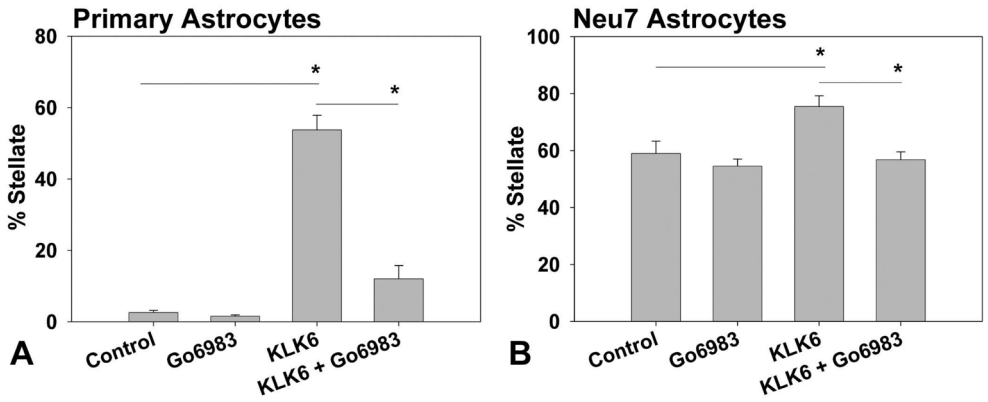

Figure 5. KLK6-induced astrocyte stellation is blocked by inhibition of PKC signaling

Histograms show that the PKC inhibitor Go6983 (60 nM) reduces the ability of KLK6 (300 nM (10 μg/ml)) to induce stellation in (A) primary astrocytes or (B) the Neu7 astrocyte cell line. (Asterisks $p<0.05$, SNK).

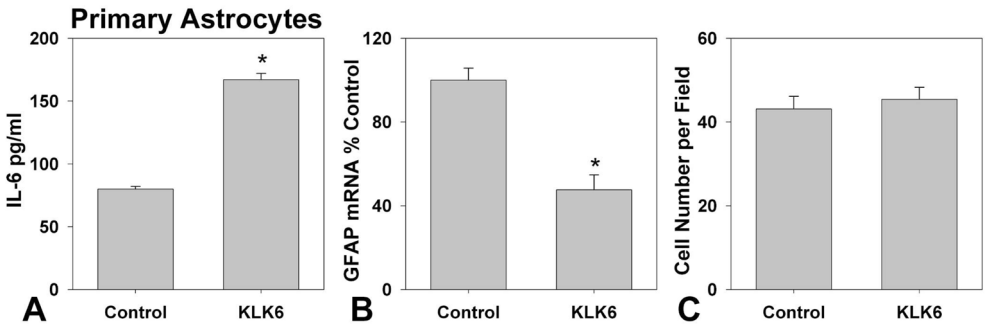

Figure 6. KLK6 promotes secretion of IL-6 but reduces expression of GFAP mRNA in primary murine astrocytes

(A) Treatment of primary astrocytes for 24 hr with KLK6 (300 nM (10 μg/ml)) resulted in a significant increase in IL-6 secretion but a decrease in expression of GFAP mRNA (B). (C) In primary astrocyte experiments (see Figure 2), counts of the mean number of DAPI labeled nuclei reveal no significant impact of KLK6-treatment (300 nM) on cell number at the 24 hr endpoint of each experiment. (Asterisks $p<0.05$, SNK).