Protease-activated receptor (PAR)-1 and PAR-2 protect rat astrocytes from apoptotic cell death via differentially regulating JNK isoform-specific release of the chemokine GRO/CINC-1
- two novel protective pathways in brain -

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1 Introduction

1.1 Protease-activated receptor (PAR)

Protease-activated receptors (PARs), a family of G protein-coupled receptors (GPCRs), are widely expressed in the central nervous system (CNS), including neurons, microglial cells, astrocytes, and oligodendrocytes (Ubl et al., 1998; Striggow et al., 2001; Wang et al., 2002a; Suo et al., 2003; Dai et al., 2004; Wang et al., 2004; Luo et al., 2005). Four members (PAR-1, -2, -3 and -4) of the PAR family have been identified. PAR-1, PAR-3 and PAR-4 are thrombin receptors, while PAR-2 is activated by serine proteases trypsin and mast cell tryptase, etc.

In contrast to the reversible activation of classical GPCRs (Coughlin, 1999), PARs are activated by the irreversible proteolytic cleavage of their N-terminus by serine proteases, such as thrombin, trypsin or tryptase (Fig. 1.1). Thus, a new N-terminus is unmasked acting as a tethered ligand, which comprises at least six amino acids. The unmasked tethered ligand domain can interact with the extracellular loop 2 of the cleaved receptor and thereby initiate multiple signal transductions (Wang and Reiser, 2003; Ossovskaya and Bunnett, 2004). So far, the tethered ligand domains for PAR-1, PAR-2 and PAR-4 in human, rat and mouse and the domain for PAR-3 in human and mouse have been clearly identified (Noorbakhsh et al., 2003) (Fig. 1.1). However, the tethered ligand domain for rat PAR-3 is still unknown. Unlike human and mouse PAR-3 tethered ligand domains, the corresponding position of rat PAR-3 is "E^{37}SFNGNE^{43}", which does not seem susceptible for the cleavage by thrombin. Synthetic peptides that mimic the tethered ligands can also activate the respective receptor, thereby bypassing the requirement for receptor proteolysis. These peptides provide useful tools for evaluating the functions of specific PARs. Activation of PAR-3, however, may vary from this model. Human PAR-3 is not activated by the peptide agonists that mimic the newly exposed amino terminus (Ishihara et al., 1997).

It is well known that PAR has seven transmembrane (TM) helices. Recently, it was reported that human PAR-1 and -2, rat/mouse PAR-1, -2 and -4, but not PAR-3, have the additional 8^{th} helix, which is anchored by Cys-palmitoylate lipids to the inner leaflet of the lipid bilayer (Swift et al., 2006) (Fig. 1.1). Studies on PAR-1 have shown that TM7 interacts with the 8^{th} helix, which in turn interacts with the intracellular loop 1, and this chain of interaction is required for PAR-1-mediated activation of G_q. Therefore, the 8^{th} helix is critical for signal transference between PAR and G protein.

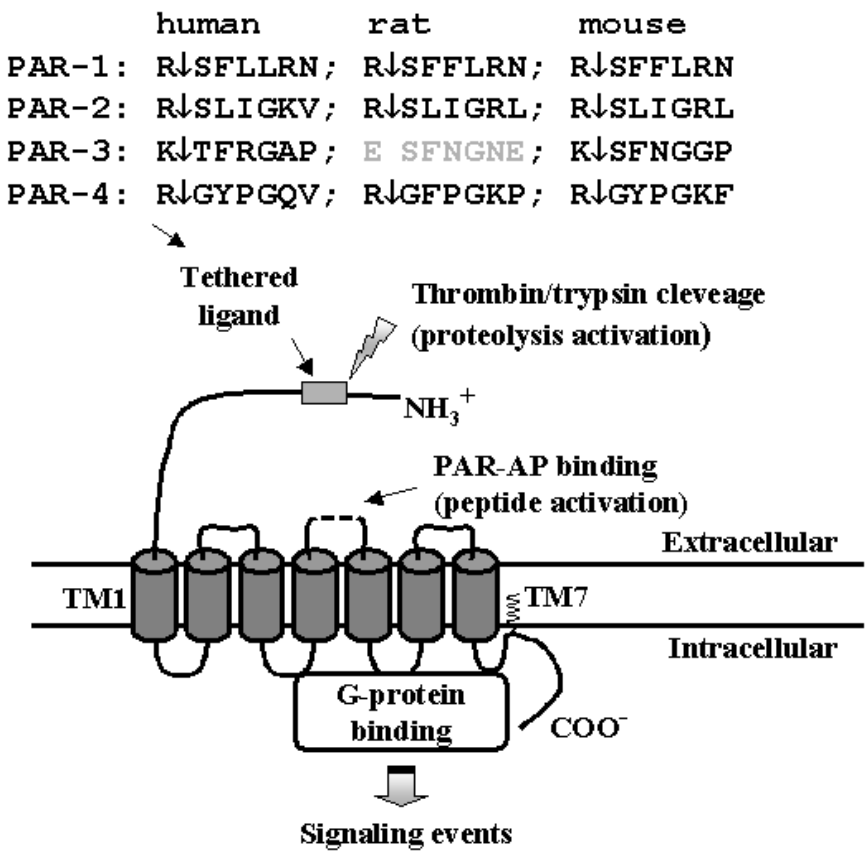


Fig. 1.1. Structure and activation mechanism of PARs [modified from (Wang and Reiser, 2003)]. PAR, a distinct subfamily of GPCR, has 7 TM helix bundles and an additional 8th intracellular C-terminal helix anchored by Cys-palmitoylate lipids to the inner leaflet of the lipid bilayer. Following irreversible proteolytic cleavage of PAR by thrombin or trypsin (depending on the receptor subtype, arrow shows the cleavage site), a new N-terminus is unmasked acting as a tethered ligand (boxed segment). The tethered ligand domains for four different PARs in human, rat and mouse are shown here (Noorbakhsh et al., 2003). However, the tethered ligand of rat PAR-3 is still unknown. Based on human and mouse PAR-3 domains, the corresponding sequence of rat PAR-3 is shown in gray, which cannot be cleaved by thrombin in principle. This tethered ligand can interact with extracellular loop-2 of the receptor (dashed). A synthetic peptide derived from the N-terminal sequence of PAR (PAR-AP) is able to activate the receptor in the absence of protease-mediated cleavage. Other proteases may also act on the extracellular N-terminus, either exposing the tethered ligand to activate PAR or disabling the tethered ligand to inactivate PARs by cleaving at a site downstream of the tethered ligand sequence (Renesto et al., 1997). The intracellular loops 2, 3 and the 8th helix have been proposed to play a pivotal role in G protein coupling for PAR-1 and subsequent signaling transductions (Hollenberg and Compton, 2002; Swift et al., 2006).

Once proteases activate PARs by the irreversible mechanism, the new exposed tethered ligand domain is always available to interact with the cleaved receptor. Therefore, this activation would result in prolonged signaling unless there are efficient mechanisms to attenuate the response. The principle mechanism, which has been supposed to terminate the signaling, is the receptor desensitization following the receptor phosphorylation by either G protein-coupled receptor kinases (GRKs) or second messenger kinases (Ossovskaya and Bunnett, 2004). Unlike classical GPCR, which recycles to the plasma membrane, activated PAR-1 in endothelial cells and fibroblasts is rapidly internalized and sorted to lysosomes for degradation. This trafficking behaviour is critical for termination of thrombin signaling (Coughlin, 1999). So far, some studies focused on the desensitization of PARs revealed that mechanisms of desensitization vary between different PARs, probably due to structural differences, especially in the intracellular loop 3 and the carboxy terminus. However, other critical aspects of PAR desensitization still remain mysterious.

1.1.1 Thrombin

Thrombin, an important serine protease generated from the proenzyme prothrombin, plays an important role in blood coagulation by cleaving fibrinogen into fibrin. Fibrin, unlike soluble fibrinogen, is insoluble and forms a mesh that creates the primary structure of the blood clot. Additional cells in the blood are trapped in the primary structure expanding the clot (Sheehan and Tsirka, 2005). In addition to the critical role in blood coagulation, thrombin has also been shown impressively to act as a multifunctional signaling molecule by activating PAR-1, PAR-3 or PAR-4 in many different types of cells.

Although prothrombin is mainly expressed in the liver, mRNA of prothrombin is also detected in the brain tissue (Dihanich et al., 1991), indicating that thrombin can be produced locally in brain. Recent data further revealed that prothrombin and thrombin are expressed in neural cells at both mRNA and protein levels, and they are associated with both extra- and intracellular neurofibrillary tangles in Alzheimer's disease (AD) and parkinsonism-dementia complex of Guam (Arai et al., 2006). Moreover, injured brain endothelial cells are also able to release thrombin (Grammas et al., 2004). On the other side, blood-brain barrier disruption, which is often associated with ischemic and traumatic brain injury, enables serine protease thrombin to infuse into the brain tissue. Thus, under certain pathophysiological conditions, such as trauma, ischemia and AD, thrombin levels are increased in the brain tissue (Xi et al., 2003; Arai et al., 2006). Consequently, neural PAR-1 is activated by thrombin.

PAR-1 and PAR-3 both contain a hirudin-like site, which binds to an anion binding site on thrombin, thereby increases the efficiency of activation by thrombin (Vu et al., 1991; Ishihara et al., 1997). In contrast to PAR-1 and PAR-3, PAR-4 lacks a hirudin-like binding site for thrombin (Kahn et al., 1998; Xu et al., 1998). Therefore, PAR-4 is a low-affinity receptor for thrombin. Interestingly, murine PAR-3 does not lead to thrombin signaling and instead serves as a cofactor for cleavage and activation of PAR-4 by thrombin (Nakanishi-Matsui et al., 2000). Accumulating evidence has demonstrated that thrombin and its receptor (mainly PAR-1) trigger a variety of effects in neurons and glial cells, as diverse as protective or detrimental.

In the CNS, the role of thrombin has received great attention. Thrombin at high concentration (100 nM) has been reported to cause apoptosis in motor neurons, hippocampal neurons and astrocytes (Donovan et al., 1997; Smirnova et al., 1998; Festoff et al., 2000; Smirnova et al., 2001; Suo et al., 2003). On the other side, thrombin might also protect neurons and astrocytes from insults (Vaughan et al., 1995; Pike et al., 1996; Donovan and Cunningham, 1998; Striggow et al., 2000). Now, it becomes clearer that thrombin at low concentrations is protective in ischemic and hemorrhagic models of stroke (Donovan and Cunningham, 1998; Masada et al., 2000; Striggow et al., 2000; Riek-Burchardt et al., 2002). However, thrombin at high concentrations can be deleterious (Striggow et al., 2000; Xi et al., 2003). Most of the above processes are known to be mediated by PARs, mainly PAR-1. However, for some thrombin-mediated effects it is still questionable whether they are mediated by PARs. It has been revealed that PAR-1 does not seem to be involved in thrombin-induced nitric oxide release from cultured microglia (Ryu et al., 2000). Moreover, it is still not clear whether PARs are involved in hippocampal neurons death in vivo caused by thrombin-induced NADPH oxidase activation in microglia (Choi et al., 2005). Recently, it was pointed out that proteases like thrombin, apart from activating or inactivating PARs, can play a hormone-like signaling role in a variety of cellular settings via non-PAR mechanisms (Hollenberg, 2005). All these data suggest a pivotal role for thrombin as an important pathophysiological modulator in brain.

1.1.2 Trypsin

Trypsin, a member of the serine protease family, was initially isolated from the pancreatic juice of animals. Human pancreas secretes three trypsinogen isoforms: mainly cationic trypsinogen (PRSS1 [protease, serine], pI=4.9) and anionic trypsinogen (PRSS2, pI=6.2), and a minor constituent mesotrypsinogen (PRSS3, pI=5.7), which are activated by

enterokinase in the small intestine (Szmola et al., 2003). Trypsinogen IV, a tissue specific alternatively spliced form of mesotrypsinogen, was originally identified in human brain (Wiegand et al., 1993). Trypsinogen IV and mesotrypsinogen have a common C-terminus of about 234 amino acids, but the amino acid sequences of the N-terminus encoded by the alternative exon 1 are different (Wiegand et al., 1993; Szmola et al., 2003). Therefore, trypsinogen IV lacks a recognizable signal sequence. After enterokinase cleavage (↓) at DDDDK↓I, the mature/active mesotrypsin and trypsin IV display exactly the same amino acid sequence. Brain trypsinogen IV has been proposed to control expression of glial fibrillary acidic protein in astrocytes and to process the amyloid precursor protein (Minn et al., 1998).

Trypsin is able to catalyze the hydrolysis of peptide bonds on the carboxyl side of lysine or argine residues, and thereby activates PAR-2, PAR-4 and PAR-1 as well (Wang et al., 2004). Our recent work has shown that both cationic and anionic trypsin, but not mesotrypsin, activate PAR-2 in HBE (human bronchial epithelial), HEK (human embryonic kidney)-293 and A549 (human pulmonary epithelial) cells (Grishina et al., 2005). The brain trypsin isoform, mesotrypsin (trypsin IV), has been revealed to selectively activate PAR-1, but not PAR-2 in primary rat astrocytes (Wang et al., 2006). The possible involvement of PAR-4 activation by mesotrypsin (trypsin IV) still needs to be investigated further. Alternatively, another group showed that trypsin IV is able to activate PAR-2 and PAR-4 in human epithelial cells (Cottrell et al., 2004). Although it is still controversial whether trypsin IV (mesotrypsin) activates PAR-1 or PAR-2, PAR activation by trypsin IV might play a role in brain.

Although cationic and/or anionic trypsin isoforms could be the main physiological agonists of PAR-2, they are not present in brain. P22, purified from rat brain slices, is a trypsin-like serine protease (Sawada et al., 2000). It can degrade matrix and signal to cells by activating PAR-2. More interestingly, the secretion of P22 appears to be enhanced after mechanical brain injury. Therefore, P22 could be a good candidate for neural PAR-2 activation.

1.1.3 Tryptase

Tryptase, which is the most abundant protease of mast cells, is a tetrameric neutral serine protease with a molecular weight of 134 kDa (Caughey, 1990; Payne and Kam, 2004). Two main types of mast cell tryptase, α-tryptase and β-tryptase, which have

approximately 90% sequence identity, have been identified. α-tryptases contain αI- and αII-tryptases, whereas β-tryptases are classified into βI-, βII- and βIII-tryptases (Schwartz, 2001; Soto et al., 2002; Peng et al., 2003). α-Protryptase is secreted constitutively from mast cells as an inactive proenzyme and is the major form of tryptase found in the blood of normal subjects. In contrast, βII-tryptase is stored in the secretory granules of mast cells (Ren et al., 1998). The tetrameric structure of β-tryptase, where the active site of each of the four monomers is orientated towards the inner face of a central pore (Fajardo and Pejler, 2003), makes the protease resistant to inactivation by biological inhibitors of serine proteases, such as α-protease inhibitor, α2 macroglobulin and aprotinin (Ren et al., 1998). β-Tryptase can activate the PAR-2 (Nystedt et al., 1994; Kawabata and Kuroda, 2000), but is considerably less potent than trypsin (Payne and Kam, 2004).

It is well known that human PAR-2 possesses two potential *N*-linked glycosylation sequons, one on the extracellular N-terminus ($N^{30}R^{31}S^{32}$) and the second on the receptor extracellular loop 2 ($N^{222}I^{223}T^{224}$). Glycosylation can directly regulate PARs functions (Compton et al., 2001; Compton et al., 2002). PAR-2 activation induced by tryptase is apparently reduced by glycosylation in close proximity to the cleavage/activation site of PAR-2 (Compton et al., 2001). However, inhibition of glycosylation at the N-terminus either by tunicamycin treatment or mutation (N30A) can enhance the ability of tryptase to activate hPAR2. Therefore, glycosylation is likely to mask human PAR-2 from tryptase. This might be one possible reason for the low potency of tryptase on PAR-2 activation.

During inflammation, tryptase can stimulate the release of granulocyte chemoattractant interleukin (IL)-8, and up-regulate expression of intercellular adhesion molecule-1 (ICAM-1) on epithelial cells (Cairns and Walls, 1996). Similar to the serine protease thrombin, the level of tryptase in the brain tissue is also elevated after brain injury. Previously, it has been shown that the number of mast cells within the CNS is significantly increased in multiple sclerosis (MS) and after trauma (Rozniecki et al., 1995; Lozada et al., 2005). Tryptase, which is released by activated mast cells, is also significantly elevated in brain under such pathological conditions. Therefore, tryptase is another potent activator of PAR-2 in the brain tissue (Sawada et al., 2000; Noorbakhsh et al., 2003).

1.1.4 Expression of PARs in brain

The tissue distribution of the four PAR members in brain has been extensively investigated. Using peroxidase-linked immunohistochemical techniques, our group has found that PAR-2, PAR-3, PAR-4 and to a lesser extent PAR-1 are widely distributed in rat brain tissue (Striggow et al., 2001). PAR-1 is abundant in the hippocampus, particularly in the pyramidal cell layers of the CA2 and CA3 region, and low-level expression is seen in cortex, thalamus, hypothalamus, striatum and amygdala. In addition, another group also showed that PAR-1 mRNA is widespread but with low intensity in the late embryonic and early postnatal nervous system, and becomes more pronounced in adult animals by *in situ* hybridization (Niclou et al., 1998). The abundant expression of PAR-2 and PAR-3 is observed in all cortical layers, hippocampus, the medial habenular nucleus, the central amygdala, ventral thalamus, hypothalamus and striatum. Similarly, the expression pattern of PAR-4 in rat brain is clear in the hippocampus, all cortical layers, thalamus, hypothalamus and amygdala (Striggow et al., 2001).

The brain contains neurons, astrocytes, oligodendrocytes and microglia. The expression of the four types of PARs in these diverse cell types has been intensively studied by different groups. Data from our laboratory show that all four PARs are abundantly expressed in primary rat astrocytes (Wang et al., 2002a). By reverse transcription-polymerase chain reaction (RT-PCR) and immunofluorescence, we show that PAR-1 and to a lesser extent PAR-2 are expressed in the rat retinal ganglion cell line RGC-5 and both receptors initiate pronounced calcium responses upon specific PAR-1 and PAR-2 activating peptide (AP) stimulation (Luo et al., 2005). In addition, functional expression of PAR-1 is also detected in primary rat oligodendrocytes and OLN-93 cells, an oligodendrocyte cell line derived from rats (Wang et al., 2004). However, PAR-3, which is expressed in OLN-93 cells, exhibits no apparent functional activity. Functional expression of PAR-1 and PAR-4 in primary mouse microglial cells and the N9 microglial cell line has been well addressed by RT-PCR, western blot, immunofluorescence and calcium imaging (Suo et al., 2002; Suo et al., 2003). No expression of any types of PARs is detected in another N11 microglial cell line (Wang, our unpublished observations). Similar to observations in rats and mice, PAR-1 mRNA is also expressed in human neurons and astrocytes, but not in human oligodendrocytes and microglia (Junge et al., 2004; Ishida et al., 2006). In addition, functional human PAR-2 is detected in primary human astrocytes by us and also by other groups (Junge et al., 2004; Luo et al., 2006). Neither PAR-3 nor PAR-4 mRNA is expressed in human neurons, astrocytes, oligodendrocytes or microglia (Ishida et al., 2006). Besides these main cell types, the brain also contains capillary endothelial

cells. It has been established that primary cultures of rat brain capillary endothelial cells express PAR-1, PAR-2 and PAR-3 mRNA (Bartha et al., 2000).

Various pathological situations have been shown to modulate the expression of PARs in brain. Recently, it was found that the number of astrocytes expressing PAR-1 is increased in substantia nigra pars compacta of Parkinson's disease (PD) brains (Ishida et al., 2006). During human immunodeficiency virus (HIV) encephalitis, the expression of PAR-1 at both mRNA and protein levels is upregulated in human astrocytes (Boven et al., 2003). These data indicate that PAR-1 activation may contribute to brain inflammation and neuronal damage (Junge et al., 2003). In addition, up-regulation of PAR-2 is observed in neurons in conjunction with neuroinflammation in the brain tissue from patients with HIV-1-associated dementia (Noorbakhsh et al., 2005). Following administration of trimethyltin, PARs (in particular PAR-1 and to a lesser extent PAR-2, PAR-3 and PAR-4) are upregulated in reactive hippocampus astrocytes (Pompili et al., 2004). Similarly, the data from our laboratory also showed that all four subtypes of PAR are upregulated after optic nerve crush (Rohatgi et al., 2003).

Different ischemic experimental conditions have been identified to differentially regulate the expression of PARs in brain. Our previous data showed that the expression of PAR-1 and PAR-3 is increased in hippocampal slices after exposure to severe experimental ischemia (oxygen-glucose deprivation, OGD) (Striggow et al., 2001). On the other side, we showed that transient focal ischemia induced by microinjection of endothelin near the middle cerebral artery results in PAR-1 and PAR-2 downregulation (Rohatgi et al., 2004b). However, PAR-4 mRNA level is increased 12 h after ischemia. Unlike the three other PARs, PAR-3 is upregulated transiently and then downregulated after transient focal ischemia (Rohatgi et al., 2004b). Recently, it was shown that focal ischemia induces expression of PAR-1 and PAR-3 on microglia and enhances PAR-4 labeling in the penumbra (Henrich-Noack et al., 2006). However, there is also evidence that the expression of PAR-1, PAR-2 and PAR-3 is not altered after transient global ischemia (Riek-Burchardt et al., 2002).

1.1.5 Physiological roles of PARs

1.1.5.1 General functional significances of PARs

Since four types of PARs were cloned, the physiological and pathological significances of PARs have been intensively studied in different systems. A growing body of evidence indicates that PARs play pivotal roles in platelet aggregation (Hung et al.,

1992; Derian et al., 2002; Kuliopulos and Covic, 2003), inflammation and pain (Cirino et al., 2000; Cocks and Moffatt, 2000; Vergnolle et al., 2001; Asokananthan et al., 2002; Derian et al., 2002; Temkin et al., 2002; Cottrell et al., 2003; Ruf, 2003; Suo et al., 2003; Vergnolle, 2003; Syeda et al., 2006), proliferation (Suo et al., 2002; Wang et al., 2002b; Sorensen et al., 2003), morphologic changes (Majumdar et al., 1999; Mahajan et al., 2000; Pai et al., 2001; Wang and Reiser, 2003), cell death (Donovan et al., 1997; Festoff et al., 2000; Striggow et al., 2000; Smirnova et al., 2001; Suo et al., 2003; Flynn and Buret, 2004) and cell survival (Vaughan et al., 1995; Pike et al., 1996; Donovan and Cunningham, 1998; Smirnova et al., 2001). So far, the roles of PARs in platelet aggregation in the cardiovascular system, inflammation in the airway and skin, modulation of the gastric functions in the gastrointestinal system have been well addressed (Macfarlane et al., 2001; Nishikawa and Kawabata, 2003; Vergnolle, 2003; Ossovskaya and Bunnett, 2004). Here, we will emphasize the roles of PARs in the CNS.

1.1.5.2 Functions of PARs in the CNS

Increasing evidence over the recent years has demonstrated that PAR-1, the main subtype of PARs, plays an important role in brain (Rohatgi et al., 2004a). We have shown previously that PAR-1 regulates astrocyte proliferation via both G_i/G_o-mediated phosphatidylinositol 3-kinase (PI3K)-extracellular signal-regulated kinase (ERK) 1/2 pathway and G_q-mediated phospholipase C (PLC)/Ca^{2+}/protein kinase C (PKC) pathway (Wang et al., 2002b). Recently, it was confirmed that thrombin via PAR-1 triggers astrogliosis after brain injury via ERK activation (Nicole et al., 2005). On the other side, another group showed that activation of the microglial PAR-1 results in a rapid cytosolic free Ca^{2+} increase and transient activation of both p38 and ERK1/2. Moreover, PAR-1 activation also contributes to thrombin-induced microglial proliferation (Suo et al., 2002). However, the relationship between the mitogen-activated protein kinase (MAPK) activation and microglial proliferation has not been described in this study. Nevertheless, these data suggest that PAR-1 plays an important role in glial proliferation.

Apart from the involvement in glial proliferation, PAR-1 activation by thrombin can also reverse the stellate morphology of cultured rat astrocytes to flat, epithelial shape and cause retraction of processes in neuronal cells (Gurwitz and Cunningham, 1988; Cavanaugh et al., 1990; Beecher et al., 1994). The small GTP-binding protein, Rho, has been implicated to play a central role in this process and thereby modulates neuron-glia interactions during the remodeling of neuronal tissues (Suidan et al., 1997). Further studies

suggest that heat shock protein 90 (Hsp90) binds to PAR-1 C-tail and mediates PAR-1-induced morphological changes (Pai et al., 2001).

One of the most striking consequences of PAR-1 activation caused by thrombin (50 pM, 1 h) or PAR-1AP is to protect hippocampal neurons in brain slices and cultured astrocytes from cell death in response to experimental ischemia (OGD), hypoglycemia or oxidative stress or β-amyloid aggregation (Vaughan et al., 1995; Pike et al., 1996; Striggow et al., 2000). On the other side, there are also evidences that PAR-1 activation induced by thrombin at high concentrations results in neurons and astrocytes death (Donovan et al., 1997; Striggow et al., 2000). Interestingly, data from Cunningham's group suggest that both beneficial and toxic effects of thrombin in astrocytes and hippocampal neurons are mediated through similar signaling machineries, with the involvement of tyrosine kinases, serine/threonine kinases and the actin cytoskeleton (Donovan et al., 1997; Donovan and Cunningham, 1998). They suggested that the cell death and protective pathways may share initial signaling proteins, but differences in the amplitude as well as the duration of the signal may result in different final consequences. ERK1/2 has also been shown to play an important role in mediating the PAR-1-induced neuroprotective effect (Jiang et al., 2002; Xi et al., 2003). However, the roles of p38 MAPK and c-Jun N-terminal kinase (JNK) have not yet been identified in this context.

PAR-1 activation induced by activated protein C (APC) has been reported to protect mouse cortical neurons from N-methyl-D-aspartate- (NMDA) and staurosporine-induced apoptosis (Guo et al., 2004), and block p53-mediated apoptosis in ischemic human brain endothelium (Cheng et al., 2003). Recently, it was found that PAR-1 activation by thrombin treatment remarkably increases levels of glutathione peroxidase in human astrocytes, which further protects neurons from the toxicity of thrombin at the high concentration (Ishida et al., 2006). However, PAR-1 activation does not alter the expression of nerve growth factor and inflammatory cytokines/chemokines (IL-1β, IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1)) in human astrocytes. These data suggest that activation of glial PAR-1 might play a role in protecting neurons from external or internal insults causing neuronal cell death, and is a restorative move of the brain to delay or block the pathological progression.

Although the inflammatory role of PAR-1 has been well addressed in many other systems, there are only few studies implicating that PAR-1 might also play a role in inflammation in brain. Previously, it was shown that cytosolic phospholipase A2 (cPLA2) participates in thrombin-induced arachidonic acid release in a human astrocytoma cell line

(Hernandez et al., 1997; Hernandez et al., 2000) and in primary rat astrocytes (Sergeeva et al., 2002). Thrombin- or PAR-1AP-induced PAR-1 activation not only significantly increases mRNA expression of IL-6, IL-1β and tumor necrosis factor (TNF)-α in rat C6 glioma cells (Fan et al., 2005), but also elevates NO produced by astroglial inducible nitric oxide synthase (iNOS) (Meli et al., 2001). Moreover, PAR-1 activation upregulates microglial CD40 expression, which indicates that PAR-1 might play an inflammatory role in microglial activation.

Very little is known about the physiological and pathophysiological importance of PAR-2 in brain compared to that of PAR-1. It was shown that PAR-2 is present in the rat hippocampus and associated with neuronal degeneration (Smith-Swintosky et al., 1997). Enhanced PAR-2 expression in neurons in conjunction with neuroinflammation in the brain tissue from patients with HIV-1-associated dementia is thought to rescue neuronal cells (Noorbakhsh et al., 2005). In addition, it was also found that the deficiency of the PAR-2 gene increases the acute ischemic cerebral injury associated with suppression of neuronal ERK activation and reactive astroglial activation (Jin et al., 2005). These data imply that PAR-2 might be involved in neuroprotection. On the other side, PAR-2 was also shown to modulate neuroinflammation in experimental autoimmune encephalomyelitis and MS (Noorbakhsh et al., 2006). So far, the functional significance of PAR-2 in brain still needs to be further investigated.

PAR-3 and PAR-4 are two other thrombin receptors (Ishihara et al., 1997; Kahn et al., 1998; Xu et al., 1998). There is evidence that murine PAR-3 functions as a cofactor for PAR-4 activation by thrombin (Nakanishi-Matsui et al., 2000). However, the expression of PAR-3 in different cells, either alone in oligodendrocytes (Wang et al., 2004) and cortical neurons (our unpublished data) or in combination with PAR-4 in astrocytes (Wang et al., 2002a), suggests that PAR-3 might play a functional role in the CNS. PAR-3 has been reported to be involved in the protective action of APC, which protects mouse cortical neurons from NMDA- and staurosporine-induced apoptosis (Guo et al., 2004).

On the other side, PAR-4 might play an inflammatory role in microglial cells. Activation of PAR-4 mediates TNF-α release from microglial cells via ERK1/2 and NF-κB activation (Suo et al., 2003). Although the wide expression of PAR-3 and PAR-4 both has been observed in the CNS (Striggow et al., 2001), the functional roles of PAR-3 and PAR-4 still remain largely unknown.

1.2 MAPK family

MAPKs, a family of serine/threonine protein kinases, mediate diverse fundamental biological processes and cellular responses to external stress signals, including PAR-induced multiple signal transductions. The best-characterized members of this family are the ERK and the two ‘‘stress-responsive’’ MAPK subfamilies, namely, the p38 MAPK and the c-Jun N-terminal kinases (JNKs), also called stress-activated protein kinase (SAPK) (Sugden and Clerk, 1998). The ERK, p38 and JNK all are activated by dual phosphorylation of a tripeptide motif (Thr-X-Tyr), Thr-Glu-Tyr, Thr-Gly-Tyr and Thr-Pro-Tyr, respectively (Davis, 2000). These three MAPK members have been identified to play a major role in regulating complex intracellular processes such as proliferation, inflammation, cell survival, differentiation or death. Besides these three best characterized MAPK members, this family also includes many other members, such as ERK3, ERK5, etc.

The classical MAPK cascades are organized in a three-kinase architecture consisting of a MEK activator (MEK kinase [MEKK] or MAPK kinase kinase [MAPKKK]), a MAPK activator (MEK, MKK or MAPK kinase [MAPKK]) and a MAPK. MAPKs are proline-directed protein kinases. They are able to phosphorylate serine or threonine residues that are neighbours to proline. Thus, MAPKs activate numerous protein kinases, nuclear proteins and transcription factors, leading to downstream signal transduction (Cuschieri and Maier, 2005). In mammalian systems five MAPK modules have been identified so far (Fig. 1.2).

ERK, the first identified MAPK family member, contains two isoforms, ERK1 (p44) and ERK2 (p42), and is commonly referred to as ERK1/2 (Cuschieri and Maier, 2005). ERK1/2 activation is initiated by the phosphorylation of Raf (MAPKKK) and MEK1/2 (MAPKK) (Kolch, 2000). Raf is a highly conserved kinase that is activated by the small guanosine triphosphate (GTP)-binding protein, Ras. Ras is activated through interaction with Grb2-SOS complex, where SOS catalyzes the formation of Ras-GTP complex. This GTP-bound complex binds to Raf and activates it in a calcium-dependent manner (Cuschieri et al., 2002). However, several lines of evidence so far suggest that enzymatic activation of kinase in a given MAPK cascade may not be sufficient for successful propagation of a specific signal. Therefore, additional factors like adapters or scaffolds are involved in facilitating the assembly of enzyme-substrate complexes. MP1 (MEK Partner 1) is identified to bind specifically to MEK1 and ERK1 and facilitates their activation. When overexpressed in cultured cells, MP1 enhances activation of both ERK1

and the genes driven by the transcription factor E-26-like protein 1 (Elk-1) (Schaeffer et al., 1998). p14 functions as an adaptor protein, which is necessary and sufficient to localize MP1 to endosomes. Reduction of MP1 or p14 protein levels by siRNA results in defective signal transduction (Teis et al., 2002). So far, it has been identified that ERK plays a pivotal role in regulating proliferation, survival and differentiation (Robinson and Cobb, 1997; Lewis et al., 1998; Wang et al., 2002b; Song et al., 2005).

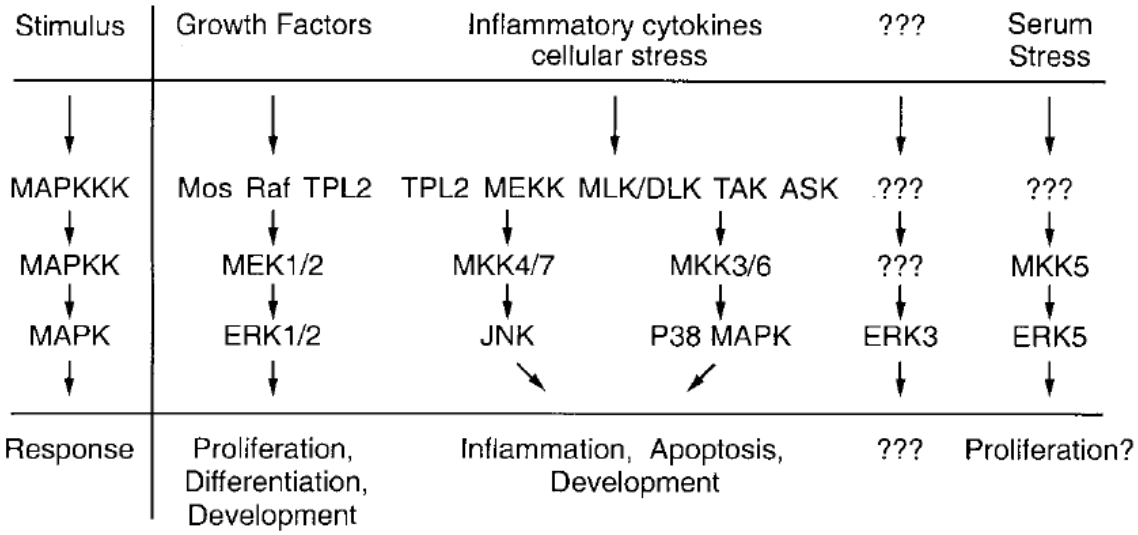


Fig. 1.2. Schematic overview of MAPK modules (Schaeffer and Weber, 1999). Mammalian MAPK modules regulate cell growth, differentiation, stress responses, and development.

The p38 kinase, which is activated in response to physiological stress, endotoxin, osmotic stress and ultraviolet exposure (Raingeaud et al., 1995), contains five isoforms: p38α (SAPK2), p38β, p38β2, p38γ (SAPK3) and p38δ. Expression of these isoforms varies among different tissues. Only p38α, p38β and p38β2 are expressed in brain with high levels (Jiang et al., 1996). MKK3 and MKK6 are two major upstream activators of p38 MAPK (Derijard et al., 1995; Lin et al., 1995; Raingeaud et al., 1996). In addition, MKK4 can also phosphorylate p38 (Guan et al., 1998; Ohtsuka and Zhou, 2002). Subsequently, p38 activation phosphorylates the activating transcription factor (ATF)-2 and ELK-1, and thus regulates the induction of c-Jun and c-fos, which might contribute to activator protein-1 (AP-1) activity (Hazzalin et al., 1996). In addition, p38α and p38β are also able to phosphorylate two other homologous protein kinases, MAP kinase-activated protein kinase 2 and 3 (MAPKAP-K2 and MAPKAPK3) (Kumar et al., 1997), which can

further activate the small Hsp-25/27 to increase the cytoprotective activity, an action that involves the stabilization of the actin cytoskeleton (Lavoie et al., 1995). Besides Hsp27, MAPKAP-2 activates cAMP response element binding protein (CREB) as well, which regulates c-fos induction (Tan et al., 1996).

It has been shown that p38 plays a pivotal role in neuronal cell death (Xia et al., 1995; Kummer et al., 1997). The p38, which is activated by nerve growth factor (NGF) withdrawal in PC12 cells, contributes to NGF-caused apoptosis (Kummer et al., 1997). It was also shown that p38 activity is increased in cerebellar neurones upon deprivation of the high concentration of potassium required for survival (Harada and Sugimoto, 1999). On the other side, previous data showed that inhibition of either NGF-mediated p38 activation or its downstream effector MAPKAPK activation eliminates NGF-induced CREB phosphorylation and neurite outgrowth, indicating p38 might be involved in neurite outgrowth (Xing et al., 1998). Although p38 is predicted to act as a stress kinase involved in apoptosis and inflammation, so far little is known about the function and activation of p38 in the CNS under physiological conditions.

The JNK kinases are encoded by three different genes, namely JNK1, JNK2 and JNK3. JNK1 and JNK2 are ubiquitously expressed in most tissues, whereas JNK3 is selectively expressed in the nervous system and with low levels in the heart and testis (Kyriakis and Avruch, 2001; Waetzig and Herdegen, 2005). Alternative splicing of the genes yields four JNK1 sub-isoforms, four JNK2 sub-isoforms and two JNK3 sub-isoforms (Gupta et al., 1996; Waetzig and Herdegen, 2005). All ten JNK isoforms are expressed in human adult brain (Gupta et al., 1996). As shown in Fig. 1.3, alternative splicing at the C-terminus generates JNK isoforms with 46 kDa and 54 kDa. To date, the functional differences ascribed to these isoforms remain to be determined.

Similar to two other MAPK members, ERK and p38, JNK is activated by dual phosphorylation on both Thr and Tyr residues (Gupta et al., 1996; Xie et al., 1998; Lisnock et al., 2000). The immediate upstream activators of JNK are the MKK4 and MKK7, known alternatively as the JNKK1 and JNKK2. MKK4 and MKK7 both are able to phosphorylate JNK on Thr and Tyr. However, MKK4 appears to preferentially phosphorylate JNK on Tyr, whereas MKK7 preferentially phosphorylates Thr (Lawler et al., 1998), suggesting that MKK4 and MKK7 may cooperate to activate JNK under some circumstances. In addition, the specificity of MKK4 and MKK7 in substrate is significantly different. MKK4, but not MKK7, can also activate p38 MAPK. Further upstream from the MKKs lie other kinases, such as mixed lineage kinases (MLKs) including MLK1-3, MEKKs including MEKK1-4,

apoptosis-regulating-signal kinase (ASK)1-2, dual leucine zipper kinase (DLK), leucine zipper-bearing kinase (LZK), TGFbeta-activated kinase-1 (TAK1) and tumour progression locus-2 (Tpl-2) (Lin et al., 1995; Tibbles et al., 1996; Davis, 2000; Barr and Bogoyevitch, 2001). Activated JNKs can further phosphorylate many substrates, including the transcription factors c-Jun (Derijard et al., 1994; Kyriakis et al., 1994), ATF2 (van Dam et al., 1995), ELK-1 (Whitmarsh et al., 1995), nuclear factor of activated T cells (NFAT) (Chow et al., 1997), as well as tumor suppressor p53 (Hu et al., 1997; Zhang et al., 1998). It has been known that JNKs are involved in numerous physiological or pathological processes, including proliferation (Sabapathy et al., 2004; Sabapathy and Wagner, 2004), differentiation (Leppa et al., 1998; Park et al., 2004; Tawadros et al., 2005) and cell death (Yang et al., 1997; Morishima et al., 2001; Keramaris et al., 2005).

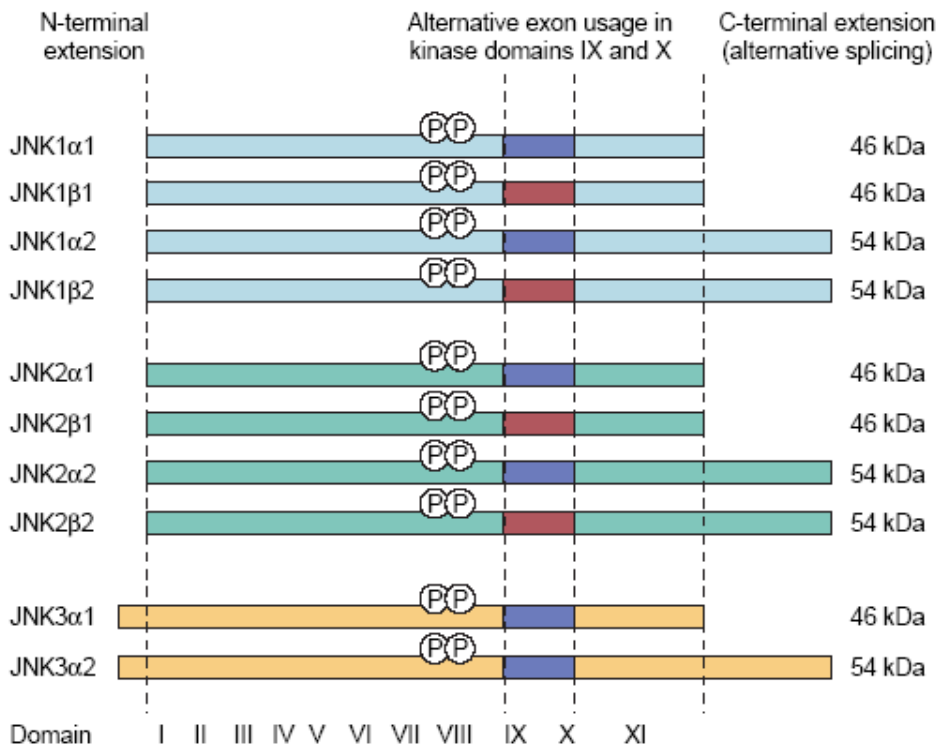


Fig. 1.3. The ten splice variants of JNK (Waetzig and Herdegen, 2005). Alternative splicing at the C-terminus generates isoforms of 46 kDa and 54 kDa. Unlike JNK1 and JNK2 isoforms, the gene that encodes JNK3 is expressed only as the α form and contains as extended N-terminal region. P indicates phosphorylation sites.

Increasing evidence shows that distinct JNK isoforms might have similar or completely different functions, depending on the physiological or pathological context. JNK2 has been identified as the main contributor for the c-Jun activation induced by lipopolysaccharide (LPS) in microglia and mediates pro-inflammatory actions (Waetzig et al., 2005). Moreover, JNK2 can mediate TNF-induced cell death in mouse embryonic fibroblasts (Dietrich et al., 2004). However, there is also evidence that JNK1, but not JNK2, which is activated by TNF-α and UV, is required for c-Jun activation and apoptosis in mouse fibroblasts (Liu et al., 2004). JNK3 appears to have an important role for ischemic apoptosis (Kuan et al., 2003). Transgenic knockouts reveal that single knockouts of *jnk1*, *jnk2* or *jnk3* and double mutants of *jnk1*/*jnk3* or *jnk2*/*jnk3* do not show clear structural abnormalities. However, a double knockout of *jnk1* and *jnk2* genes leads to embryonic lethality (Kuan et al., 1999). To date, the precise roles of each JNK isoform still remain largely unknown.

1.3 Proinflammatory cytokines and chemokines

Cytokines are pleiotropic molecules that are involved in immune responses, inflammation and angiogenesis. Previously, it was shown that thrombin via PAR-1 activation significantly enhances the release of IL-1β and IL-6 in human mononuclear cells (Naldini et al., 2002). In the CNS, normally IL-6 levels remain low. However, elevated expression occurs during injury, infection, stroke and inflammation. The physiological function of IL-6 within the CNS is complex. IL-6 can exert neurotrophic and neuroprotective effects, and can also function as a mediator of inflammation, demyelination and astrogliosis, depending on the cellular context (Van Wagoner and Benveniste, 1999). TNF-α, another important proinflammatory cytokine, is produced locally in the CNS via PAR-4 activation (Suo et al., 2003). Like TNF-α, IL-1β can also be produced in the CNS from microglia, astrocytes, neurons and endothelium (Rothwell, 1991). After brain injury, the levels of IL-1β are increased (Minami et al., 1991; Akiyama et al., 2000; Fan et al., 2005).

Chemokines form a group of more than 50 relatively small proteins (8-14 kDa) that are shown to have a role in inflammation in the CNS, and are thought to be involved in pathological conditions including MS and AD (Hesselgesser and Horuk, 1999; Mennicken et al., 1999; Xia and Hyman, 1999). Based on the N-terminal cystein motif, the

chemokines are classified into four groups: CXC, CX3C, CC and C (Murdoch and Finn, 2000). Rat chemokine growth-regulated oncogene/cytokine-induced neutrophil chemoattractant (GRO/CINC)-1, the member of the C-X-C family, is a counterpart of the human GRO and IL-8, which are not produced in rat (Ramos et al., 2003). GRO/CINC-1 is expressed by inflammatory cells at the site of inflammation and is involved in neutrophil infiltration (Watanabe et al., 1989). In ischemic brain areas, GRO/CINC-1 is transiently increased and mediates granulocyte infiltration into the brain in response to focal ischemia in rats (Yamasaki et al., 1995). CXCR2, which is a unique receptor for GRO/CINC-1, has also been shown to be increased in AD in a subpopulation of neuritic plaques (Xia and Hyman, 2002). Thus, GRO/CINC-1 seems to play an important role in inflammatory processes in brain. In addition, GRO/CINC-1 has been found to exhibit trophic and mitotic effects on oligodendrocyte precursors (Robinson et al., 1998; Wu et al., 2000). There is also evidence that GRO/CINC-1 is a potent trigger for tau hyperphosphorylation (Xia and Hyman, 2002), indicating a pathophysiological role of GRO/CINC-1 in neurodegenerative diseases.

1.4 Ceramide and brain injury

Ceramide, a membrane sphingolipid, now is also viewed as an important second messenger that regulates several cellular processes, including differentiation, proliferation and apoptosis. So far, the role of ceramide specifically in apoptosis has attracted much attention in recent years. It has been applied as a cell injury model to induce apoptosis in numerous studies. The exogenous addition of the cell-permeable ceramide analogue C_2-ceramide mimics the ceramide-induced apoptosis in a variety of cells. However, the naturally occurring ceramide precursor dihydroceramide does not induce apoptosis. Therefore, to investigate the protective effects of PAR activation in neural cells, we might use C_2-ceramide to mimic cell injury conditions and dihydroceramide as a negative control in the present study.

Ceramide is comprised of an N-acylated (14-26 carbons) sphingosine (18 carbons). Carbons 1-5 of the sphingosine backbone are biologically important and consist of hydroxyl groups at C1 and C3, a trans double bond across C4 and C5, and an amido group that serves as the fatty acyl linkage at C2 (Fig. 1.4) (Pettus et al., 2002). Ceramide can be synthesized in the plasma membrane, endoplasmic reticulum (ER), Golgi, nucleus membranes and mitochondrial membranes by multiple different pathways. Because of the

hydrophobic property, ceramide tends to remain within the membrane bilayer and may exert its function exclusively at the subcellular site of production.

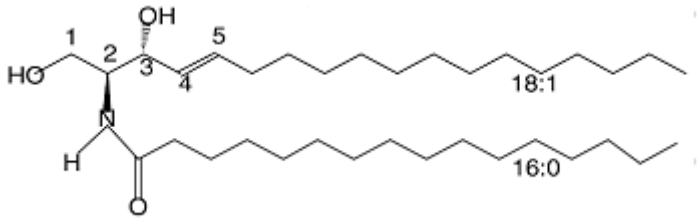


Fig. 1.4. General structure of ceramide.

Increasing evidence shows that a number of different enzymatic mechanisms exist for the production of ceramide (Kolesnick and Kronke, 1998). The ceramide metabolic pathways are summarized in Fig. 1.5. First, the catabolic pathway for ceramide involves the action of sphingomyelinases (SMases), which hydrolyze the sphingomyelin yielding ceramide and phosphorylcholine. SMases are classified into acid SMases and neutral SMases. Acid SMases are localized mainly in lysosomes and have optimal enzymatic activity at about pH 4.5-5. Neutral SMases have optimal activity at a neutral pH and are mainly located in the plasma membrane, cytosol, ER or nuclear membranes (Siskind, 2005). SMases are considered as principal pathways for the production of ceramide in early signal transduction. Second, ceramide can be synthesized de novo by condensation of serine and palmitoyl-CoA, which is subsequently reduced to dihydrosphingosine. The acylation of dihydrosphingosine to dihydroceramide is catalyzed by ceramide synthase. Ceramide is eventually formed by the action of dihydroceramide reductase. This process, which occurs in the ER and mitochondria (Mandon et al., 1992; Bionda et al., 2004), is another important pathway for ceramide generation. Third, ceramide can also be generated by breakdown of complex glycosphingolipids through acid hydrolases. Finally, ceramide is also subject to extensive modifications. Ceramide can be converted to ceramide-1-phosphate and can also be glycosylated by glucosylceramide synthase in the Golgi apparatus.

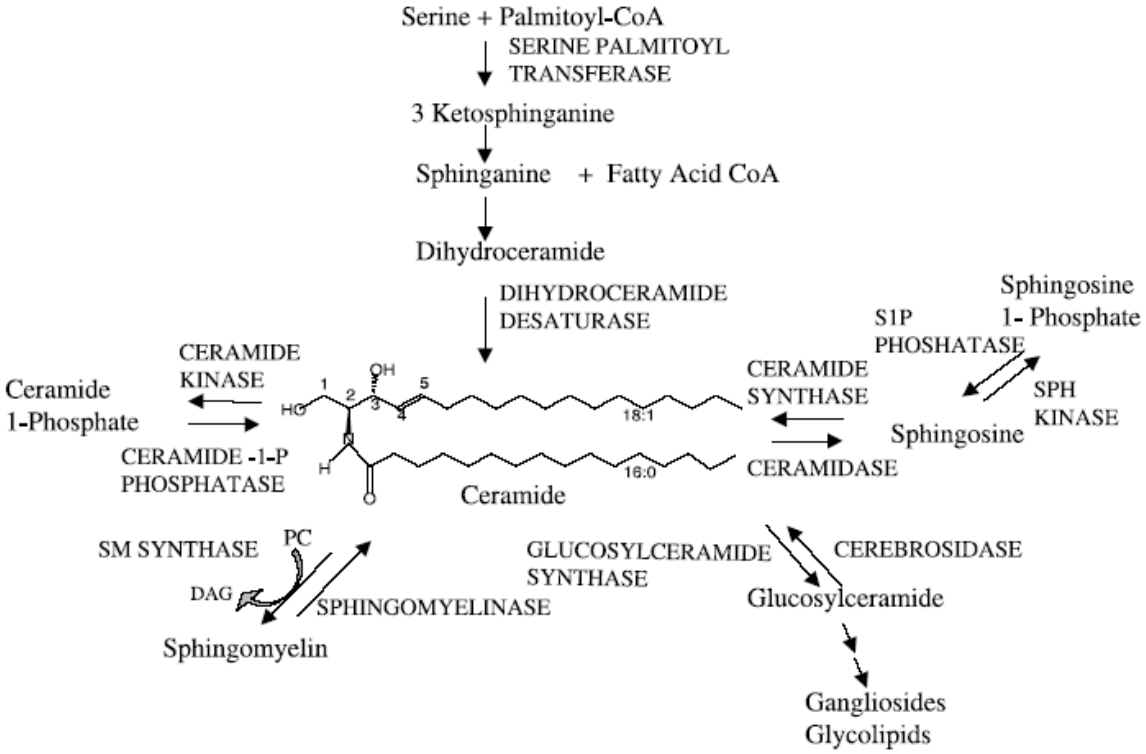


Fig. 1.5. Ceramide metabolic pathways.

Several putative and direct targets of ceramide action in apoptosis have been identified (Fig. 1.6). They are ceramide-activated protein kinase (CAPK) (Lozano et al., 1994; Muller et al., 1995), cathepsin D (Heinrich et al., 2000), and serine/threonine protein phosphatases 1 (PP1) and PP2A (Dobrowsky et al., 1993; Wolff et al., 1994). Activation of phosphatases could explain the ability of ceramide to induce apoptosis via dephosphorylation/inactivation of AKT, a kinase that phosphorylates and inactivates proapoptotic protein BAD. Dephosphorylation of BAD results in the release of reactive oxygen species (ROS) and cytochrome c from mitochondria and activation of caspase-9 and caspase-3. These processes are summarized in Fig. 1.6.

Previously, it was shown that after lethal ischemia, sphingomyelin levels are decreased and in parallel ceramide levels are apparently increased in the gerbil hippocampus (Nakane et al., 2000). In addition, fatty acids are able to enhance the de novo synthesis of ceramide and cause apoptosis in primary astrocytes (Blazquez et al., 2001). There are some more evidences showing that under certain pathophysiological situations, for example exposure to stress stimuli, as well as in neurodegenerative disorders such as

AD, PD, epilepsy and ischemia/stroke, the accumulation of the apoptotic ceramide in neural astrocytes causes apoptosis (Ariga et al., 1998; Blazquez et al., 2000; Gomez del Pulgar et al., 2002; Barrier et al., 2005). Therefore, these data support an important proapoptotic role for ceramide in neural cell apoptosis.

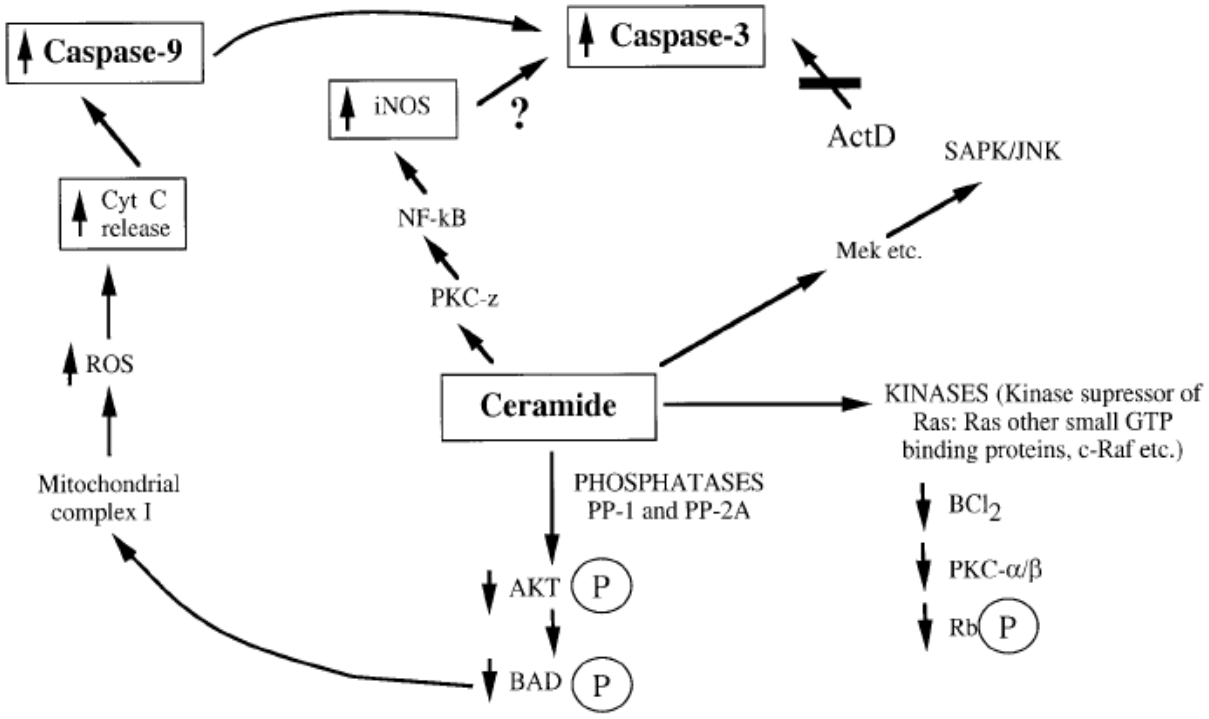


Fig. 1.6. Biological role of ceramide (Goswami and Dawson, 2000). Ceramide can directly inhibit AKT kinase by activating PP2A and PP1, which results in the dephosphorylation of AKT and BAD. Subsequently, ROS and cytochrome c are released from mitochondria, which further activates caspase-9 and caspase-3. It has also been claimed that ceramide promotes the formation of inactive Ras-Raf-1 complexes that may sequester active Ras required for anti-apoptotic signaling pathways. Ceramide also potently activates the MEK-JNK pathway that induces de novo synthesis of caspase-3, inhibited by Actinomycin D (Act D). Ceramide can induce iNOS via PKC and NF-κB, but this might not be pro-apoptotic.

1.5 Aims for the thesis project

Astrocytes, the most abundant cell type within the CNS, are involved in a variety of tasks critical for normal brain functioning (Kirchhoff et al., 2001; Chen and Swanson, 2003). Recently accumulating evidence showed that glial cells, in particular astrocytes, play an active and important role in the demise of brain tissue after brain injury. We have shown previously that all four types of PAR are functionally expressed in rat astrocytes and that PAR-1 regulates astrocyte proliferation via the ERK pathway (Wang et al., 2002a; Wang et al., 2002b). It was confirmed recently that thrombin via PAR-1 triggers astrogliosis after brain injury (Nicole et al., 2005). In addition, PAR-1 also contributes to microglia activation (Suo et al., 2003). So far, some functions have already been attributed to PAR-1 in the CNS. However, it is still not clear whether PAR-1 or any of the other PARs is involved in the release of cytokines/chemokines, such as IL-6, GRO/CINC-1, IL-1β and TNF-α, in rat astrocytes.

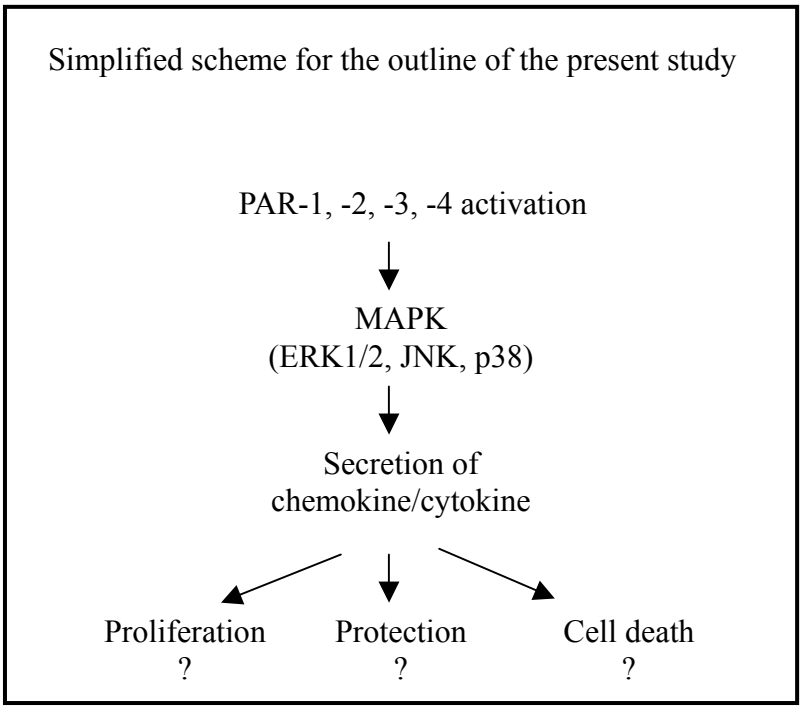
If PAR activation results in the release of chemokine GRO/CINC-1 or other cytokines, it is of high interest to investigate further what mechanisms are beneath PAR-induced chemokine/cytokine release. We focused on MAPKs, since MAPKs have been reported to be involved in diverse physiological processes, including proliferation, inflammation, cell death and survival.

Most importantly, we further aimed at shedding light on the physiological or pathophysiological significance of chemokine/cytokine secretion. It is clear that thrombin at low concentrations via PAR-1 is protective in ischemic and hemorrhagic models of stroke (Donovan and Cunningham, 1998; Masada et al., 2000; Striggow et al., 2000). However, the mechanism of thrombin-induced cell protection remains largely unknown. Under certain pathological conditions, like ischemia, trauma or AD, the apoptotic mediator ceramide accumulates in neural astrocytes and causes apoptosis; on the other hand, the levels of serine proteases, like thrombin and tryptase, are increased. Subsequently, PAR-1 and/or PAR-2 will be activated to release chemokines or cytokines. However, the role of PAR activation and mediator secretion in the process of ceramide-induced cell death is obscure.

Therefore, in the present study, I will explore whether PAR activation and chemokine/cytokine secretion have protective or deleterious effects on astrocytes, using exogenous C_2-ceramide to induce cell injury. In addition, I will also investigate the feedback effects of mediator secretion on PAR-induced astrocyte proliferation, since astrocyte proliferation was observed at 24 or 48 h after the exposure to PAR agonists.

The outline of the present study is as follows:

1. Investigating whether activation of the four different PARs increases the levels of the chemokine GRO/CINC-1, cytokine IL-6, IL-1β and TNF-α at both mRNA and protein.
2. Elucidating the signaling pathways of PAR-induced chemokine or cytokine secretion. The focus is on the role of three MAPK members, ERK, JNK and p38.
3. Clarification of the differences of the biochemical mechanisms among different PARs, if activation of different PARs stimulates the same chemokine or cytokine release.
4. Using recombinant chemokine or cytokine to study the feedback effects of chemokine or cytokine secretion on astrocyte proliferation, or to detect whether chemokine or cytokine has protective/deleterious effects on astrocytes, when cells are exposed to exogenous C_2-ceramide.
5. Studying whether PAR activation has similar physiological effects on cell death/survival as chemokine/cytokine secretion.



2 Materials and methods

2.1 Materials

2.1.1 Instruments

Applied Biosystems	ABI PRISMTM^{TM} 310 Genetic Analyzer
(Foster City, CA, USA)	
Bandelin electronic	Ultrasonic homogenizer
(Berlin, Germany)	
Biometra	T3 Thermocycler
(Göttingen, Germany)	
Bio-Rad	Electrophoresis power supply
(Herts, UK)	Gel document system and Gene pulser II
	GS-800 Calibrated Densitometer
	Semi-dry Transfer Cell
Eppendof	Thermomixer comfort
(Hamburg, Germany)	
GE Healthcare	Mighty Small II and UV/visible Spectrophotometer
(Munich, Germany)	
Heraeus	Biofuge pico and 13 R centrifuges
(Hanau, Germany)	Megafuge 1.0 R centrifuge
	Cell culture incubator; Refrigerate (-80^{o}C)
Integra Biosciences	Tecnoflow bench
(Fernwald, Germany)	
Labinco	Rotator
(BA, Netherlands)	
Bachofer	Waterbath
(Reutlingen, Germany)	
Liebherr	Refrigerates (4^{o}C and -20^{o}C)
(Hamburg, Germany)	
Millipore	Millipore purification system and ultra-pure water system
(Schwalbach, Germany)	
Molecular Devices	Microplate reader
(Sunnyvale, CA, USA)	

Tecan Deutschland GmbH (Crailsheim, Germany)	GENios, GENios FL, and GENios Plus.
Sartorius (Göttingen, Germany)	Balance (analytical and preparative)
WTW (Weilheim, Germany)	pH Meter (pH526)
Whatman GmbH (Dassel, Germany)	Nitrocellulose membrane Protran BA83

2.1.2 Chemicals and reagents

Applied Biosystems (Foster city, CA, USA)	Template Suppression Reagent
Bachem (Heidelberg, Germany)	Human PAR-3AP (TFRGAP, H-Thr-Phe-Arg-Gly-Ala-Pro-OH)
Biochrom (Berlin, Germany)	Dulbecco's modified Eagle's medium (DMEM) Fetal calf serum (FCS) Penicillin and streptomycin
Biomol (Hamburg, Germany)	HEPES Tris base
Bio-Rad (Munich, Germany)	Precision Plus Protein All Blue Standard Bio-Rad protein assay dye reagent concentrate
CALBIOCHEM (La Jolla, CA, USA)	D-erythro-Sphingosine, N-Acetyl- (C_2-ceramide) D-erythro-Sphingosine, Dihydro-, N-Acetyl-(C_2-dihydroceramide); Bisindolylmaleimide I (GF109203X) Pertussis toxin (PTX); PD 98059; SB203580; SKF-96365, hydrochloride; JNK inhibitor II (SP600125); U0126; U73122; U73343; Wortmannin; 2-aminoethoxydiphenylborate (2-APB)
Cell sciences (Canton, MA, USA)	Recombinant rat GRO/KC
Fluka (Buchs, Switzerland)	Ammonium peroxodisulfate Sodium azide

IBA GmbH	Magnet assisted transfection
(Göttingen, Germany)	
Invitrogen	SeeBlue® Plus2 Pre-Stained Standard
(Karlsruhe, Germany)	NuPAGE® Novex 10% Bis-Tris Pre-Cast Gel
	NuPAGE® Antioxidant
	NuPAGE® Transfer Buffer (20X)
	NuPAGE® LDS Sample Buffer (4X)
	NuPAGE® MOPS SDS Running Buffer (20X)
MBI Fermentas	100 bp DNA ladders
(St. Leon-Rot, Germany)	
NeoMPS SA	TRag (Ala-pFluoro-Phe-Arg-Cha-HomoArg-Tyr-NH_2)
(Strasbourg, France)	Rat PAR-2AP (SLIGRL, Ser-Leu-Ile-Gly-Arg-Leu-OH)
	Mouse PAR-4AP (GYPGKF, Gly-Tyr-Pro-Gly-Lys-Phe-OH)
	α-Thrombin antagonist in human platelets (YFLLRNP, Tyr-Phe-Leu-Leu-Arg-Asn-Pro)
Qiagen	Non-silencing siRNA, Fluorescein
(Hilden, Germany)	JNK1-3 siRNA
Roche Diagnostics	Trypsin; Protease inhibitor cocktail tablets
(Mannheim, GERMANY)	Ponceau S solution (0.2% in acetic acid)
Carl Roth	Albumin Fraction V, protease free
(Karlsruhe, Germany)	a-D(+)-Glucose Monohydrat; D(+)-Saccharose
Santa Cruz Biotechnology	Blotto Non-fat dry milk
(Heidelberg, Germany)	
SERVA	Acrylamide (2 ×); N, N'-Methylenbisacrylamide (2 ×)
(Heidelberg, Germany)	Triton X-100
Sigma	Human thrombin; LY294002; Tween 20; Bromphenol blue;
(Deisenhofen, Germany)	TEMED; Dimethyl sulfoxide (DMSO); β-mercaptoethanol; Igepal CA630; Protein G-Agarose; Poly-L-lysine

SB-332235 was generously provided by Dr. H. Sarau from Glaxo Smith Kline (King of Prussia, USA).

2.1.3 Antibodies

Abcam (cambridge, UK)	Rabbit polyclonal CXCR2 antibody
Calbiochem (La Jolla, CA, USA)	Rabbit anti-Porin (Ab-5)
Cell Signaling Technology (Beverly, MA, USA)	Rabbit anti-phospho-JNK (Thr183/Tyr185) antibody Rabbit anti-JNK antibody Rabbit anti-phospho-p38 (Thr180/Tyr182) antibody Rabbit anti-p38 antibody Rabbit anti-phospho-c-Jun (Ser63) antibody Rabbit anti-c-Jun antibody antibody
Dianova (Hamburg, Germany)	Mouse monoclonal anti-cytochrome c Ab-2 (clone 7H8.2C12) Goat anti-mouse-HRP IgG Goat anti-rabbit-HRP IgG
Santa Cruz (Heidelberg, Germany)	Mouse monoclonal anti-JNK1 (F-3) antibody Mouse monoclonal anti-JNK2 (D-2) antibody
Sigma (Deisenhofen, Germany)	Mouse monoclonal anti-β-tubulin I (clone SAP.4G5) antibody
Upstate (Charlottesville, VA, USA)	Rabbit monoclonal anti-JNK3/SAPK1b (clone C05T)

2.1.4 Kits

BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA)

Supersignal West Pico Chemiluminescent Substrate (Pierce, Bonn, Germany)

HotStarTaq™ Master Mix kit (Qiagen, Hilden, Germany)

MinElute PCR Purification kit (Qiagen, Hilden, Germany)

Omniscript™ Reverse Transcription kit (Qiagen, Hilden, Germany)

RNeasy Mini kit (Qiagen, Hilden, Germany)

Rat IL-6 Immunoassay Kit (Biosource, California, USA)

Rat IL-1β ELISA kit (Pierce, Rockford, USA)

Rat tumor necrosis factor (TNF)-α ELISA kit (Pierce, Bonn, Germany)

Rat GRO/CINC-1 ELISA kit (GE Healthcare, Freiburg, Germany)
Cytotoxicity detection kit (LDH) (Roche Diagnostics, Penzberg, Germany)
Nonradioactive SAPK/JNK assay kit (New England Biolab, Beverly, MA, USA).
Cell proliferation ELISA, Brdu (Roche Molecular Biochemicals, Mannheim, Germany)

2.1.5 Buffers

60% Acrylamide/Bis	58.4% Acrylamide (2 ×), 1.6% N,N'-Methylen-bisacrylamide (2 ×)
Coomassie brilliant blue solution	0.25% Coomassie brilliant blue R 250, 45% methanol, 10% acetic acid
Coomassie gel fixing solution	20% ethanol, 10% glycerol
Destaining solution	30% methanol, 10% acetic acid
Hypotonic buffer	10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and Protease Inhibitor Cocktail
4 × Laemmli buffer	500 mM Tris/HCl, pH6.8, 8% SDS, 40% glycerol, 0.01% bromphenol blue, 20% β-mecaptoethanol (fresh)
Modified RIPA buffer	50 mM Tris/HCl pH 7.4, 1% Igepal, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1mM Na_3VO_4, 1 mM NaF and Protease Inhibitor Cocktail
PBS	137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2PHO_4, 1.4 mM KH_2PO_4, pH 7.4
Puck's D1 solution	137 mM NaCl, 5.4 mM KCl, 0.2 mM KH_2PO_4, 0.17 mM Na_2HPO4, 5.0 mM glucose, 58.4 mM sucrose, pH 7.4
Resolving buffer	750 mM Tris/HCl, pH 8.8
Stacking buffer	250 mM Tris/HCl, pH 6.8
Stripping buffer	62.5 mM Tris, pH 6.8, 100 mM β-mercaptoethanol, 2% SDS
1 × TAE buffer	40 mM Tris, 20 mM acetic acid, 1 mM Na_2EDTA
0.5 × TBE buffer	44.5 mM Tris, 44.5 mM Boric acid, 1 mM Na_2EDTA, pH 8.0
TBST	20 mM Tris/HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.6
10 × TE buffer	100 mM Tris/HCl, 10 mM EDTA, pH 7.5

2.2 Methods

2.2.1 Cell culture

Primary astrocyte-enriched cell cultures were obtained from several newborn rats according to a previously published method (Hamprecht and Loffler, 1985). All experiments conformed to guidelines from Sachsen-Anhalt, Germany on the ethical use of animals and all efforts were made to minimize the number of animals used. In brief, two newborn rats were decapitated, total brains were removed and collected in ice-cold Puck's-D1 solution. The brains were gently passed through nylon mesh (256 μM and 136 μm diameter) and centrifuged at 4°C for 5 min at 350 g. The cells were resuspended in 10 ml DMEM containing 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were plated in 60-mm diameter culture dishes at a density of 2.5-5.0 $\times 10^5$ cells /dish, 1.0-2.7 $\times 10^5$ cells/well in 6-well plates or 0.5-1 $\times 10^5$ cells /well in 24-well plates. The cells were cultured in the humidified incubator with 10% CO_2 at 37 °C. The medium was changed for the first time after 5 days and thereafter every 2-3 days, depending on the cell density. For experiments cells were used between day 10 and 13 in culture.

2.2.2 RT-PCR

Serum-starved astrocytes were stimulated by agonists of PAR-1, PAR-2, PAR-3 or PAR-4 with increasing concentrations for 3 h. Total RNA was extracted with RNeasy mini kit. Two micrograms of RNA was reverse-transcribed using Omniscript™ RT kit, and the resulting cDNA was amplified by PCR using HotStarTaq™ Master Mix kit for 15 min at 95°C, followed by repeat cycles, 30 s at 94°C, 90 s at 53-60°C, 60 s at 72°C, then a final 10 min extension at 72°C. Specific primers, annealing temperatures, reaction cycles and length of PCR products for IL-6, TNF-α, IL-1β, GRO/CINC-1, CXCR2, JNK1, JNK2, JNK3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table 2.1. The reaction products were analyzed by electrophoresis with 1% agarose gel containing ethidium bromide, and visualized by Bio-Rad gel document system. The intensity of PCR products was semi-quantified by Quantity one Quantification software (Bio-Rad).

Table 2.1. Primer pairs for IL-6, IL-1β, TNF-α, GRO/CINC-1, JNK1, JNK2, JNK3, CXCR2 and GAPDH. PCR reactions were performed as described in Materials and Methods using the following primers.

	Primers	Annealing (T °C)	Cycles	Product length (bp)
IL-6	F: 5'-CCACTGCCTTCCCTACTTC-3' R: 5'-TTGGTCCTTAGCCACTCCT-3'	53°C	30	499
IL-1β	F: 5'-TGATGTTCCCATTAGACAGC-3' R: 5'-GAGGTGCTGATGTACCAGTT-3'	56°C	31	378
TNF-α	F: 5'-GTAGCCCACGTCGTAGCAAA-3' R: 5'-CCCTTCTCCAGCTGGAAGAC-3'	56°C	28	346
GRO/CINC-1	F: 5'-CTCCAGCCACACTCCAACAGA-3' R: 5'-CACCCTAACACAAAACACGAT-3'	60°C	28	670
JNK1	F: 5'-ATGCTAAGCGAGCCTACCG-3' R: 5'-TCTCAAAGCTATAGCCAGCG-3'	55°C	30	621
JNK2	F: 5'-ACCTCCTCTACCAGATGCT-3' R: 5'-TGAACTCTGCGGATGGTG-3'	58°C	27	357
JNK3	F: 5'-ATGCCAAGAGGGCTTACCG-3' R: 5'-CGTAGTTTCTGACGGTGGG-3'	55°C	30	582
CXCR2	F: 5'-CATCCTGCCTCAGACCTA-3' R: 5'-AAGCCAAGAATCTCAGTAGC-3'	53°C	35	276
GAPDH	F: 5'-GTGAAGGTCGGTGTCAAC-3' R: 5'-CAACCTGGTCCTCAGTGTAGC-3'	53°C	31	835

2.2.3 DNA Sequencing

According to the manufacturer's protocol, JNK1, JNK2 and JNK3 PCR products were purified and dissolved in 50 µl prewarmed TE buffer (70°C) using MinElute PCR purification kit. For DNA sequencing, 150-200 ng DNA plus 1 µl single primer and 4 µl Bigdye, add H_2O to the final volume of 20 µl. The respective forward or reverse primer for RT-PCR was used separately for sequencing of the respective DNA fragments by PCR with fluorescent dye-labled dideoxynucleotides (DNA sequencing Kit, Applied Biosystems). The PCR condition was: denaturation for 2 min at 98°C, 25 cycles at 96°C for 30 sec,

52°C for 30 sec, 60°C for initially 4 min. With each cycle the extension time was increased by 25 sec. DNA was precipitated and dissolved in Template Suppression Reagent, and denatured at 92°C for 2 min before sequencing. The sequence of nucleotides was determined by a ABI PRISM^{TM} 310 Genetic Analyzer.

2.2.4 Rat IL-6, TNF-α, IL-1β and GRO/CINC-1 protein determination

According to the manufacturers' protocol, extracellular IL-6, TNF-α, IL-1β and GRO/CINC-1 protein were measured using rat IL-6, IL-1β, TNF-α and GRO/CINC-1 ELISA kits, respectively. Briefly, serum-starved astrocytes were stimulated with thrombin, TRag, trypsin or PAR-2AP for indicated time intervals (2 h, 3 h, 6 h, 12 h, and 24 h), and then the supernatant medium was collected for ELISA analysis. For inhibitor studies, astrocytes were pretreated with the inhibitors as indicated in Results for 30 min prior to thrombin, TRag, trypsin or PAR-2AP stimulation. The concentration of inhibitors used was based on previous studies (Favata et al., 1998; Sodhi and Biswas, 2002; Chaban et al., 2004; Kim et al., 2005). The levels of IL-6, TNF-α, IL-1β and GRO/CINC-1 were assayed at 450 nm. In parallel, cells were lysed for measuring the amount of total cellular protein.

2.2.5 Preparation of whole cell lysate

Serum-starved astrocytes were treated with agonists or inhibitors at 37 °C as indicated in Results. After stimulation, cells were washed with ice-cold PBS, then lysed in modified RIPA buffer. After brief sonification, the cell lysate was centrifuged at 15 000 g for 15 min at 4 °C. The protein content of the resulting supernatant was determined by the Bradford method using bovine serum albumin (BSA) as standard.

2.2.6 Preparation of cell cytosol fraction

Serum-starved astrocytes were treated with 20 μM C_2-ceramide in the absence or presence of thrombin, TRag, PAR-2AP or recombinant GRO/CINC-1 at the indicated concentrations for 7 h. After stimulation, cells were washed with ice-cold PBS, then lysed by 30 strokes of Dounce homogenizer in the hypotonic buffer. The samples were centrifuged for 30 min at 50 000 g, 4°C, to remove nuclear, membrane and mitochondria fractions. The supernatant was transferred to a fresh tube and stored at –20°C. The determination of protein concentration was performed as mentioned above.

2.2.7 Western blot

Samples containing equal amounts of protein were separated on a 10% NuPAGE Novex bis-tris Gel, 10% or 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to nitrocellulose membrane Protran BA83 and blocked by incubation with 3% BSA for 1 h at room temperature. The membrane was then incubated with the antibody against phosphorylated JNK (phospho-SAPK/JNK, Thr183/Tyr185, 1:2000), phospho-p38 MAP kinase (Thr180/Tyr182, 1:1000), phospho-c-Jun (Ser63) II (1:2000), JNK1 (1:1000), JNK2 (1:2500), JNK3 (1:5000) or cytochrome c Ab-2 (1:1000), overnight at 4 °C, followed by incubation with peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1: 10 000) for 60 min at room temperature. After washing with TBST, the target proteins were detected by the SuperSignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL, USA).

After stripping, the membranes were reprobed with anti-JNK (1:2000), p38 MAP kinase (1:1000), c-Jun (1:2000), or β-tubulin (1:20 000) antibody. Films were analyzed densitometrically by the GS-800 Calibrated densitometer (Bio-Rad) referring the optical density values to a range of preset gray values. The intensity of the western blot bands was quantified by Quantity one Quantitation software (Bio-Rad). Values of phosphorylation of target proteins are given in a quantitative manner by normalizing signals corresponding to the total amount of the respective protein or to the constitutively expressed β-tubulin.

2.2.8 Kinase assays

Serum-starved astrocytes were treated with 10 μM TRag or 500 μM PAR-2AP for indicated times, then whole cell lysate was harvested as described above. The ‘‘Pull down’’ assay with c-Jun fusion beads was carried out according to the manufacturer’s protocol. One hundred micrograms of protein diluted in 200 μl lysis buffer were incubated with 20 μl GST-c-Jun (1-89) fusion protein immobilized on glutathione agarose beads at 4°C overnight. After washing two times with 500 μl cell lysis buffer and twice with 500 μl kinase buffer, the bound proteins were incubated with the kinase buffer (50 μl) containing ATP (200 μM) for 30 min at 30°C. Afterwards, the reaction was terminated with 25 μl laemmli buffer and the proteins (25 μl) were separated on 10% SDS-PAGE. The JNK activity was determined by western blot using phospho-c-Jun (Ser 63) antibody (provided in the kit).

2.2.9 Small interfering RNA (siRNA) transfection

JNK1, JNK2 and JNK3 siRNA were designed and synthesized by Qiagen. Non-silencing siRNA with fluorescein was taken as the scrambled control. All siRNA sequences are provided in Table 2.2. Rat astrocytes seeded on the 6-well or 24-well plate at 80% density were transfected with siRNA using Magnet Assisted Transfection for adherent cells (MATra-A) reagent according to the manufacturer's protocol. JNK knockdown was determined by both RT-PCR and western blot at 48 h after transfection.

Table 2.2. Sequences for JNK1 siRNA, JNK2 siRNA, JNK3 siRNA and scrambled siRNA. Gene bank accession number and the location of siRNA target sequence are also given in the Table.

	siRNA sequence	Accession number	Target sequence (bp)
JNK1 siRNA#1	5' GAAGCUCAGCCGGCCAUUUdTdT 3'	L27129	338-358
JNK1 siRNA#2	5' GCGAGCCUACCGAGAACUAdTdT 3'		377-397
JNK2 siRNA#1	5' GCGCCACCACCUCAAAUUUdTdT 3'	NM_017322	1008-1028
JNK2 siRNA#2	5' GCCUUGCGCCACCCGUAUAdTdT 3'		957-977
JNK2 siRNA#3	5' CGCUAGAAGAAUUCCAAGAdTdT 3'		304-324
JNK3 siRNA#1	5' GCCAGGGACUUGUUGUCAAdTdT 3'	NM_012806	1241-1261
JNK3 siRNA#2	5' CUCCGUGUCCAGAAUUCAUdTdT 3'		1089-1109
JNK3 siRNA#3	5' GUGGGAGACUCAACCUUCAdTdT 3'		404-424
Scrambled siRNA	5' UUCUCCGAACGUGUCACGUdTdT 3'	--	--

2.2.10 Lactate dehydrogenase (LDH) release assessment

Serum-starved astrocytes in phenol red-free DMEM were treated with C_2-ceramide in the absence or presence of thrombin, TRag, PAR-2AP, recombinant GRO/CINC-1 or JNK inhibitor at the indicated concentrations for 7 h. The cell culture medium of each well was removed for LDH release assay using cytotoxicity detection kit, according to the manufacturer's protocol. Cells without treatment served as low level

reference value, whereas cells incubated with 2% Triton X-100 were regarded as high level reference value. Inactive C_2-ceramide analog (C_2-dihydroceramide) was applied as negative control. The relative absorbance of all samples was measured at 490 nm. Cytotoxicity was calculated with the following formula: Cytotoxicity (%) = $(A_{\text{test sample}} - A_{\text{low control}})/(A_{\text{high control}} - A_{\text{low control}})$.

2.2.11 Cell proliferation ELISA

Astrocytes were plated at a density of 1×10^4 cells per well in 96-well plates, and were serum-starved for 24 h before test. The cells were treated with thrombin, TRag or recombinant GRO/CINC-1 for a certain period of time (1 h - 6 h), depending on the individual assay system. Subsequently, BrdU was added to the cells reincubated for 24 h. During this labelling period, the pyrimidine analogue BrdU was incorporated in place of thymidine into the DNA of proliferating cells. After removing the culture medium the cells were fixed and the DNA was denatured in one step by adding FixDenat (the denaturation of the DNA is necessary to improve the accessibility of the incorporated BrdU for detection by the antibody). The anti-BrdU-POD bound to the BrdU incorporated in newly synthesized cellular DNA. The cell proliferation was detected by measuring the light emission of the samples in a microplate luminometer (Tecan Deutschland GmbH, Crailsheim, Germany).

2.3 Statistical analysis

Statistical evaluation was carried out by Student's *t*-test between two groups and by one-way analysis of variance (ANOVA) with GraphPrism software within multiple groups. Data were given as means ± SEM and $p < 0.05$ was considered significant.

3 Results

3.1 PAR-1 and PAR-2 have comparable capacity to increase mRNA and protein levels of GRO/CINC-1

3.1.1 Activation of PAR-1 and PAR-2 both increase GRO/CINC-1 mRNA level

Thrombin acting on PAR-1 exerts multiple biological functions in astrocytes (Wang and Reiser, 2003). Here, we first determined the effects of PAR-1 activation on the expression of the chemokine GRO/CINC-1 at the mRNA level. We treated serum-starved astrocytes with thrombin and TRag at increasing concentrations for 3 h, then extracted the total RNA. As Fig. 3.1 A shows, the treatment with thrombin or TRag significantly increased the GRO/CINC-1 mRNA level. The concentration dependence is depicted in the quantitative analysis in Fig. 3.1 B. In parallel, the effects of the three other PARs, besides PAR-1, on GRO/CINC-1 mRNA level were also investigated here. We treated serum-starved astrocytes with trypsin, PAR-2AP (SLIGRL), PAR-3AP (TFRGAP) and PAR-4AP (GYPGKF) for 3 h. As Fig. 3.1 C & D shows, PAR-2 activation induced by either trypsin or PAR-2AP increased the GRO/CINC-1 mRNA level in a concentration-dependent manner. However, PAR-3 and PAR-4 activation failed to upregulate GRO/CINC-1 mRNA expression (data not shown). Taken together, these results suggest that PAR-1 and PAR-2 activation are both able to upregulate GRO/CINC-1 mRNA level.

3.1.2 Activation of PAR-1 and PAR-2 both upregulate secretion of GRO/CINC-1 protein

Next, the protein secretion of GRO/CINC-1 induced by either PAR-1 or PAR-2 was investigated. We found that the secretion of GRO/CINC-1 was significantly increased by thrombin (1 U/ml), TRag (10 μM) or PAR-2AP (500 μM) in a time-dependent manner (Fig. 3.2 A & B). Moreover, we also exposed cells to increasing concentrations of thrombin, TRag, trypsin or PAR-2AP in a 3 h challenge. As shown in Fig. 3.2 C & D, thrombin, TRag, trypsin and PAR-2AP concentration-dependently induced GRO/CINC-1 release from astrocytes.

To our surprise, 5 nM trypsin significantly induced GRO/CINC-1 secretion, while no GRO/CINC-1 could be detected after treating with 50 nM trypsin for 2, 3 or 6 h (Fig. 3.2 B & D). In this context it should be noted that trypsin cleaves peptides C-terminally to Arg or Lys residues, and prefers Gly or Pro residues in the P2 position (Wang et al., 2004). Sequence analysis of rat GRO/CINC-1 (accession number: NM_030845) revealed one potential cleavage site for trypsin: Gly^{71}-Arg^{72}. Thus, it is likely that trypsin degrades

GRO/CINC-1 by cleaving after position Gly^{71}-Arg^{72}. To test this, we stimulated cells with 10 μM TRag for 2 h, then further treated the cells with 50 nM trypsin together with TRag for another 1 h. Cells treated with TRag (10 μM) alone for 3 h served as positive control, and cells without any treatment were taken as basal control. As Fig. 3.2 E shows, the application of trypsin abolished TRag-induced GRO/CINC-1 release, which confirms that trypsin has the capability to degrade GRO/CINC-1. Fig. 3.2 F shows that thrombin, TRag and PAR-2AP induced similar amounts of GRO/CINC-1 release from astrocytes by about 15-25 pg/μg protein. This level was much higher than that seen with untreated cells. These results demonstrate that PAR-1 and PAR-2 activation both are able to increase chemokine GRO/CINC-1 release with a similar capacity.

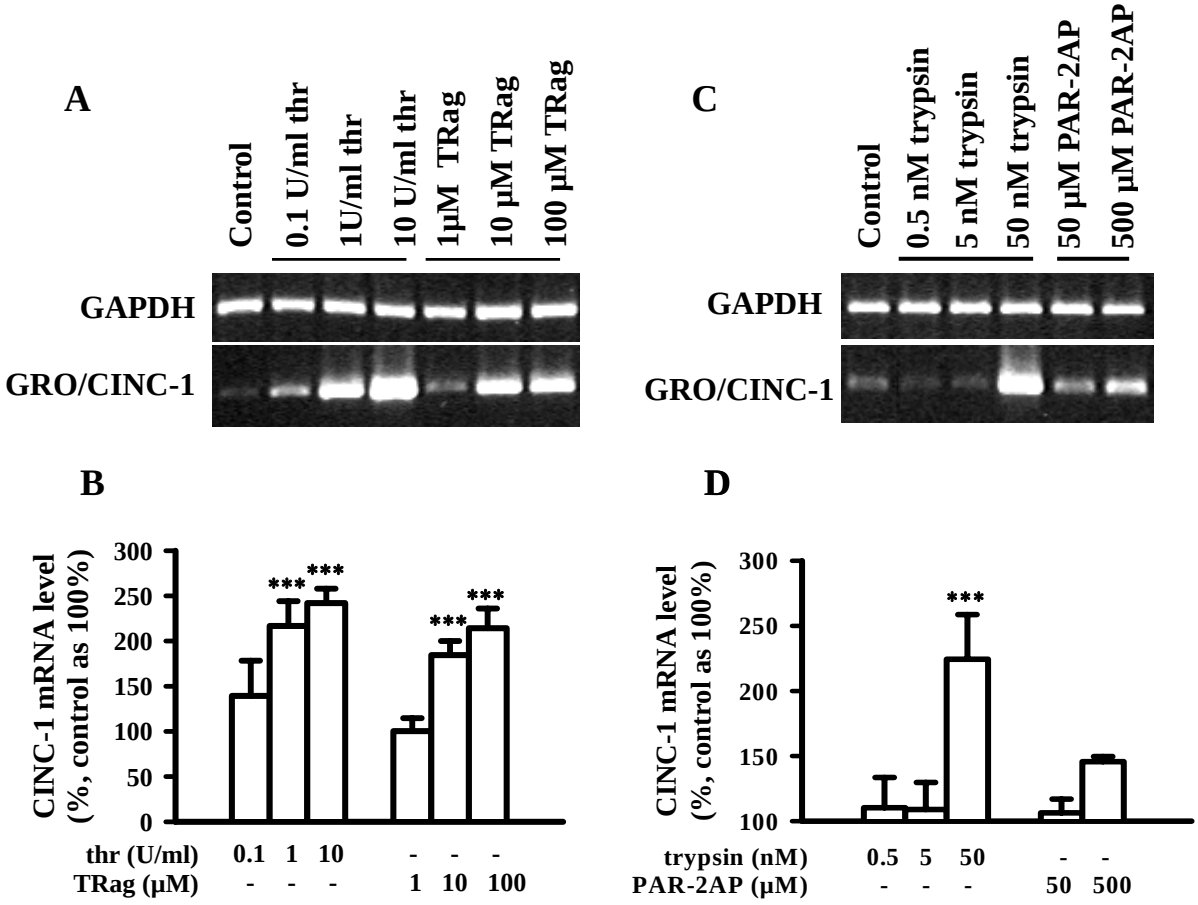


Fig. 3.1. GRO/CINC-1 mRNA levels in astrocytes after PAR-1 and PAR-2 activation. Serum-starved astrocytes were stimulated by increasing concentrations of PAR-1 agonists (thrombin and TRag, A & B) and PAR-2 agonists (trypsin and PAR-2AP, C & D) for 3 h. **A & C**. Representative gels of GRO/CINC-1 mRNA levels induced by thrombin (thr, 0.1-10 U/ml), TRag (1-100 μM), trypsin (0.5-50 nM) or PAR-2AP (50-500 μM). **B & D.** The amounts of GRO/CINC-1 were normalized by the corresponding GAPDH signal. Data shown in B & D represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$ as compared with control.

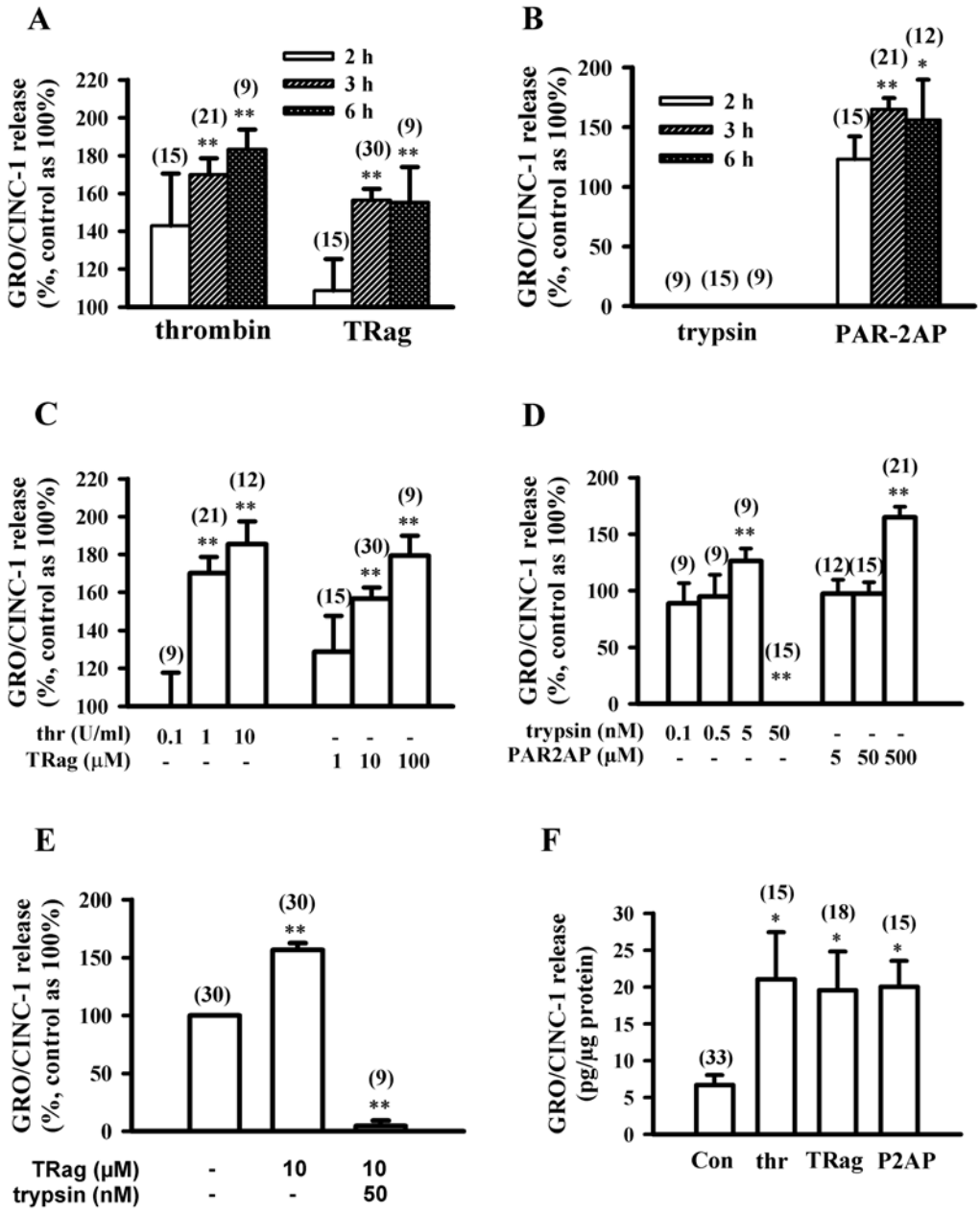


Fig. 3.2. PAR-1- and PAR-2-mediated GRO/CINC-1 release from astrocytes. A & B. Time dependence of GRO/CINC-1 release induced by 1 U/ml thrombin, 10 μM TRag, 50 nM trypsin or 500 μM PAR-2AP for 2 h, 3 h or 6 h. **C & D**. Concentration dependence of GRO/CINC-1 release induced by 3 h-incubation with thrombin (thr, 0.1-10 U/ml), TRag (1-100 μM), trypsin (0.1-50 nM) or PAR-2AP (50-500 μM). **E**. Degradation of GRO/CINC-1 by trypsin. The secretion of GRO/CINC-1 induced by 3 h-incubation with 10 μM TRag can be degradated by 50 nM trypsin in 1 h. **F**. The amounts of GRO/CINC-1 release from astrocytes at control (Con) level or by 3 h-incubation with 1 U/ml thrombin, 10 μM TRag or 500 μM PAR-2AP (P2AP). Data in A-F show the mean ± SEM. Numbers given above the respective column represent the number of samples. ** $p < 0.01$, * $p < 0.05$ as compared with control.

3.1.3 Effects of PAR-1 and PAR-2 activation on other cytokines at both mRNA and protein levels

The expression of proinflammatory cytokines IL-6, TNF-α and IL-1β after activation of four types of PARs was also investigated here. We found that the treatment with PAR-1 agonists (thrombin or TRag) and PAR-2 agonists (trypsin or PAR-2AP) also concentration-dependently upregulated the mRNA levels of TNF-α and IL-1β (Fig. 3.3 A-C), whereas the treatment with PAR-3AP and PAR-4AP did not affect the mRNA levels of TNF-α and IL-1β (data not shown). The IL-6 mRNA level, however, was significantly upregulated only by thrombin, but not by TRag (Fig. 3.3 D & E). The ELISA results further demonstrate that the treatment with 1 U/ml thrombin, 10 μM TRag, 5 nM trypsin or 500 μM PAR-2AP for 2 h to 24 h failed to increase the release of IL-6, TNF-α and IL-1β in astrocytes (data not shown). LPS (50 ng/ml), which served as a positive control, was applied to astrocytes for 6 h or 24 h. We found that LPS clearly increased the release of IL-6 and TNF-α protein, thousand times above that seen with non-stimulated cells (data not shown). This confirms the validity of our experimental working conditions. Therefore, our data suggest that PAR-1 and PAR-2 activation are able to increase only mRNA levels of TNF-α or IL-1β, but not the protein secretion. IL-6 mRNA level is only regulated by the serine protease thrombin, which is independent of PAR-1 activation.

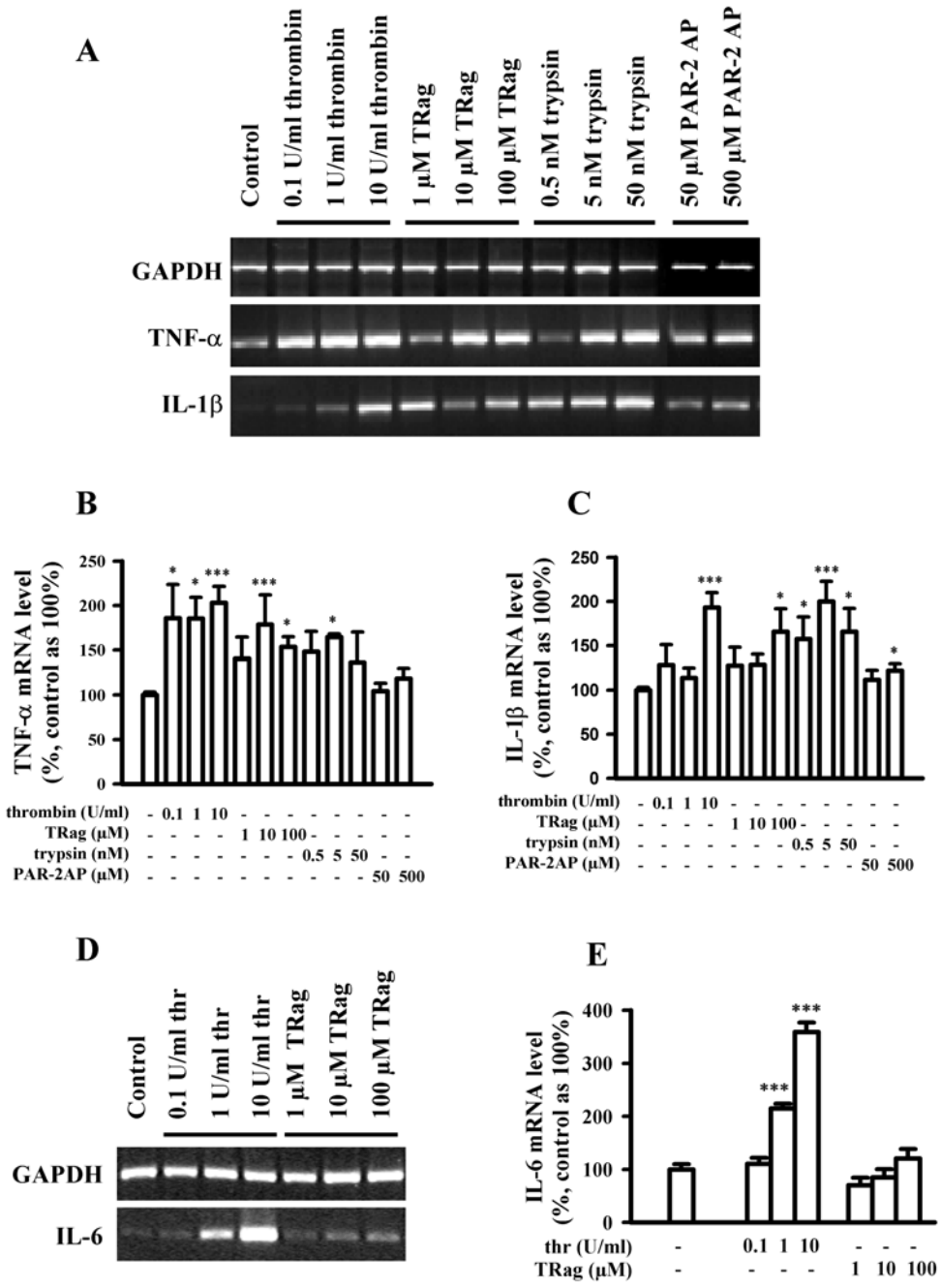


Fig. 3.3. TNF-α, IL-1β and IL-6 mRNA levels in astrocytes after PAR-1 and PAR-2 activation. Serum-starved astrocytes were stimulated by increasing concentrations of PAR-1 agonists (thrombin and TRag) and PAR-2 agonists (trypsin and PAR-2AP) for 3 h. **A & D**. Representative gels of TNF-α, IL-1β and IL-6 mRNA levels induced by 3 h-incubation with thrombin (thr, 0.1-10 U/ml), TRag (1-100 µM), trypsin (0.5-50 nM) or PAR-2AP (50-500 µM). **B, C & E.** The amounts of TNF-α, IL-1β and IL-6 were normalized by the corresponding GAPDH signal. Data shown in B, C & E represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, * $p < 0.05$ as compared with control.

3.2 The mechanisms of GRO/CINC-1 release from astrocytes

3.2.1 Roles of MAPKs in PAR-1- and PAR-2-induced GRO/CINC-1 release

3.2.1.1 Activation of JNK and ERK1/2, but not p38, is responsible for PAR-1-induced GRO/CINC-1 release

MAPK have been reported to be involved in diverse physiological processes. Here, we investigated whether the three MAPK family members mediate PAR-1-induced GRO/CINC-1 release. Our previous results have already shown that treatment with both thrombin and TRag could time- and concentration-dependently induce the phosphorylation of ERK1/2 in astrocytes (see Fig. 1 and Fig. 2 in (Wang et al., 2002b)). The most pronounced ERK1/2 activation, induced by 10 U/ml thrombin or 10 μM TRag, was obtained at 5 min. Moreover, the ERK1/2 phosphorylation was remarkably blocked by the MEK1/2 inhibitor PD98059. Both U0126 and PD98059 are known as specific inhibitors for MEK1/2 (Alessi et al., 1995; Favata et al., 1998), an upstream kinase of ERK1/2. Here, our ELISA data demonstrated that the MEK1/2 inhibitor U0126 almost completely abolished PAR-1-induced GRO/CINC-1 secretion (Fig. 3.4). These results suggest that ERK1/2 is likely to be involved in PAR-1-mediated GRO/CINC-1 release.

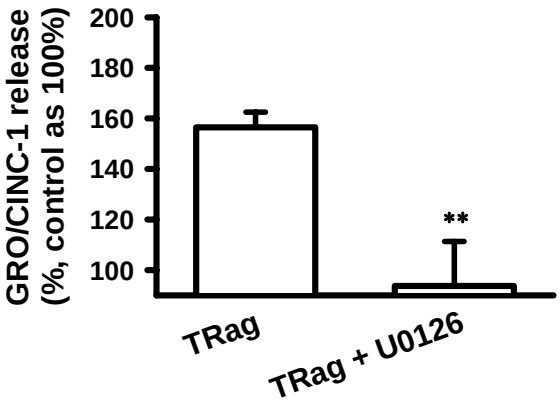


Fig. 3.4 The effect of U0126 treatment on PAR-1-induced GRO/CINC-1 release from astrocytes. Serum-starved cells were preincubated with MEK1/2 inhibitor U0126 (25 μM) for 30 min prior to 3 h-stimulation with 10 μM TRag. Astrocytes without any treatment were taken as baseline (100%). Cells treated only with TRag served as positive control for GRO/CINC-1 release. Data represent the mean ± SEM. ** $p < 0.01$ as compared to the cells exposed to TRag alone.

Activation of the two other MAPK members, JNK and p38, induced by thrombin or TRag were investigated with western blot analysis in the present study. We challenged serum-starved astrocytes with thrombin (1 U/ml) or TRag (10 μM) for times ranging from 10 min to 2 h. As shown in Fig. 3.5 A and B, PAR-1 activation time-dependently induced JNK phosphorylation in astrocytes. The strongest JNK activation was obtained at 10 min. Afterwards this phosphorylation decreased gradually but persisted for at least 2 h. As previous data have shown, the MAPK JNK has ten different isoforms resulting from three alternatively spliced genes (JNK1, JNK2 and JNK3), which mainly yield proteins of 46 kDa and 54 kDa (Sugden and Clerk, 1998; Barr and Bogoyevitch, 2001; Enomoto et al., 2003). In the present study, we found that the JNK isoforms with 46 kDa, but not the 54 kDa isoforms, were phosphorylated due to PAR-1 activation. Interestingly, there was another band of about 43 kDa, which was time-dependently phosphorylated after treatment with thrombin or TRag (Fig. 3.5 A). Moreover, the phosphorylation of this band was even much stronger than that of 46 kDa JNKs, although its total protein content was almost undetectable by western blot analysis. These results are in line with another report which showed that both p46 JNKs and JNK isoforms with about 43 kDa could be phosphorylated in primary cultured mice astrocytes during prolonged OGD (Yung and Tolkovsky, 2003).

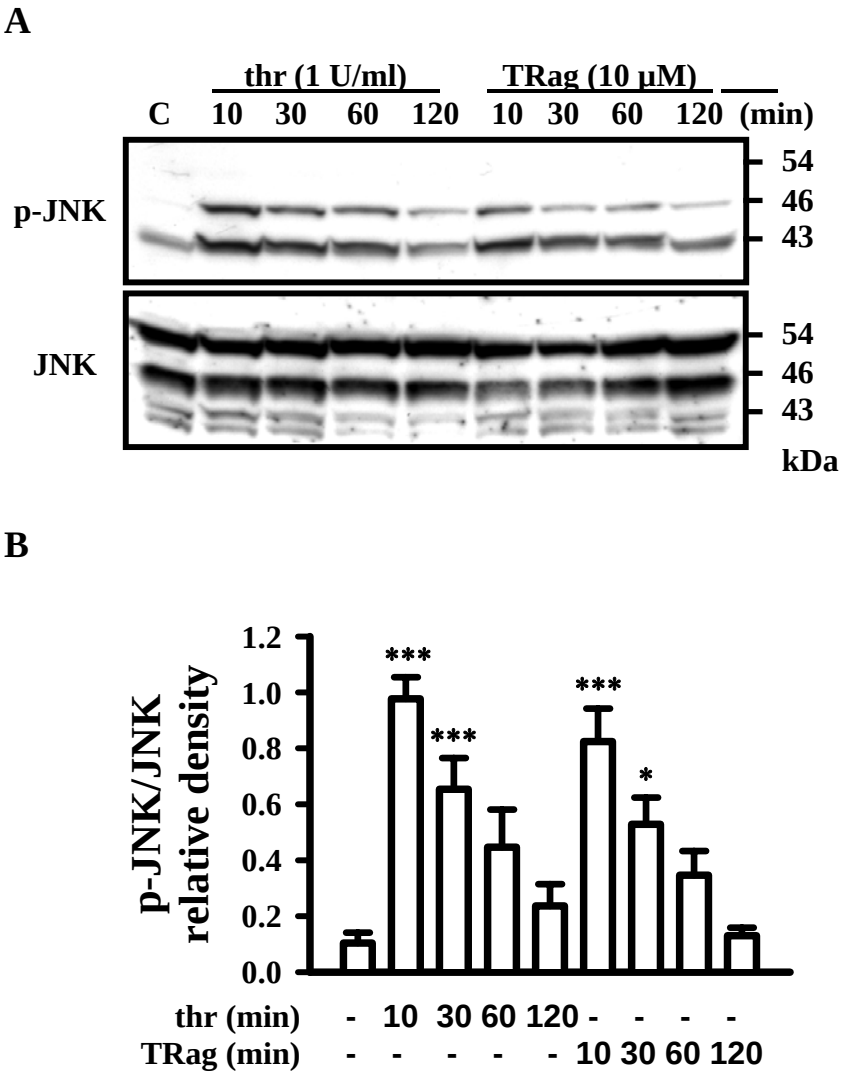


Fig. 3.5. Thrombin- and TRag-induced JNK phosphorylation in astrocytes. Serum-starved cells were exposed to 1 U/ml thrombin (thr) or 10 µM TRag for 10 min, 30 min, 60 min or 120 min. **A**. Representative blots are shown for the time-dependency of JNK phosphorylation (upper panel) and total JNK (lower panel). The molecular mass values for the respective JNK isoforms are given here and in the following figures in kDa. **B**. The phosphorylation of 46 kDa JNK isoform was normalized relative to the total 46 kDa JNK isoform. Data shown in B represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, * $p < 0.05$ as compared with control.

When serum-starved astrocytes were exposed to different concentrations of thrombin (0.001-1 U/ml) or TRag (0.01-10 μM), JNK was significantly phosphorylated even with very low concentrations of thrombin (0.001 U/ml) or TRag (0.01 μM), as shown in Fig. 3.6 A. Concentration-effect curves for the 46 kDa JNK isoform phosphorylation obtained with thrombin and TRag are shown in Fig. 3.6 B. They give EC_{50} values of 0.05 nM (0.01 U/ml) for thrombin and 0.3 μM for TRag. In parallel with western blot

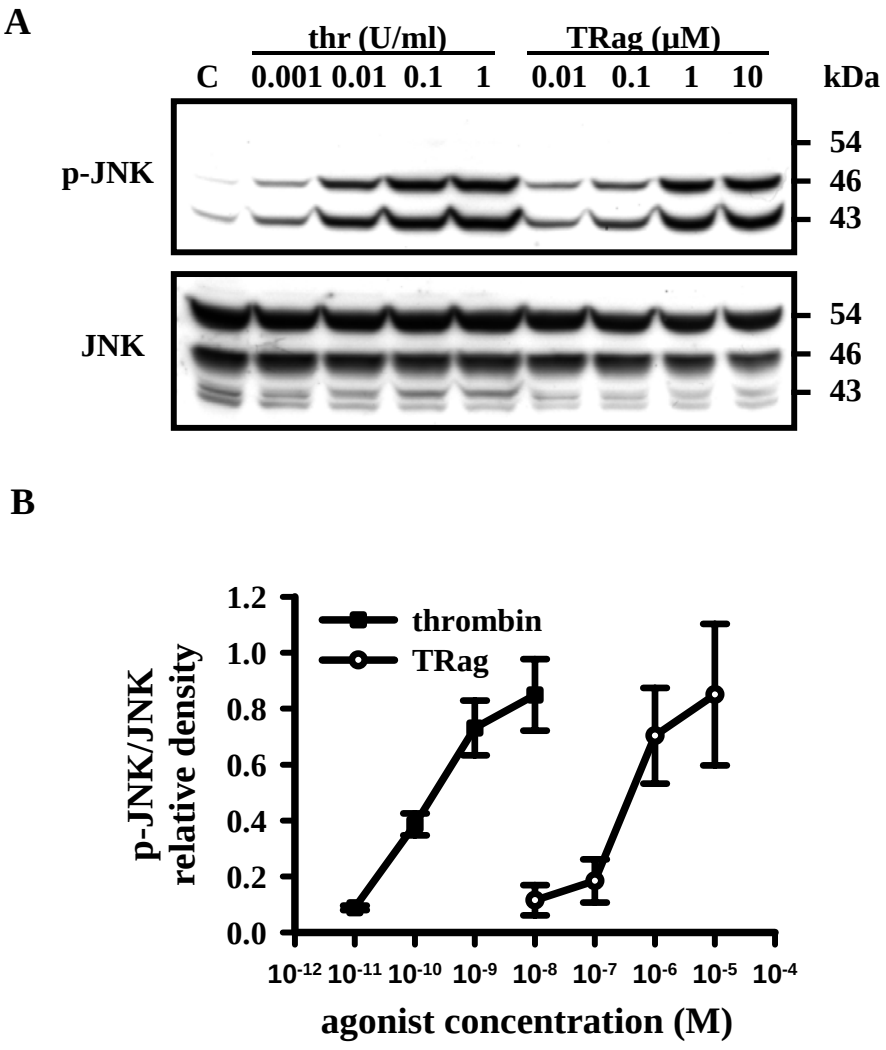


Fig. 3.6. Concentration-dependence of thrombin- and TRag-induced JNK activation. Serum-starved cells were stimulated by thrombin (thr, 0.001-1 U/ml) or TRag (0.01- 10 μM) for 10 min. **A**. Representative blots from one experiment are shown for JNK phosphorylation (upper panel) and total JNK (lower panel). **B.** Concentration-effect curves for thrombin and TRag on 46 kDa JNK phosphorylation. The phosphorylation of 46 kDa JNK isoform was normalized relative to the total 46 kDa JNK isoform.

experiments, the direct role of JNK activation in GRO/CINC-1 release was studied by ELISA. We found that the specific JNK inhibitor SP600125 significantly reduced PAR-1-induced GRO/CINC-1 release by 76% (Fig. 3.7). The observations from both western blot analysis and ELISA data clearly indicate that JNK plays a pivotal role in mediating PAR-1-induced GRO/CINC-1 release.

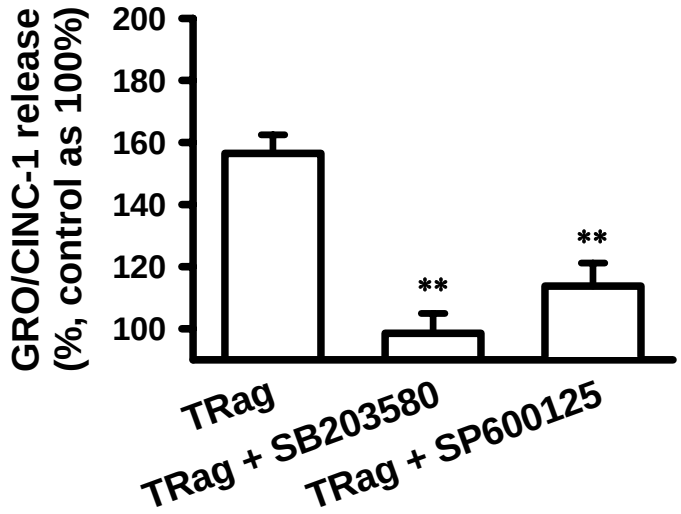


Fig. 3.7 Effects of JNK and p38 MAPK activation on PAR-1-induced GRO/CINC-1 release from astrocytes. Serum-starved cells were preincubated with p38 MAPK inhibitor SB203580 (10 μM) or JNK inhibitor SP600125 (30 μM) for 30 min prior to 3 h-stimulation with 10 μM TRag. Astrocytes without any treatment were taken as baseline (100%). Data represent the mean ± SEM. ** $p < 0.01$ as compared to the cells exposed to TRag alone.

In contrast to JNK activation, no thrombin- and TRag-induced phosphorylation of p38 was detectable (Fig. 3.8 A), although the p38 MAPK inhibitor SB203580 could significantly reduce GRO/CINC-1 release (Fig. 3.7). Some previous reports have shown that SB203580 can also inhibit JNK activation (Lisnock et al., 2000; Harper and LoGrasso, 2001). To investigate whether SB203580 has cross-inhibition of JNK activation, we performed additional experiments to study the effects of SB203580 on JNK phosphorylation. As Fig. 3.8 B & C shows, 10 μM SB203580 did not significantly affect JNK phosphorylation, although there was a slight reduction. These data reveal that SB203580 at the concentration used has no cross-inhibition of JNK phosphorylation. However, we still cannot exclude that this substance might block downstream factors of JNK activation, since the specificity of SB203580 is still a critical point. Nevertheless, our

western blot data reveal that p38 is apparently not involved in the PAR-1 signaling pathway.

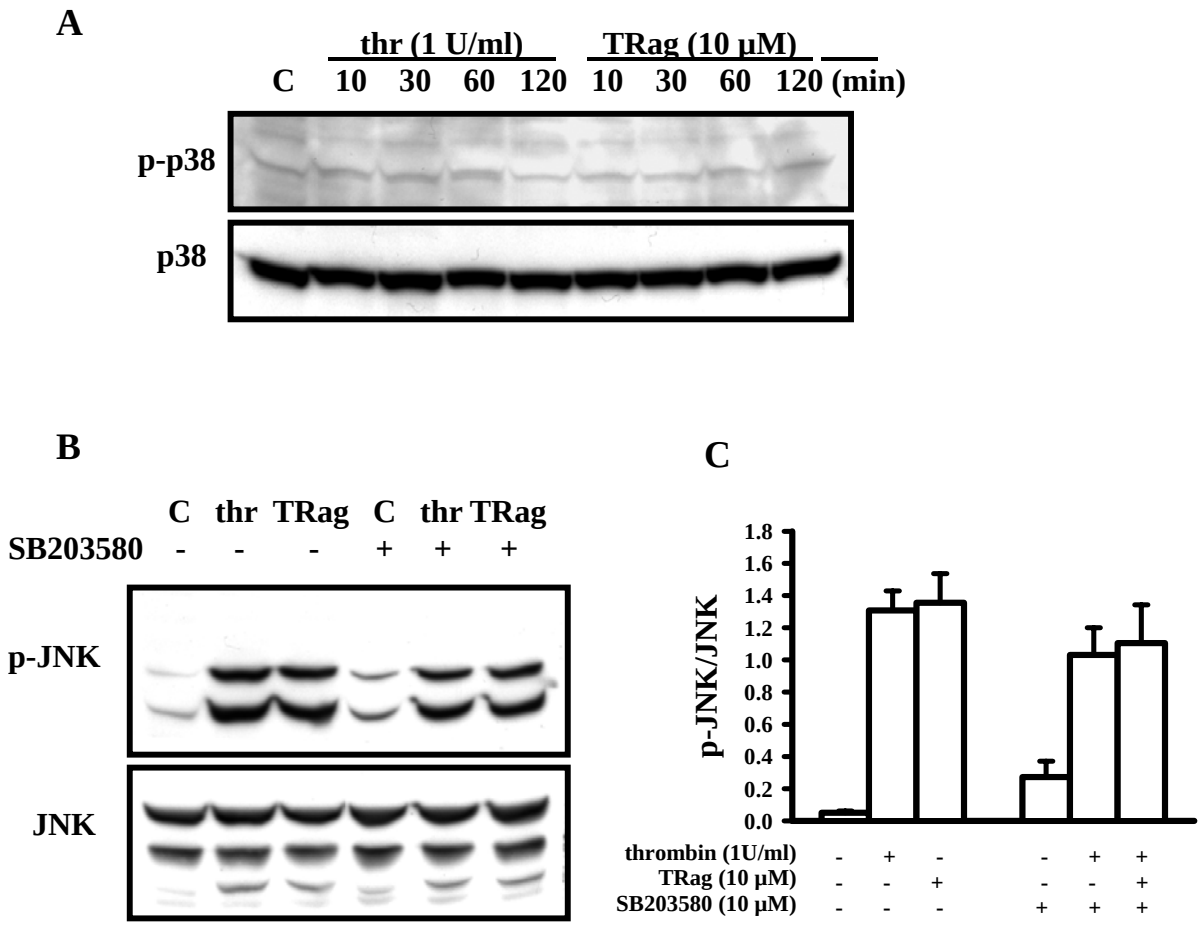


Fig. 3.8 p38 MAPK activation and the effect of SB203580 on PAR-1-induced JNK phosphorylation in astrocytes. A. Serum-starved cells were exposed to 1 U/ml thrombin (thr) or 10 µM TRag for 10 min, 30 min, 60 min or 120 min. Representative blots are shown for p38 phosphorylation (upper panel) and total p38 (lower panel). **B.** Serum-starved cells were preincubated with p38 MAPK inhibitor SB203580 (10 µM) for 30 min prior to 10 min stimulation with 1 U/ml thrombin (thr) or 10 µM TRag. Representative blots are shown for JNK phosphorylation (upper panel) and total JNK (lower panel). **C**. The phosphorylation of 46 kDa JNK isoform was normalized relative to the total 46 kDa JNK isoform. Data shown in C represent the mean ± SEM of at least three independent experiments.

3.2.1.2 JNK activation, but not p38 or ERK1/2, is involved in PAR-2-induced GRO/CINC-1 release

To investigate whether PAR-2-induced GRO/CINC-1 release was mediated by a similar mechanism, we applied U0126 (MEK1/2 inhibitor), SB203580 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor) to astrocytes for 30 min prior to a 3 h PAR-2AP stimulation. As Fig. 3.9 shows, PAR-2-induced GRO/CINC-1 release was almost completely abolished by the JNK specific inhibitor SP600125. However, inhibitors for MEK1/2 and p38 MAPK did not significantly affect PAR-2-induced GRO/CINC-1 release, which clearly differed from that of PAR-1-induced GRO/CINC-1 secretion. These results suggest that JNK, but not MEK1/2 or p38 MAPK, is an important mediator for PAR-2-induced GRO/CINC-1 release.

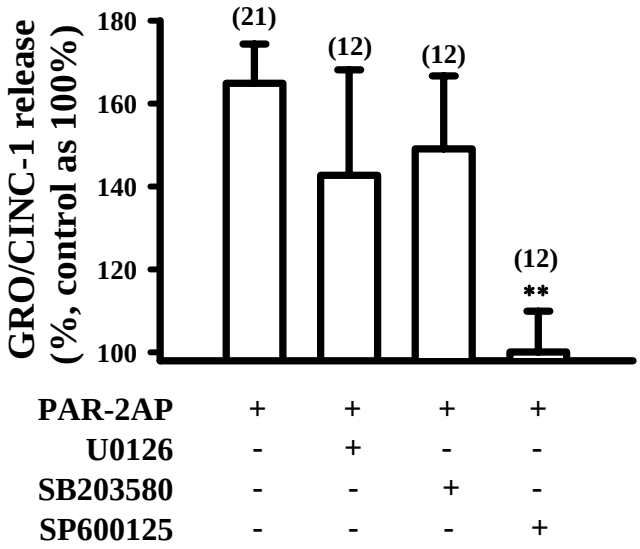


Fig. 3.9 JNK activation mainly contributes to PAR-2-induced GRO/CINC-1 release from astrocytes. Serum-starved cells were preincubated with MEK1/2 inhibitor U0126 (25 μM), p38 MAPK inhibitor SB203580 (10 μM) or JNK inhibitor SP600125 (30 μM) for 30 min prior to 3 h-stimulation with 500 μM PAR-2AP. Astrocytes without any treatment were taken as baseline (100%). Data represent the mean ± SEM. Numbers given above the respective column represent the number of samples. ** $p < 0.01$ as compared to the cells exposed to PAR-2AP alone.

Next, we tested JNK activation with PAR-2 agonist stimulation in astrocytes to further confirm that JNK is involved in the PAR-2 signaling pathway. As Fig. 3.10 A-C shows, both JNK isoforms with 43 kDa and 46 kDa were time-dependently phosphorylated upon treatment with trypsin (10 nM). However, the JNK isoform with 54 kDa was not phosphorylated after trypsin treatment. These results are in line with our data that PAR-1 activation induced by thrombin resulted in 43 kDa and 46 kDa JNK isoforms phosphorylation, but not 54 kDa JNK isoform (Fig. 3.5). Trypsin, a main agonist of PAR-2, has been reported to activate PAR-1 as well (Ossovskaya and Bunnett, 2004). To rule out any unspecific proteolytic activity of trypsin and its possible effect on PAR-1 activation, the specific PAR-2AP was also applied here. PAR-2AP (500 μM) stimulation significantly induced the phosphorylation of JNK isoform with 43 kDa, but not 46 kDa, which occurred in a time-dependent manner. The pronounced activation of JNK isoforms induced by PAR-2AP and trypsin were both observed at 10 min. Unlike PAR-2AP-induced JNK phosphorylation, the PAR-1 specific stimulation with TRag activated JNK isoforms with both 46 kDa and 43 kDa (Fig. 3. 10D). These results suggest that the 43 kDa JNK isoform, but not the 46 kDa isoform, is activated upon PAR-2 activation, revealing a clear difference between PAR-1- and PAR-2-induced JNK activation.

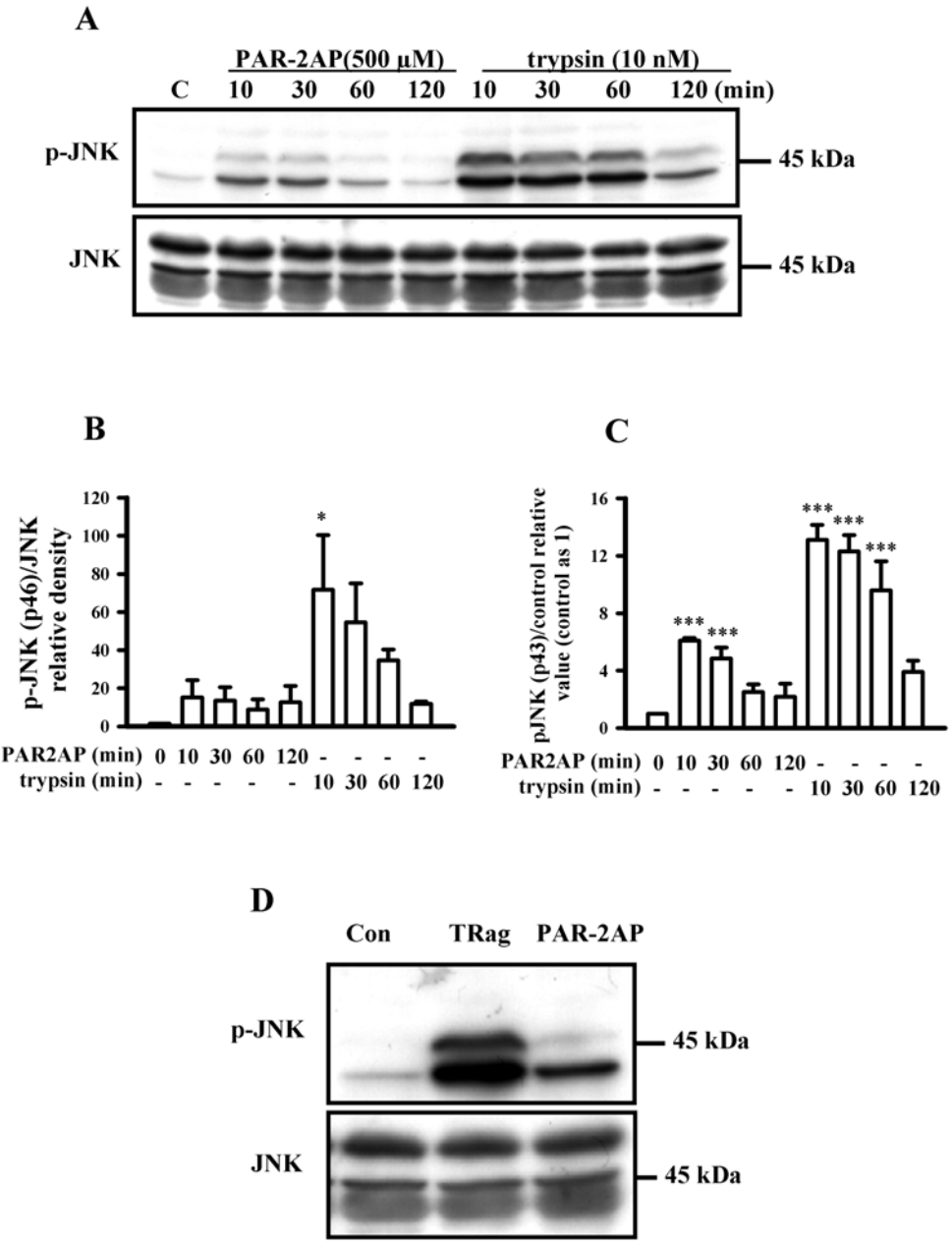


Fig. 3.10. PAR-2AP- and trypsin-induced JNK phosphorylation in rat astrocytes. Serum-starved cells were exposed to 500 μM PAR-2AP or 10 nM trypsin for 10 min, 30 min, 60 min or 120 min. **A**. Representative blots are shown for JNK phosphorylation (upper panel) and total JNK (lower panel). **B**. The phosphorylation of 46 kDa JNK isoform was normalized relative to the total 46 kDa JNK isoform. **C**. The phosphorylation of 43 kDa JNK isoform was normalized relative to that of control. **D**. Serum-starved cells were stimulated by 10 μM TRag or 500 μM PAR-2AP for 10 min. Representative blots are shown for JNK phosphorylation (upper panel) and total JNK (lower panel). Data shown in B & C represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, * $p < 0.05$ as compared to control.

3.2.2 Different upstream components mediate PAR-1- and PAR-2-induced JNK activation

3.2.2.1 Upstream components of JNK activation in PAR-1-mediated GRO/CINC-1 release

PKC and PI3K are two important activators for MAPK activation in many different cells including astrocytes (Wang et al., 2002b; Muscella et al., 2003). It has been known that PTX can inactivate the G_i/G_o family but does not affect G_s, G_q and $G_{12/13}$. To examine the cellular mechanisms of PAR-1-dependent JNK activation, astrocytes were pretreated with PTX for 24 h, GF109203X (PKC inhibitor), wortmannin or LY294002 (PI3K inhibitors) for 30 min prior to thrombin or TRag stimulation. As shown in Fig. 3.11, PTX had no effect on JNK phosphorylation. In contrast, wortmannin apparently blocked PAR-1-induced JNK phosphorylation (Fig. 3.11). In addition, LY294002 (50 μM) had similar effects as wortmannin did on JNK phosphorylation (data not shown). Also GF109203X (Fig. 3.12) inhibited the PAR-1-mediated JNK phosphorylation. These results demonstrate that PKC and PI3K play important roles in PAR-1-induced JNK activation.

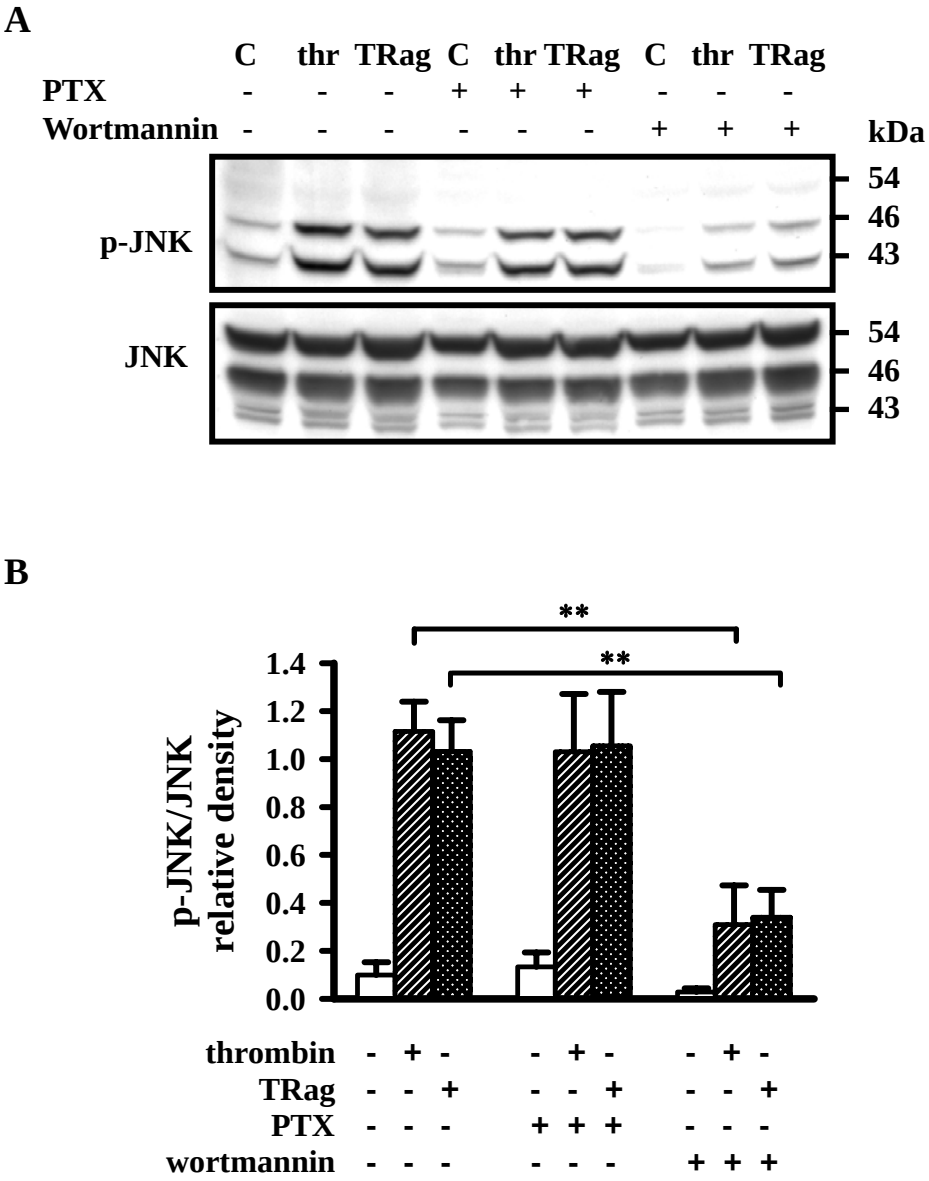


Fig. 3.11. Effects of PTX and wortmannin on thrombin- and TRag-induced JNK activation in astrocytes. Serum-starved cells were preincubated with PTX (200 ng/ml) for 24 h and PI3K inhibitor wortmannin (5 μM) for 30 min prior to 10 min-incubation with 1 U/ml thrombin (thr) or 10 μM TRag. **A**. Representative blots of phosphorylated JNK and total JNK from one experiment. **B**. The phosphorylation of 46 kDa JNK isoform was normalized relative to the total 46 kDa JNK isoform. Data shown in **B** represent the mean ± SEM of at least three independent experiments. ** $p < 0.01$ as compared to the cells exposed to thrombin or TRag alone.

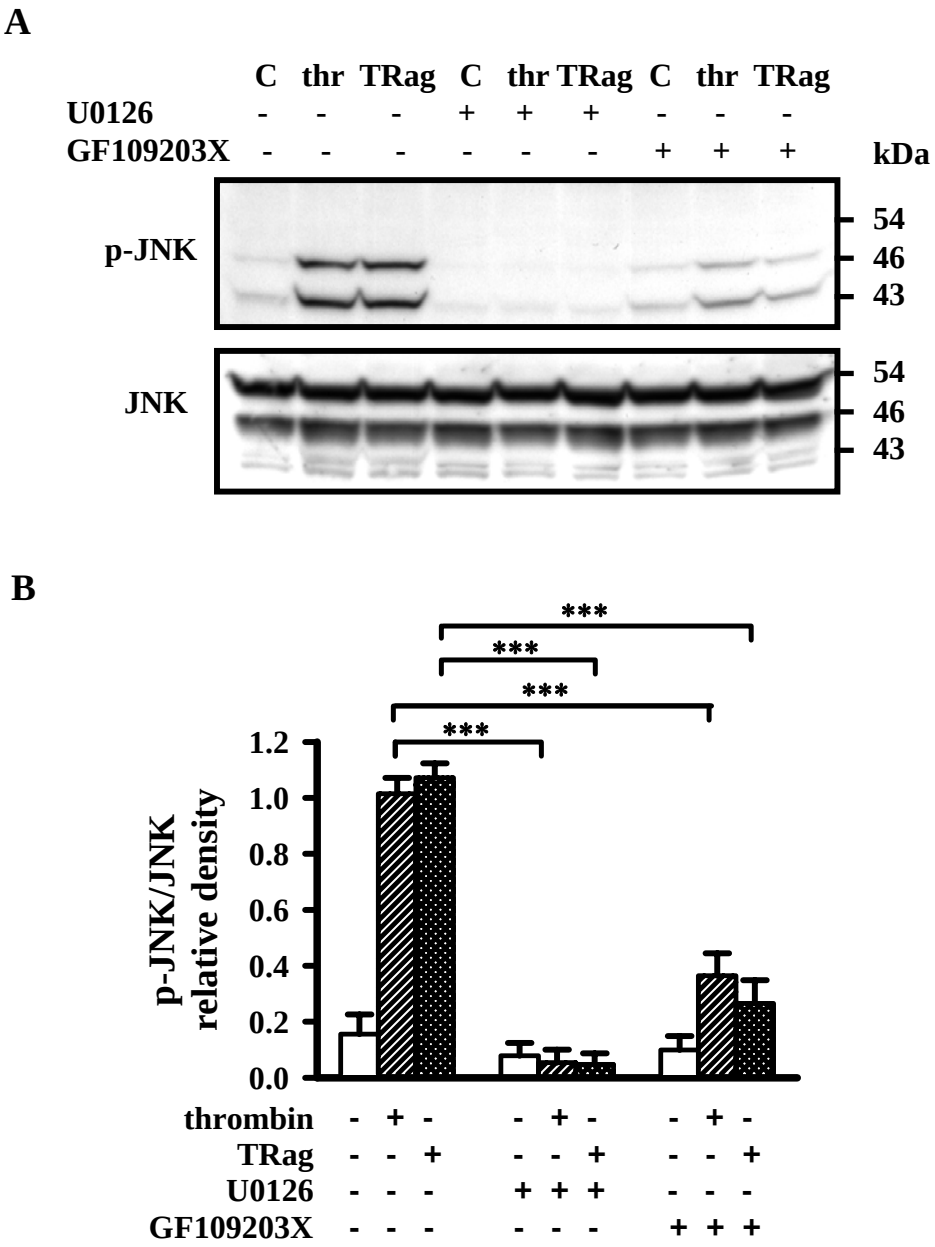


Fig. 3.12. Effects of U0126 and GF109203X on thrombin- and TRag-induced JNK activation in astrocytes. Cells were preincubated with MEK1/2 specific inhibitor U0126 (25 μM) or PKC inhibitor GF109203X (5 μM) for 30 min prior to 10 min-treatment with thrombin (thr, 1 U/ml) or TRag (10 μM). **A**. Representative blots of phosphorylated JNK and total JNK from one experiment. **B**. The phosphorylation of 46 kDa JNK isoform was normalized relative to the total 46 kDa JNK isoform. Data shown in **B** represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$ as compared to the cells exposed to thrombin or TRag alone.

In parallel, the effects of PKC and PI3K on PAR-1-induced GRO/CINC-1 release were also studied, by treating astrocytes with GF109203X and wortmannin prior to TRag. As shown in Fig. 3.13, both GF109203X and wortmannin almost completely abolished the TRag-induced GRO/CINC-1 release. These results suggest that PKC- and PI3K-mediated JNK activation is essential for PAR-1-induced GRO/CINC-1 secretion.

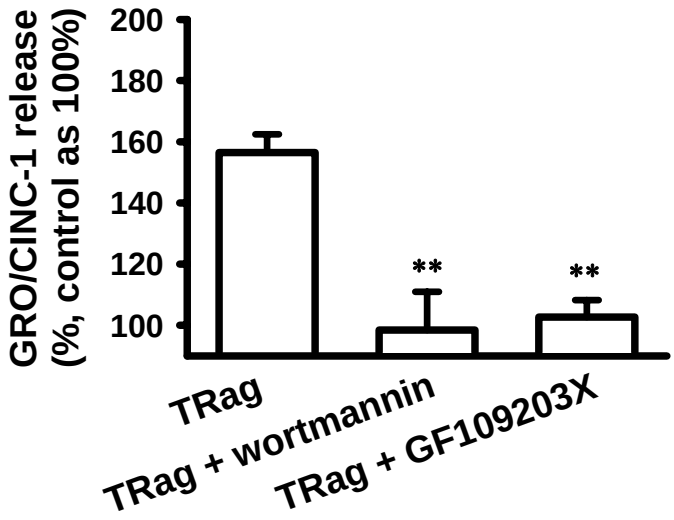


Fig. 3.13. Treatment with wortmannin and GF109203X prevents the release of GRO/CINC-1 induced by PAR-1 activation. Serum-starved cells were preincubated with PI3K inhibitor wortmannin (5 μM) or PKC inhibitor GF109203X (5 μM) for 30 min prior to 3 h-stimulation with 10 μM TRag. Astrocytes without any treatment were taken as baseline (100%). Cells treated only with TRag served as positive control for GRO/CINC-1 release. Data represent the mean ± SEM. ** $p < 0.01$ as compared to the cells exposed to TRag alone.

In Fig. 3.4, we show that blocking the activity of MEK1/2 by U0126 reduced GRO/CINC-1 release to the level in untreated cells. It was reported previously that the specific MEK1/2 inhibitor U0126 could inhibit its downstream kinase ERK1/2, but not JNK (Alessi et al., 1995; Favata et al., 1998). Thus, blocking the activity of ERK1/2 induced by U0126 should be responsible for the reduction of GRO/CINC-1 release.

However, recently it was reported that U0126 treatment blocked both ERK1/2 and JNK phosphorylation by affecting their common upstream kinase MEKK1 (Waetzig and Herdegen, 2005). To clarify whether the U0126-induced reduction of GRO/CINC-1 release is dependent on the JNK pathway, we investigated possible effects of U0126 on JNK phosphorylation. Our data in Fig. 3.12 show that U0126 treatment almost completely abolished PAR-1-mediated JNK activation, indicating that U0126-mediated JNK inhibition should be also responsible for the reduction of GRO/CINC-1 release. In summary, these results suggest that JNK activation, mediated by PTX-insensitive G protein, PI3K and PKC, is the main contributor for PAR-1-induced GRO/CINC-1 release.

3.2.2.2 Upstream components of JNK activation in PAR-2-mediated GRO/CINC-1 release

PKC and PI3K inhibition, but not PTX, significantly reduced PAR-1-induced JNK phosphorylation, suggesting that PKC and PI3K are two main upstream activators of PAR-1-induced JNK phosphorylation (Figs. 3.11 and 3.12). However, PAR-2-induced JNK activation was apparently not affected by treatment with the PKC inhibitor GF109203X (Fig. 3.14). Application of the PI3K inhibitor wortmannin only partially reduced PAR-2-induced JNK activation (Fig. 3.14), and PTX treatment also slightly reduced PAR-2-induced JNK phosphorylation (Fig. 3.14). These results suggest that PAR-2-induced JNK activation was partially mediated by PTX-sensitive G protein and PI3K activation, but apparently independent of PKC activation. This indicates that the upstream components of JNK activation induced by PAR-1 and PAR-2 are remarkably different.

Both ELISA and western blot data above show that PAR-1-dependent PKC and PI3K activation not only mediated JNK phosphorylation, but also contributed to GRO/CINC-1 release (Figs. 3.11, 3.12 and 3.13). However, inhibitors for PKC and PI3K did not significantly affect PAR-2-induced GRO/CINC-1 release (Fig. 3.15). These results suggest that PAR-2-induced GRO/CINC-1 release is apparently independent of either PKC or PI3K activation. Therefore, PI3K-mediated partial JNK activation should not be essential for PAR-2-induced GRO/CINC-1 release.

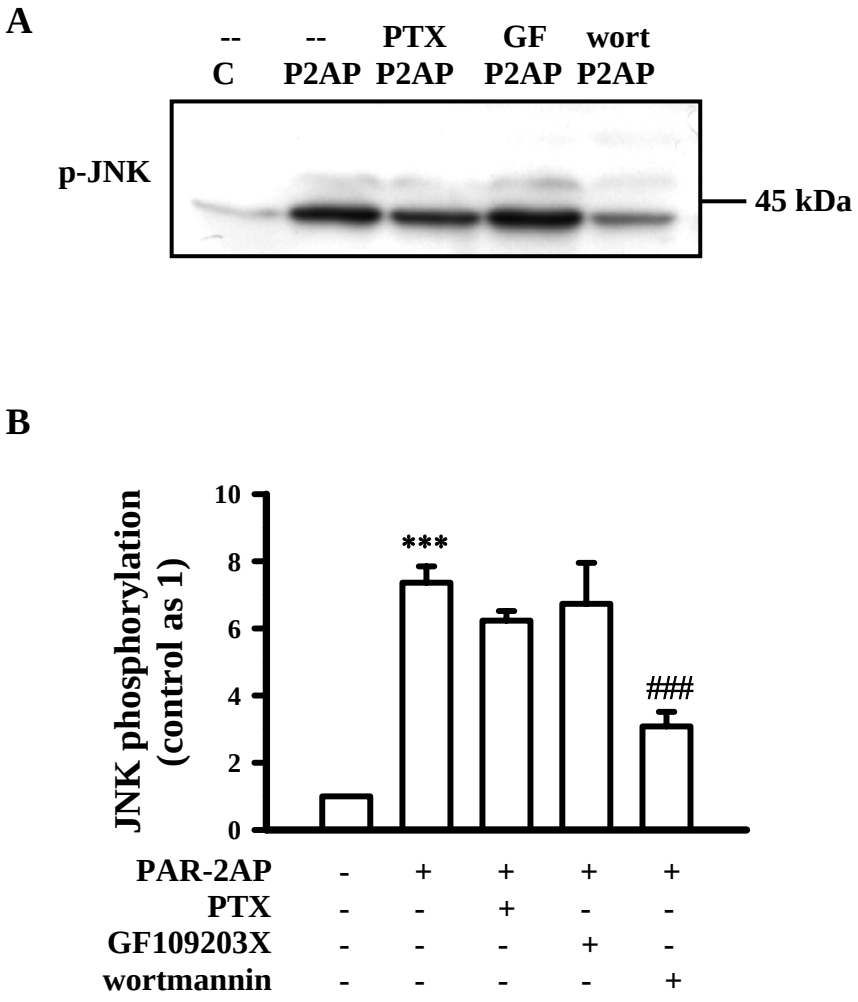


Fig. 3.14. Effects of PTX, GF109203X and wortmannin on PAR-2AP-induced JNK activation in astrocytes. Serum-starved cells were preincubated with PTX (200 ng/ml) for 24 h and PKC inhibitor GF109203X (5 μM) or PI3K inhibitor wortmannin (5 μM) for 30 min prior to 10 min-incubation with 500 μM PAR-2AP. **A**. Representative blot of JNK phosphorylation from one experiment. **B**. The phosphorylation of 43 kDa JNK isoform was normalized relative to that of control. Data shown in **B** represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$ as compared to control; ### $p < 0.001$ as compared to the cells exposed to PAR-2AP alone.

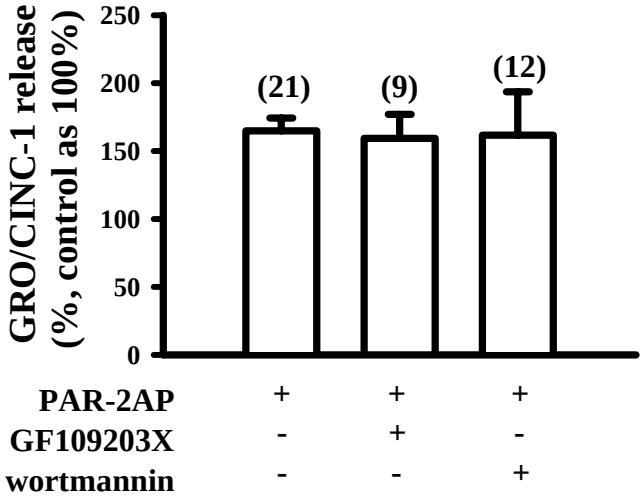


Fig. 3.15. Activation of PI3K and PKC is not involved in PAR-2-induced GRO/CINC-1 release from astrocytes. Serum-starved cells were preincubated with PI3K inhibitor wortmannin (5 μM) or PKC inhibitor GF109203X (5 μM) for 30 min prior to 3 h-stimulation with 500 μM PAR-2AP. Astrocytes without any treatment were taken as baseline (100%). Data represent the mean ± SEM. Numbers given above the respective column represent the number of samples.

3.2.3 PAR-1- and PAR-2-induced JNK activation have different effects on c-Jun phosphorylation

We have found that both PAR-1 agonists (low concentrations of thrombin and TRag) and PAR-2 agonists (trypsin and PAR-2AP) could significantly activate JNK. JNK has been reported in many other studies to further stimulate c-Jun. Therefore, here we investigated whether c-Jun can be activated by thrombin, TRag, trypsin or PAR-2AP treatment, and further studied whether c-Jun directly regulated GRO/CINC-1 expression.

As shown in Fig. 3.16 A and B, c-Jun was time-dependently phosphorylated by thrombin and TRag stimulation. The strongest activation was obtained at 60 min. Moreover, the total content of c-Jun protein was also upregulated following PAR-1 activation (Fig. 3.16 A, C). The expression of c-Jun was increased at 30 min, peaked at 60 min, and then c-Jun started to decrease at 120 min stimulation. Further experiments revealed that c-Jun phosphorylation was concentration-dependently reduced by the specific JNK inhibitor SP600125 (Fig. 3.17 A, B). However, the total amount of c-Jun protein was not affected by the JNK blocker (Fig. 3.17 A). These results reveal that PAR-1 is able to activate c-Jun, which is mainly mediated by JNK activation.

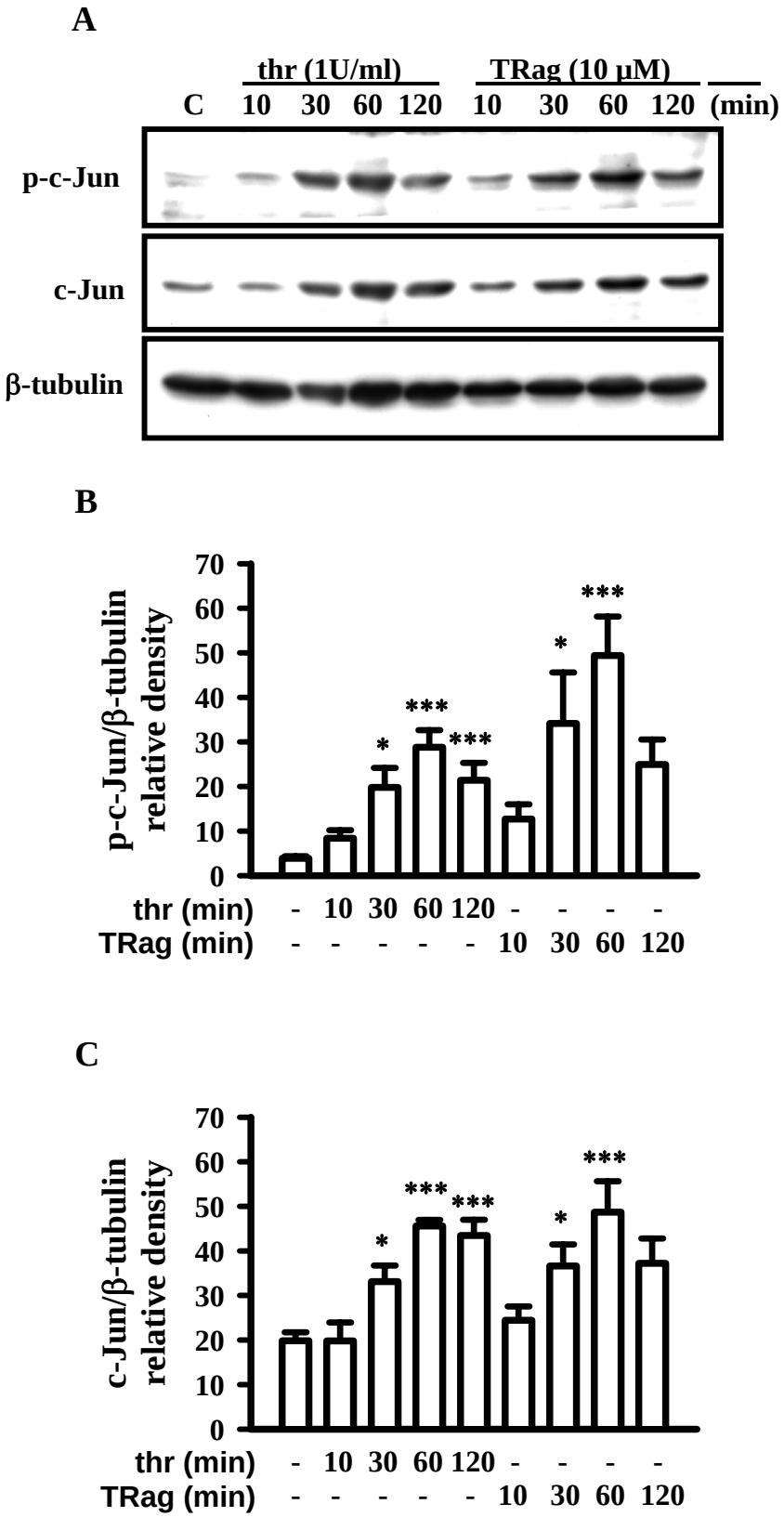


Fig. 3.16. Activation of c-Jun induced by thrombin and TRag. Serum-starved cells were exposed to 1 U/ml thrombin (thr) and 10 μM TRag for 10 min, 30 min, 60 min or 120 min. **A**. Representative blots from one experiment are shown for c-Jun phosphorylation (upper panel), total c-Jun (middle panel) and β-tubulin (lower panel). **B & C**. The amount of c-Jun phosphorylation (B) and total c-Jun (C) were normalized relative to β-tubulin. Data shown in **B** and **C** represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, * $p < 0.05$ as compared to control.

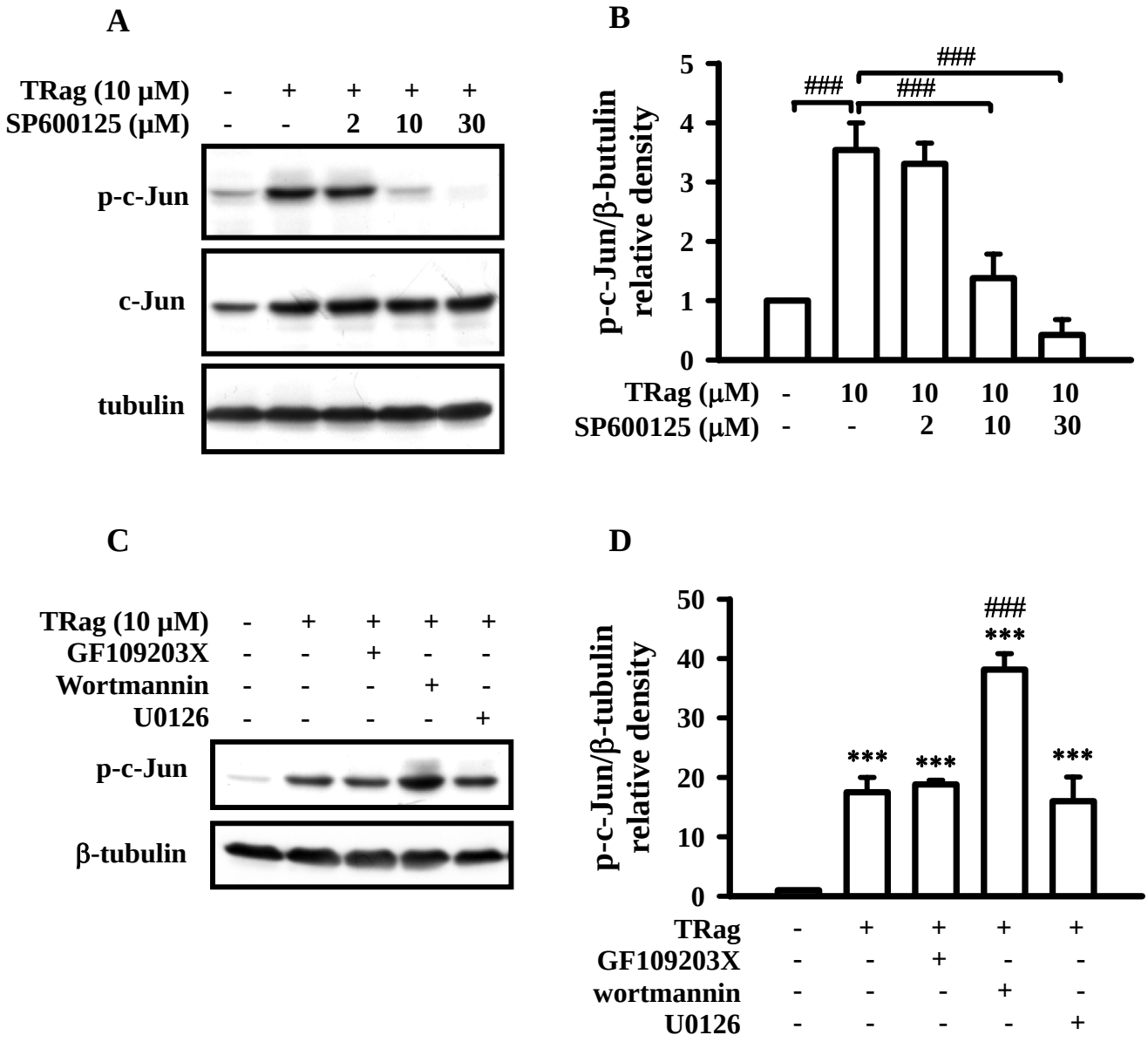


Fig. 3.17. Effects of SP600125, GF109203X, wortmannin and U0126 on TRag-induced c-Jun activation. Serum-starved cells were preincubated with specific JNK inhibitor SP600125 (2 μM, 10 μM and 30 μM), PKC inhibitor GF109203X (5 μM), PI3K inhibitor wortmannin (5 μM) or MEK1/2 inhibitor U0126 (25 μM) for 30 min prior to 1 h-treatment with 10 μM TRag. Astrocytes without any treatment were taken as control, cells treated only with TRag served as positive control. Representative blots from one experiment (**A** & **C**) and quantification (**B** & **D**). Data shown in B and D represent the mean ± SEM of at least three independent experiments. ### $p < 0.001$ as compared to the cells exposed to TRag alone, *** $p < 0.001$ as compared to control.

However, inhibitors of PKC, PI3K and MEK1/2, which apparently blocked JNK activation, did not reduce TRag-induced c-Jun phosphorylation (Fig. 3.17 C & D). In contrast, the PI3K inhibitor wortmannin even doubled the TRag-induced c-Jun phosphorylation. As the ELISA data above showed, PKC, PI3K and MEK1/2 were required for the PAR-1-induced release of GRO/CINC-1 (Fig. 3.4, 3.13). Taken together, these results imply that PAR-1-induced c-Jun activation might be not essential for GRO/CINC-1 release, which is in line with the fact that the potential AP-1 binding site does not exist in the promoter region of GRO/CINC-1 (Shibata et al., 1998).

Unlike PAR-1-induced JNK activation, we found that JNK activation induced by the specific PAR-2AP failed to further phosphorylate c-Jun, although the serine protease trypsin could slightly induce c-Jun phosphorylation (Fig. 3.18). These results imply that different JNK isoforms might be activated in the PAR-1 and PAR-2 signaling pathways.

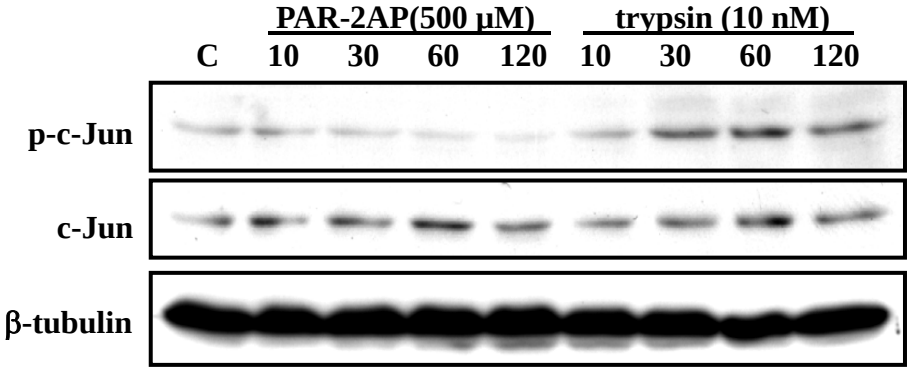


Fig. 3.18. c-Jun phosphorylation after PAR-2 activation. Serum-starved cells were exposed to 500 μM PAR-2AP or 10 nM trypsin for 10 min, 30 min, 60 min or 120 min. Representative blots from one experiment are shown for c-Jun phosphorylation (upper panel), total c-Jun (middle panel) and β-tubulin (lower panel).

To further investigate whether different JNK isoforms with distinct properties were activated in the PAR-1 and PAR-2 signaling pathways, we examined and compared the JNK activity by using JNK kinase assay. We stimulated astrocytes for different times varying from 10 min to 2 h. Then the cell extracts were incubated with c-Jun-GST fusion protein immobilized on glutathione agarose beads, which was used to pull down JNK enzyme from cell extracts. Upon addition of kinase buffer and ATP, activated JNK could

phosphorylate the substrate c-Jun. The JNK activity was determined by checking c-Jun phosphorylation by immunoblotting. As shown in Fig. 3.19, TRag-induced JNK activation time-dependently phosphorylated c-Jun. However, PAR-2AP-induced JNK activation was unable to phosphorylate c-Jun. These results indicate that different JNK isoforms with distinct activities are possibly involved in either PAR-1 or PAR-2 signaling pathways. Therefore, the roles of different JNK isoforms in the PAR-1 and PAR-2 pathways were investigated further.

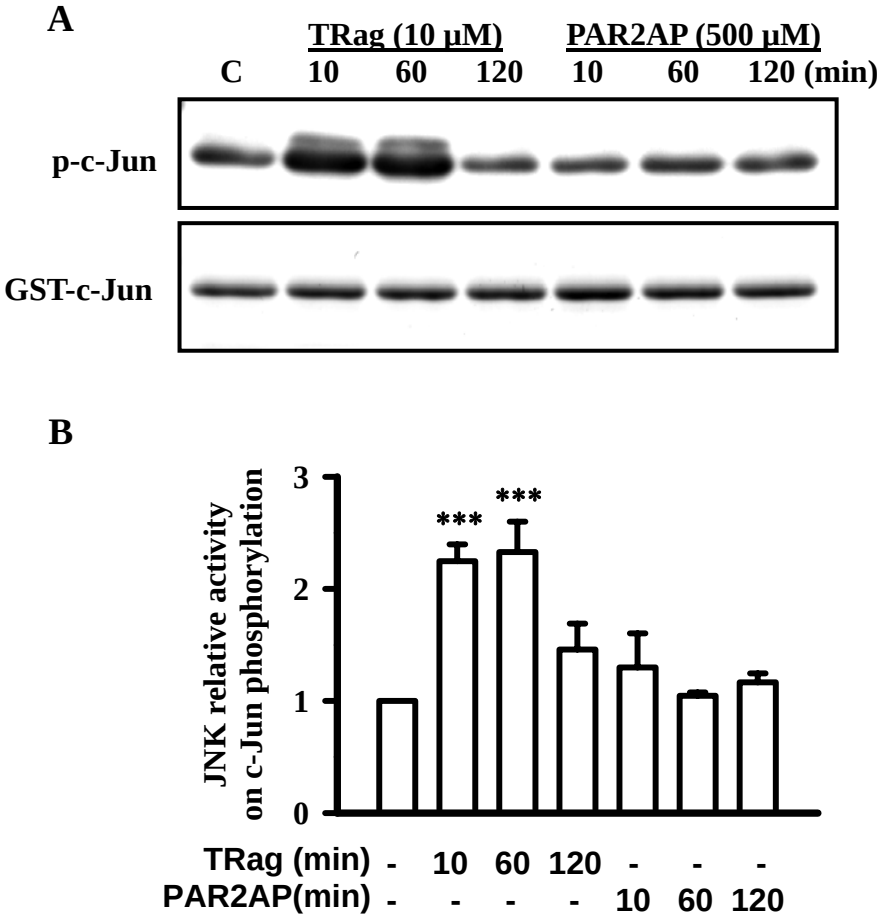


Fig. 3.19. Time course of JNK activity in response to TRag or PAR-2AP in rat astrocytes. A. JNK activity in response to 10 μM TRag or 500 μM PAR-2AP for indicated time was evaluated by nonradioactive kinase assay. Upper panel shows the phosphorylation of c-Jun induced by TRag and PAR-2AP time-dependently; lower panel demonstrates the equal loading of each sample by coomassie blue staining. **B**. The amount of c-Jun phosphorylation was normalized relative to that of control. Data shown in B represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$ as compared to the control group.

3.2.4 The role of calcium in GRO/CINC-1 release from astrocytes

Previous studies from our laboratory have already shown that PAR activation could increase the cytosolic Ca^{2+} level by calcium release from intracellular stores and calcium influx from the extracellular space in astrocytes as well as in oligodendrocytes (Ubl et al., 1998; Wang et al., 2002a; Wang et al., 2004). To investigate the role of calcium in PAR-1- and PAR-2-induced GRO/CINC-1 release in rat astrocytes, we applied U73122 (PLC antagonist, 10 μM) and 2-APB (the inositol 1, 4, 5-trisphosphate ($InsP_3$) receptor antagonist, 100 μM) to prevent calcium release from intracellular stores, and SKF96365 (calcium entry inhibitor, 50 μM) to prevent calcium influx from the extracellular space. U73343 (10 μM), an inactive control analog of U73122, served as the negative control. In addition, the role of calcium-dependent PKC was also studied here using its inhibitor Go6976 (1 μM). All inhibitors or antagonists were applied to astrocytes for 30 min prior to 3 h stimulation with TRag (10 μM) or PAR-2AP (500 μM).

As shown in Fig. 3.20 A, U73122, 2-APB, and SKF96365 did not attenuate PAR-1-induced GRO/CINC-1 release. In contrast, blocking the calcium release from intracellular stores especially by the inhibitor U73122, doubled the PAR-1-induced GRO/CINC-1 release from astrocytes. 2-APB, however, did not exert any effect. Preventing calcium influx by SKF96365 also significantly increased PAR-1-induced GRO/CINC-1 release. On the other side, we found that PAR-2-induced GRO/CINC-1 release was significantly elevated by the treatment with U73122, 2-APB or SKF96365 (Fig. 3.20 B). However, the calcium dependent PKC inhibitor Go6976 did not exert any effect in both PAR-1- and PAR-2-induced GRO/CINC-1 secretion (Fig. 3.20 B). It seems that a calcium rise probably could negatively regulate GRO/CINC-1 release. However, the connection between the mechanism underlying the calcium regulation and the other biochemical pathways described here still has to be investigated in further detail.

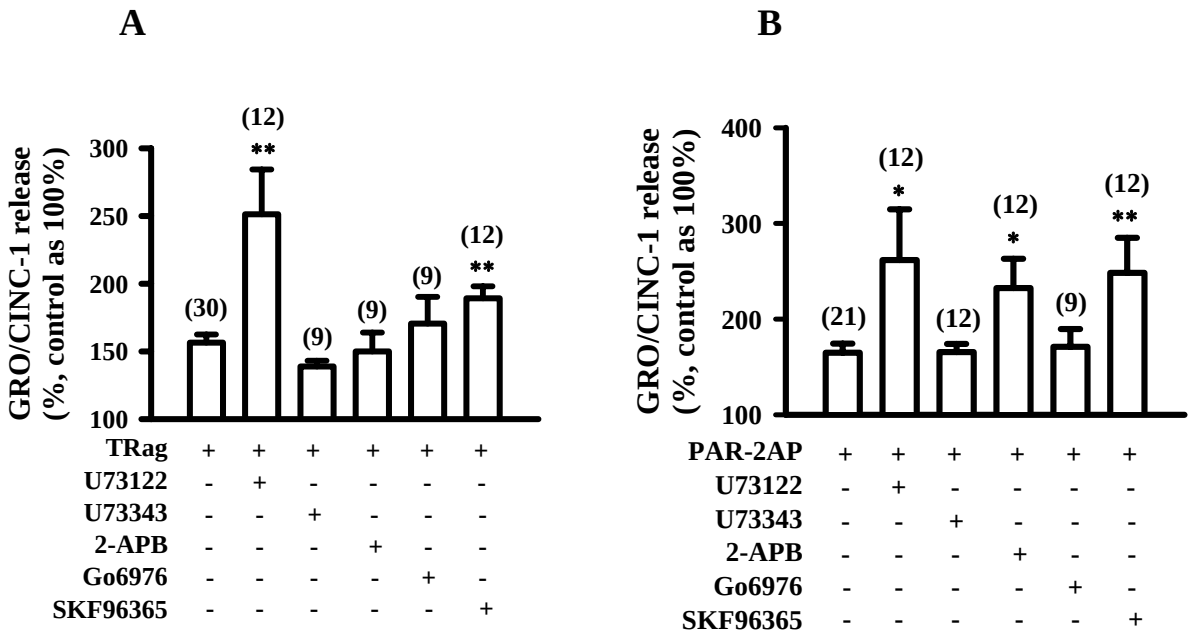


Fig. 3.20. The role of calcium in GRO/CINC-1 release from astrocytes. Serum-starved cells were preincubated with U73122 (PLC antagonist, 10 μM), U73343 (an inactive control analog of U73122, 10 μM), the $InsP_3$ receptor antagonist 2-APB (100 μM), calcium-dependent PKC inhibitor Go6976 (1 μM) and calcium entry inhibitor SKF96365 (50 μM) for 30 min prior to 3 h-stimulation with 10 μM TRag or 500 μM PAR-2AP. Astrocytes without any treatment were taken as baseline (100%). Cells treated only with TRag or PAR-2AP served as positive control for GRO/CINC-1 release. Data represent the mean ± SEM. Numbers given above the respective column represent the number of samples. ** $p < 0.01$, * $p < 0.05$ as compared to the cells exposed to TRag or PAR-2AP alone.

3.3 Three JNK isoforms differentially regulate PAR-1- and PAR-2-induced GRO/CINC-1 secretion

3.3.1 Expression of JNK isoforms in astrocytes

JNK activation apparently plays a pivotal role in both PAR-1- and PAR-2-induced GRO/CINC-1 release from astrocytes, as our data above show. It is well known that there are three JNK genes (*jnk1*, *jnk2* and *jnk3*) expressed in different tissues (Sugden and Clerk, 1998; Barr and Bogoyevitch, 2001; Enomoto et al., 2003). However, it is still not known whether all three JNK isoforms are expressed in astrocytes. Therefore, before investigating which JNK isoforms are specifically activated in the PAR-1 and PAR-2 signaling pathways, here we firstly study the expression of three JNK isoforms in astrocytes. RT-

PCR was performed using specific primers designed according to the respective conserved motif for *jnk1*, *jnk2* and *jnk3*, which could recognize different sub-isoforms of the individual gene but could not cross-recognize isoforms of the two other genes (Table 2.1). As shown in Fig. 3.21 A, all three JNK isoforms are expressed in astrocytes. The PCR products of JNKs were confirmed by DNA sequencing. In addition, all three JNK isoforms were also detected at the protein level in astrocytes (Fig. 3.21 B).

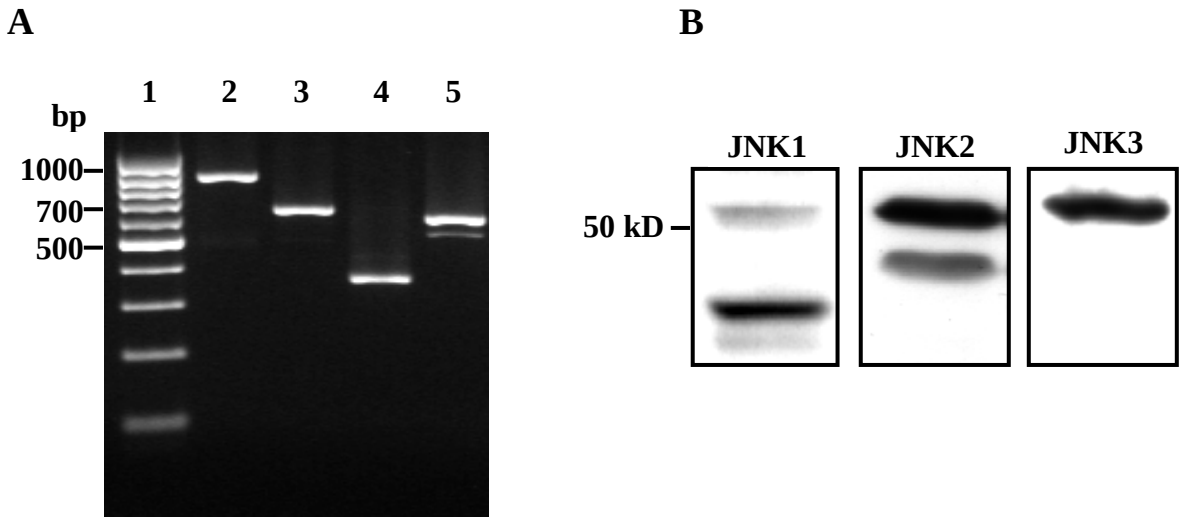


Fig. 3.21. Expression of JNK isoforms in rat astrocytes. A. RT-PCR determination of JNK1, JNK2 and JNK3 expression. Lane 1-5 represent Marker, GAPDH, JNK1, JNK2 and JNK3, respectively. **B**. Protein expression of JNK1, JNK2 and JNK3 in rat astrocytes by western blot.

3.3.2 Knocking down of the three JNK isoforms in astrocytes

To investigate the distinct physiological roles of JNK isoforms, we applied JNK siRNA to knock down the respective JNK isoforms. Specific JNK siRNAs were designed from respectively conserved motifs for JNK1, JNK2 and JNK3, which could recognize different sub-isoforms of the respective gene but could not cross-recognize isoforms of the two other genes (Table 2.2). Primary cultured astrocytes were transfected with JNK siRNAs using Magnet Assisted Transfection. As Fig. 3.22 A shows, JNK1 siRNA#1 significantly knocked down the expression of JNK1 without interfering with JNK2 or JNK3 mRNA expression. Similarly, JNK2 siRNA#1 and JNK3 siRNA#1 also only specifically knocked down their own mRNA expression. The transfection of non-silencing siRNA, which was given as scrambled siRNA control, did not disturb the mRNA

expression of either of the three JNK isoforms or the house-keeping gene GAPDH. On the other hand, the effects of JNK siRNAs were also studied at the protein level by western blot. As shown in Fig. 3.22 B, JNK1 siRNA#1, JNK2 siRNA#1 and JNK3 siRNA#1 concentration-dependently reduced the expression of the respective JNK isoform, without interfering with the expression of the two other JNK isoforms. Scrambled siRNA control also did not affect the expression of the three JNK isoforms, confirming the specificity of three JNK siRNAs. Similar to JNK1-3 siRNA#1, other JNK siRNAs (including JNK1

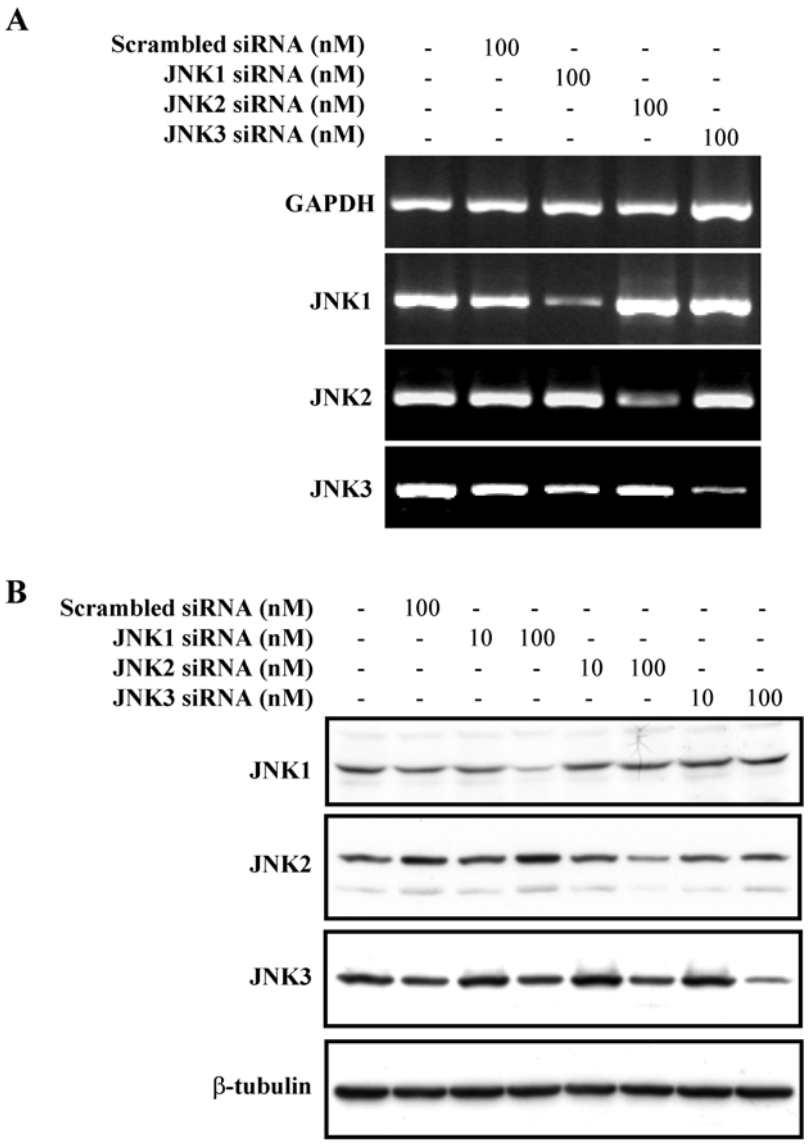


Fig. 3.22. Down-regulation of JNK1, JNK2 and JNK3 expression by specific siRNAs in astrocytes. A. The mRNA levels of JNK isoforms detected by RT-PCR after transfection with scrambled siRNA or JNK1-3 siRNAs for 48 h. **B**. The protein levels of JNK isoforms detected by western blot after transfection with scrambled siRNA or JNK1-3 siRNAs for 48 h.

siRNA#2, JNK2 siRNA#2-3 and JNK3 siRNA#2-3), which target to different sites of the three JNK isoforms, also specifically reduced the respective JNK isoforms at both mRNA and protein levels (Fig. 3.23). This further confirmed the specificity of the three JNK siRNAs. Taken together, these results indicate that siRNA for the three JNK isoforms at the indicated concentrations could specifically knock down the respective JNK isoforms at both mRNA and protein levels.

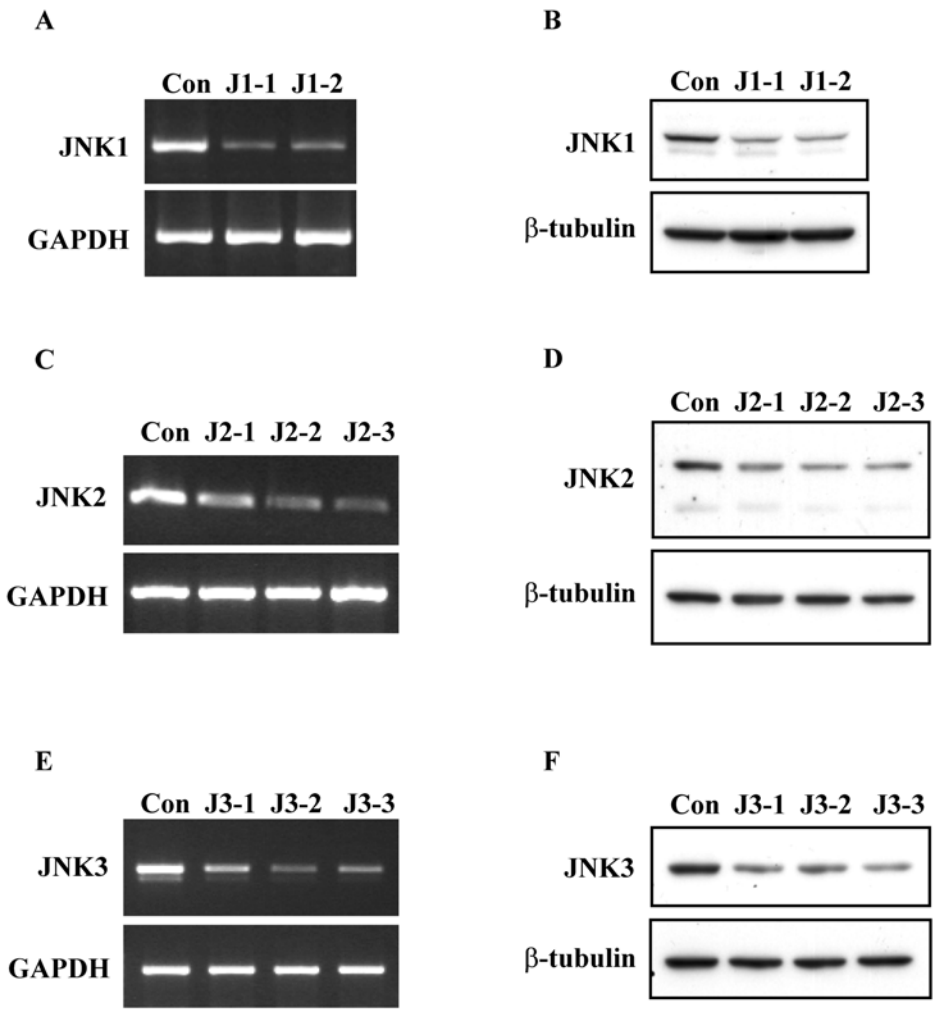


Fig. 3.23. Silencing of JNK1, JNK2 and JNK3 in astrocytes. The mRNA levels of JNK1 (A), JNK2 (C) and JNK3 (E) were detected by RT-PCR after transfection with JNK1 siRNA#1-2 (J1-1, J1-2, 100 nM), JNK2 siRNA#1-3 (J2-1, J2-2, J2-3, 100 nM) or JNK3 siRNA#1-3 (J3-1, J3-2, J3-3, 100 nM) for 48 h. The protein levels of JNK1 (B), JNK2 (D) and JNK3 (F) were detected by western blot after transfection with JNK1 siRNA#1-2 (100 nM), JNK2 siRNA#1-3 (100 nM) or JNK3 siRNA#1-3 (100 nM) for 48 h.

3.3.3 PAR-1 and PAR-2 activate different JNK isoforms in astrocytes

To investigate which JNK isoforms are involved in the PAR-1 signaling pathway, we examined JNK activation after knocking down certain JNK isoforms by siRNA. As Fig. 3.24 A1 & A2 shows, TRag-induced JNK phosphorylation of both 46 kDa and 43 kDa isoforms was apparently impaired in JNK1-deficient cells. Similarly, TRag-induced JNK phosphorylation, especially with the 46 kDa isoform, was remarkably eliminated in both JNK2-deficient and JNK3-deficient cells (Fig. 3.24 B1, B2, C1 and C2). These results suggest that all three JNK isoforms (JNK1, JNK2 and JNK3) are activated by PAR-1 agonist stimulation.

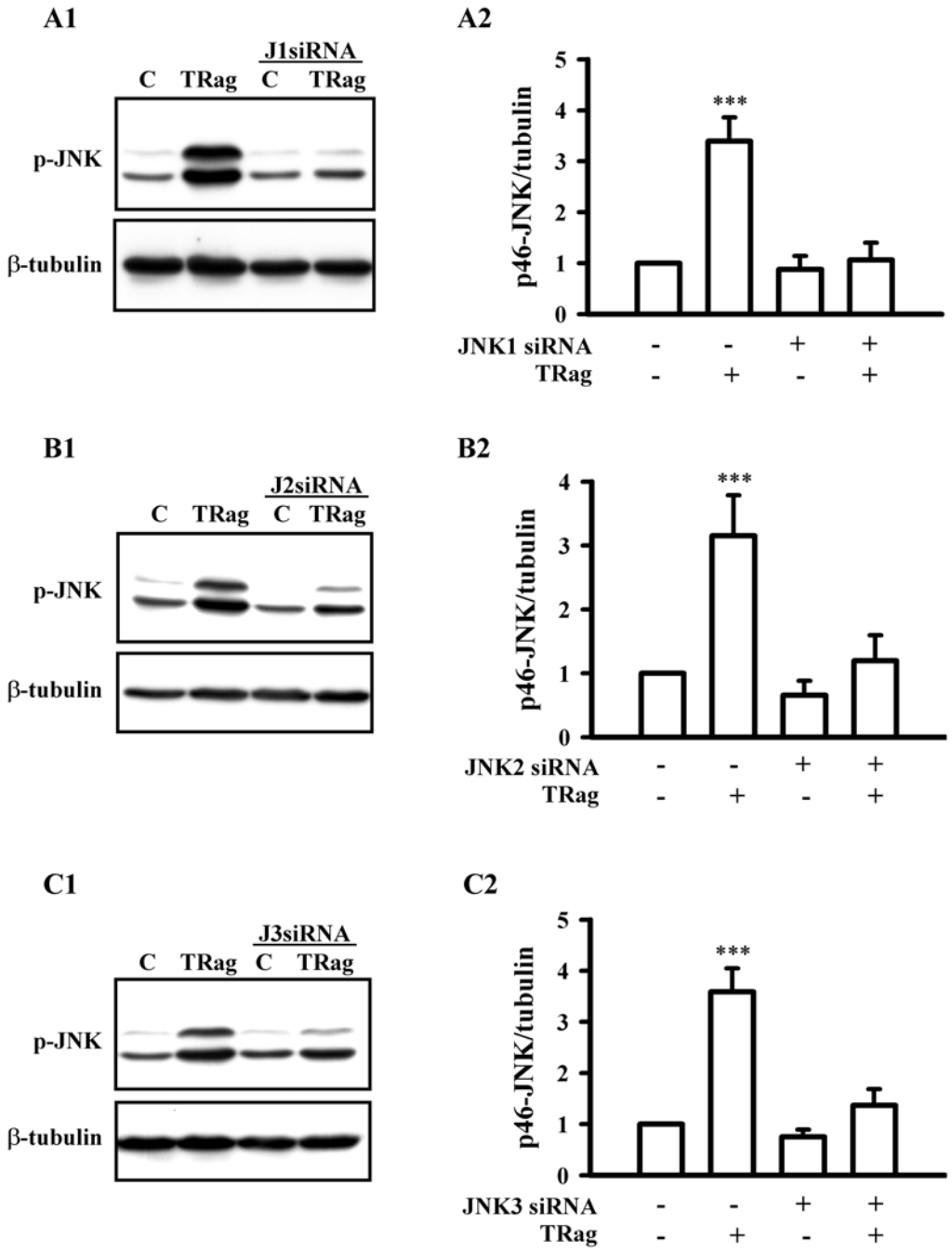


Fig. 3.24. Three different JNK isoforms are activated by PAR-1 in rat astrocytes. Cells, transfected with JNK1 siRNA#1 (J1 siRNA, 100 nM, **A1** & **A2**), JNK2 siRNA#1 (J2 siRNA, 100 nM, **B1** & **B2**) or JNK3 siRNA#1 (J3 siRNA, 100 nM, **C1** & **C2**) for 48 h, were treated with or without 10 μM TRag for 10 min. Cells without transfection served as control (C). **A1**, **B1** and **C1**, the representative blots for JNK phosphorylation (p-JNK, upper panel) and β-tubulin (lower panel). **A2**, **B2** and **C2,** the relative value of 46 kDa JNK phosphorylation normalized relative to β-tubulin. Data represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$ as compared to the control cells.

Similarly, by knocking down JNK expression with siRNA, we investigated which JNK isoforms were activated in the PAR-2 signaling pathway. As Fig. 3.25 A1 and A2 shows, the 43 kDa JNK isoform was significantly phosphorylated after 10 min PAR-2AP treatment in wild-type (WT) cells. However, PAR-2AP-induced JNK activation in JNK1-deficient cells was strikingly reduced compared to that in WT cells, although JNKs still can be significantly phosphorylated in these cells. Unlike the role of JNK1 in the PAR-2 signaling pathway, JNK2 knockdown with siRNA did not significantly affect PAR-2AP-induced JNK phosphorylation, although the phosphorylation of JNK induced by PAR-2 was slightly reduced compared to that of WT (Fig. 3.25 B1 & B2). However, PAR-2AP-induced JNK activation was significantly reduced by JNK3 knockdown (Fig. 3.25 C1 and C2). The reduction was comparable to that in JNK1-deficient cells. Taken together, these results indicate that JNK1 and JNK3, but not JNK2, are involved in the PAR-2 signaling pathway.

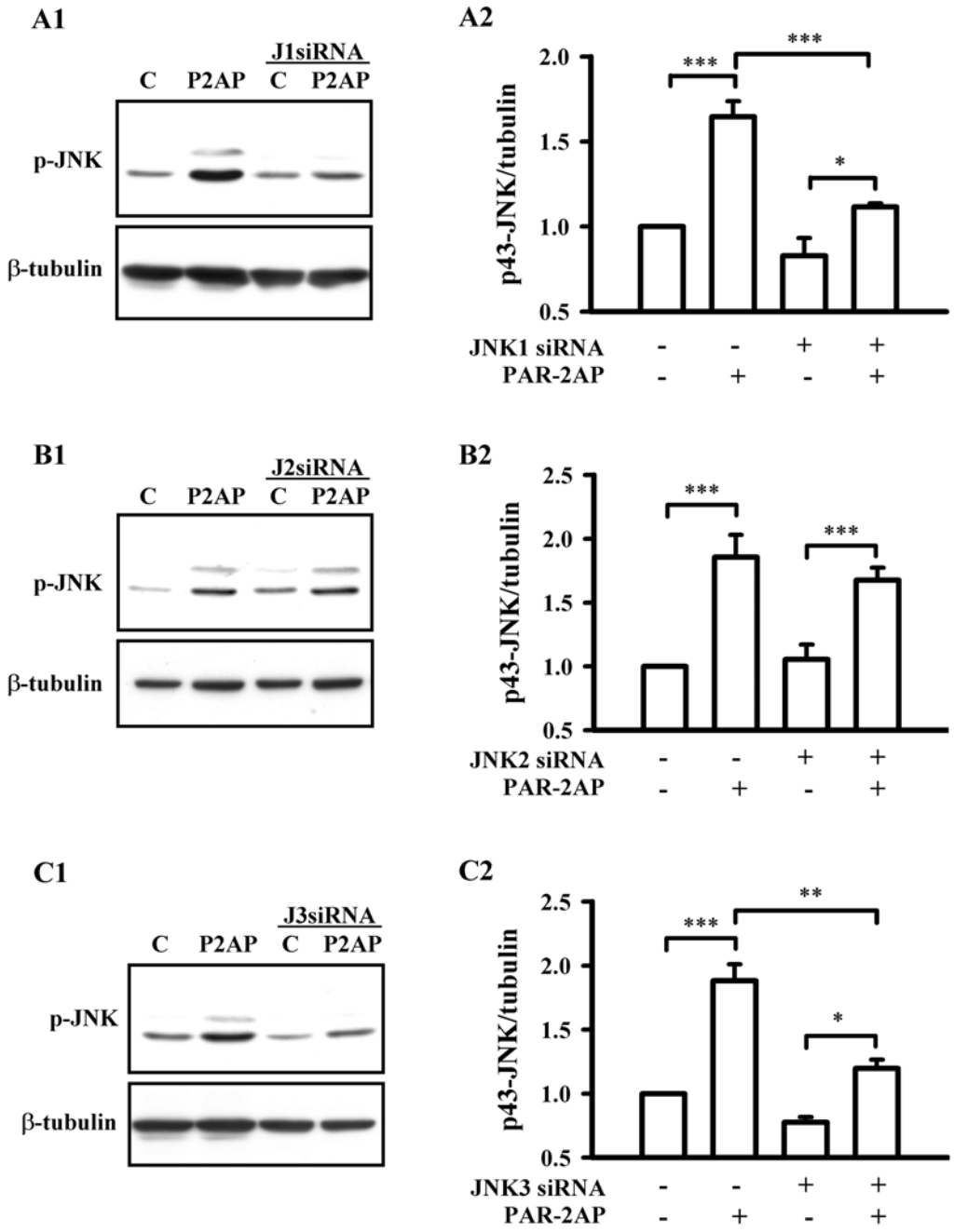


Fig. 3.25. JNK1 and JNK3 isoforms are activated by PAR-2 in rat astrocytes. Cells, transfected with JNK1 siRNA#1 (J1 siRNA, 100 nM, **A1** & **A2**), JNK2 siRNA#1 (J2 siRNA, 100 nM, **B1** & **B2**) or JNK3 siRNA#1 (J3 siRNA, 100 nM, **C1** & **C2**) for 48 h, were treated with or without 500 μM PAR-2AP (P2AP) for 10 min. Cells without transfection served as control (C). **A1**, **B1** and **C1,** the representative blots for JNK phosphorylation (p-JNK, upper panel) and β-tubulin (lower panel). **A2**, **B2** and **C2,** the relative value of 43 kDa JNK phosphorylation normalized relative to β-tubulin. Data represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

3.3.4 PAR-1-dependent JNK1 activation is responsible for c-Jun phosphorylation

To further elucidate which JNK isoform was responsible for PAR-1-dependent c-Jun phosphorylation, we compared the TRag-induced c-Jun phosphorylation between WT cells and certain JNK isoform-deficient cells. As Fig. 3.26 A1 & A2 shows, TRag stimulation (1 h) significantly increased c-Jun phosphorylation in WT cells, which was notably eliminated in JNK1-deficient cells. In JNK2-deficient cells, TRag stimulation was still able to significantly activate c-Jun (Fig. 3.26 B1 & B2). However, compared to that in WT cells, TRag-induced c-Jun phosphorylation in JNK2-deficient cells was slightly decreased. Interestingly, c-Jun phosphorylation was significantly enhanced even in non-stimulated JNK3-deficient cells compared to that in WT cells (Fig. 3.26 C1 & C2). Treatment with TRag could further slightly increase the phosphorylation of c-Jun in JNK3-deficient cells. These results suggest that PAR-1-induced JNK1 activation mainly mediates c-Jun phosphorylation, whereas JNK3 is able to negatively regulate c-Jun activation.

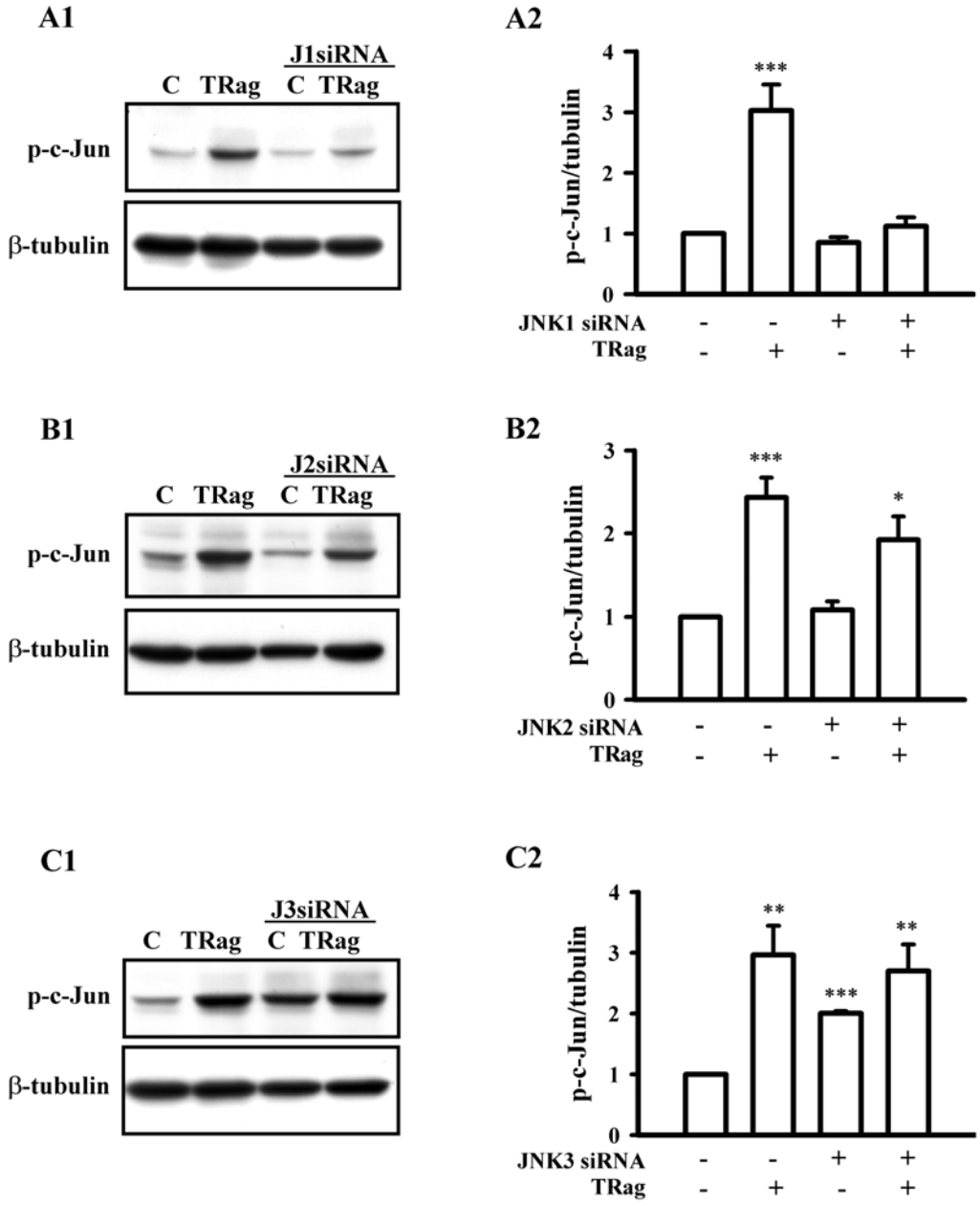


Fig. 3.26. Different effects of JNK isoforms on PAR-1-dependent c-Jun activation in rat astrocytes. Cells, transfected with JNK1 siRNA#1 (J1 siRNA, 100 nM, **A1** & **A2**), JNK2 siRNA#1 (J2 siRNA, 100 nM, **B1** & **B2**) or JNK3 siRNA#1 (J3 siRNA, 100 nM, **C1** & **C2**) for 48 h, were treated with or without 10 μM TRag for 1 h. Cells without transfection served as control (C). **A1**, **B1** and **C1, t**he representative blots for c-Jun phosphorylation (p-c-Jun, upper panel) and β-tubulin (lower panel). **A2**, **B2** and **C2,** the relative value of c-Jun phosphorylation normalized relative to β-tubulin. Data represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ as compared to the control cells.

3.3.5 Distinct JNK isoforms are essential for PAR-1- and PAR-2-induced GRO/CINC-1 secretion

Considering that diverse JNK isoforms are activated in both PAR-1 and PAR-2 signaling pathways, as our data above show, here we further investigated the effects of individual JNK isoforms on the chemokine GRO/CINC-1 secretion by knocking down different JNK isoforms. As expected, activation of PAR-1 and PAR-2 both significantly upregulated the release of the chemokine GRO/CINC-1 in WT cells as well as cells, which were transfected with scrambled siRNA (Fig. 3.27).

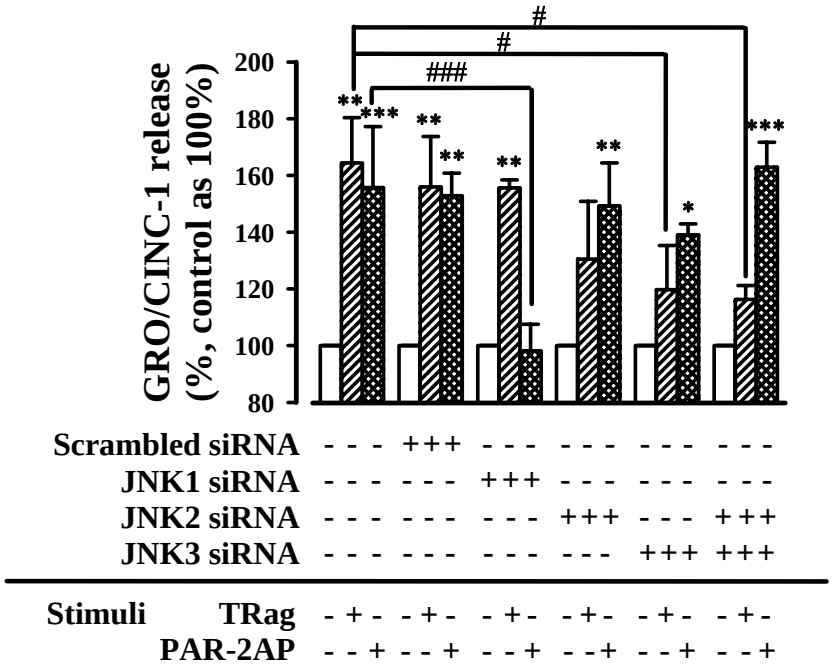


Fig. 3.27. Distinct JNK isoforms are essential for PAR-1- and PAR-2-induced the release of GRO/CINC-1. Astrocytes were transfected with scrambled siRNA (100 nM), JNK1 siRNA#1 (100 nM), JNK2 siRNA#1 (100 nM) or JNK3 siRNA#1 (100 nM) for 48 h. Then, transfected and non-transfected cells were stimulated with or without 10 μM TRag or 500 μM PAR-2AP for 6 h. The amount of GRO/CINC-1 release from non-stimulated cells (including transfected and non-transfected cells) was regarded as baseline (100%). Data represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ as compared to the respective control cells. ### $p < 0.001$, # $p < 0.05$ as compared to non-transfected cells exposed to TRag or PAR-2AP alone, respectively.

Importantly, JNK1 knockdown with siRNA did not affect PAR-1-induced GRO/CINC-1 release (Fig. 3.27). However, PAR-2-induced GRO/CINC-1 release was greatly reduced in JNK1-deficient cells compared to that in WT cells. Unlike the stimulation in JNK1-deficient cells, PAR-1-induced GRO/CINC-1 release was apparently eliminated in both JNK2-deficient and JNK3-deficient cells. This effect was even much clearer in JNK2/3-double-deficient cells (Fig. 3.27). However, PAR-2-induced GRO/CINC-1 release was not affected by JNK2 or JNK3 silencing or JNK2/3 double silencing. Similar biological effects were also observed when using other JNK siRNAs (Fig. 3.28). These results demonstrate that PAR-1-induced GRO/CINC-1 release is apparently mediated by JNK2 and JNK3 activation, whereas PAR-1-induced JNK1/c-Jun activation is not essential for the secretion of GRO/CINC-1. In contrast, in the PAR-2 signaling pathway, JNK1 activation mainly contributes to GRO/CINC-1 release.

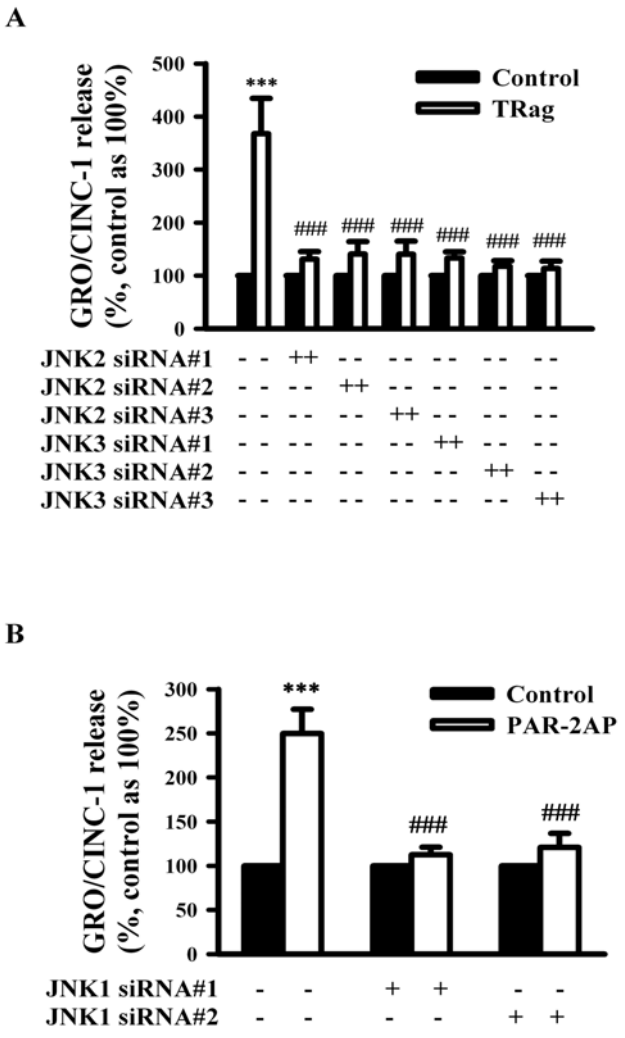


Fig. 3.28. Distinct JNK isoforms are essential for PAR-1- and PAR-2-induced the release of GRO/CINC-1. Astrocytes were transfected with 100 nM JNK1 siRNA#1-2, JNK2 siRNA#1-3 or JNK3 siRNA#1-3 for 48 h. Then, transfected and non-transfected cells were stimulated with or without 10 μM TRag (A) or 500 μM PAR-2AP (B) for 6 h. The amount of GRO/CINC-1 release from non-stimulated cells (including transfected and non-transfected cells) was regarded as baseline (100%). Data in A & B represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$ as compared to the respective control cells; ### $p < 0.001$ as compared to non-transfected cells exposed to TRag or PAR-2AP alone.

3.4 GRO/CINC-1, PAR-1 and PAR-2 activation rescue astrocytes from C_2-ceramide-induced cell death

3.4.1 Expression of CXCR2 in astrocytes

GRO/CINC-1 is the major chemokine involved in neutrophil recruitment to the brain and spinal cord, which follows after an inflammatory challenge in rat (Campbell et al., 2003). GRO/CINC-1 exerts similar functions as those caused by the human C-X-C chemokines IL-8 and GRO-α. GRO/CINC-1 signals via its unique receptor CXCR2 (Shibata, 2002). We are interested to see whether this receptor CXCR2 might be involved in a feedback response to GRO/CINC-1. Therefore, we investigated the expression of CXCR2 in astrocytes. As Fig. 3.29 shows, CXCR2 is abundantly expressed in astrocytes. These results imply that GRO/CINC-1 is likely to mediate some physiological functions on astrocytes by its cognate receptor.

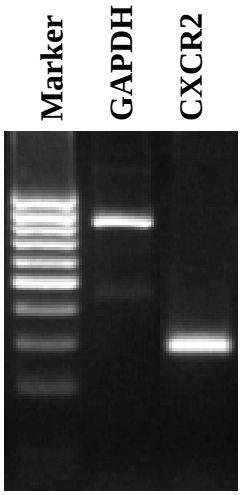


Fig. 3.29. Expression of chemokine GRO/CINC-1 receptor CXCR2 in rat astrocytes.

3.4.2 GRO/CINC-1 protects astrocytes from C_2-ceramide-induced cell death

Our previous studies had demonstrated that activation of PAR-1 induced astrocyte proliferation via the ERK1/2 pathway (Wang et al., 2002b). Here we investigated whether the chemokine GRO/CINC-1 is involved in PAR-1-induced cell proliferation. As Fig. 3.30 shows, the treatment with thrombin or TRag concentration-dependently increased cell proliferation. However, recombinant GRO/CINC-1 treatment at the concentration indicated failed to induce cell proliferation. These results suggest that the chemokine GRO/CINC-1 is apparently not involved in PAR-1-induced cell proliferation. Therefore, next we investigate whether GRO/CINC-1 has other biological functions.

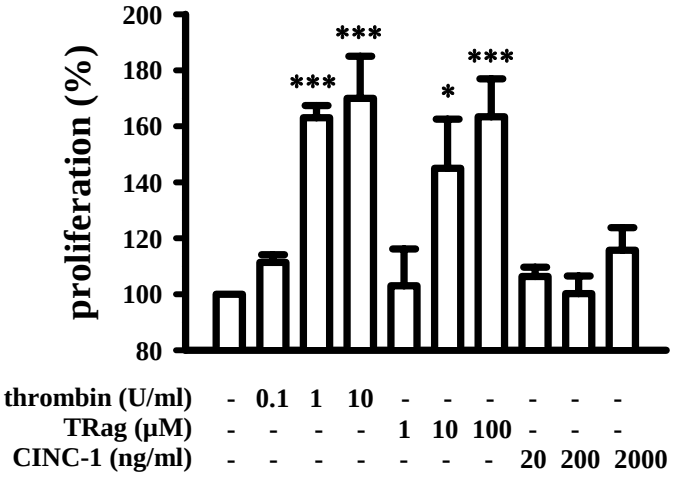


Fig. 3.30. The effect of GRO/CINC-1 treatment on astrocyte proliferation. Serum-starved cells were treated with thrombin, TRag or recombinant GRO/CINC-1 protein for 24 h. The proliferation was assessed using cell proliferation ELISA, Brdu (chemiluminescence). Proliferation is expressed as percent change compared to control. Data shown represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, * $p < 0.05$ as compared to control.

It is well known that release of LDH, a stable cytoplasmic enzyme present in all cells, is a cell death marker. Upon damage of the plasma membrane, LDH is rapidly released into the cell culture supernatant. To further study the possible physiological effects of GRO/CINC-1 on astrocytes, we stimulated cells with the pro-apoptotic lipid C_2-ceramide. Thus, we aimed to investigate the potential protective effect of GRO/CINC-1 on astrocytes by checking LDH activity in the cell culture medium. As shown in Fig. 3.31, C_2-ceramide significantly increased LDH release from astrocytes. However, C_2-ceramide-induced LDH release was notably reduced by treatment with the recombinant GRO/CINC-1. This occurred in a concentration-dependent manner. At the concentrations indicated, GRO/CINC-1 could mimic the effects resulting from PAR-1 activation induced by thrombin or TRag. These results suggest that GRO/CINC-1 could rescue astrocytes from C_2-ceramide-induced cell death.

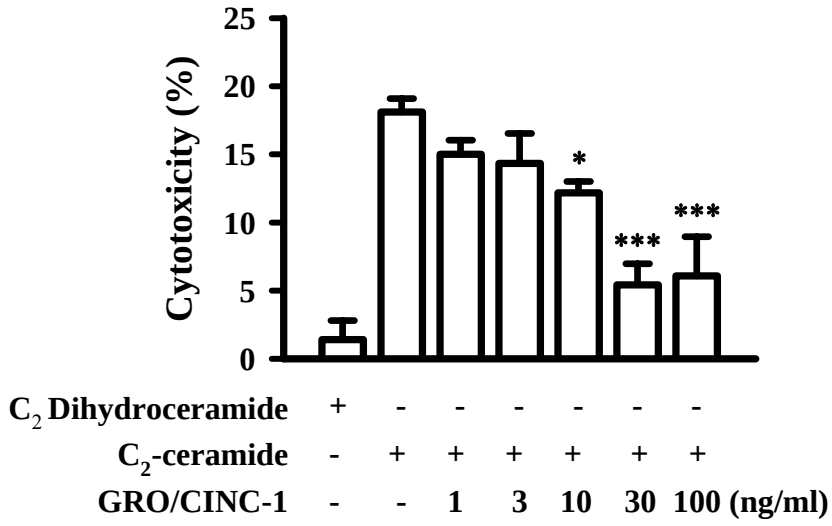


Fig. 3.31. GRO/CINC-1 protects primary astrocytes from C_2-ceramide-induced cell death. Serum-starved cells were treated with 20 μM C_2-ceramide in the absence or presence of GRO/CINC-1 for 7 h. Then, the cell culture supernatant was collected for LDH measurement. Cells only treated with C_2-dihydroceramide (inactive C_2-ceramide analog) served as negative control. Data shown represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, * $p < 0.05$ as compared to the cells exposed to C_2-ceramide alone.

3.4.3 Thrombin and TRag treatments both protect astrocytes from C_2-ceramide-induced cell death

Next, we further investigated whether PAR-1 activation could also rescue astrocytes from C_2-ceramide-induced cell death. Therefore, we stimulated cells with the pro-apoptotic lipid C_2-ceramide in the absence or presence of thrombin or TRag at different concentrations. As expected, C_2-ceramide, but not its inactive analog C_2-dihydroceramide, pronouncedly increased the cytotoxicity. Importantly, here we found that PAR-1 activation induced by thrombin or TRag, similar to treatment with the recombinant GRO/CINC-1, significantly protects astrocytes from ceramide-induced cell death (Fig. 3.32). This protection became more obvious with increasing PAR-1 agonist concentration. These results suggest that PAR-1 activation could rescue astrocytes from C_2-ceramide-induced cell death possibly via regulating GRO/CINC-1 release.

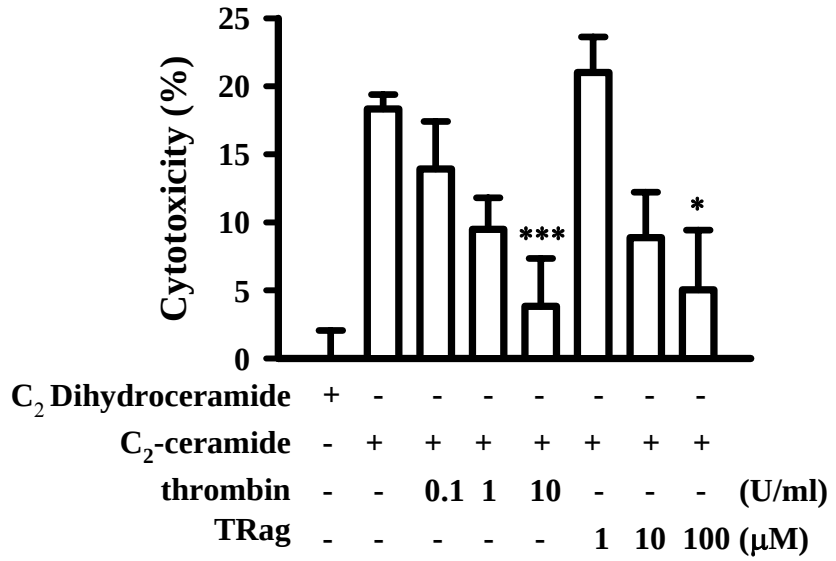


Fig. 3.32. PAR-1 agonists protect primary astrocytes from C_2-ceramide-induced cell death. Serum-starved cells were treated with 20 μM C_2-ceramide in the absence or presence of thrombin or TRag at indicated concentrations for 7 h. Then, the cell culture supernatant was collected for LDH measurement. Cells only treated with C_2-dihydroceramide (inactive C_2-ceramide analog) served as negative control. Data shown represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, * $p < 0.05$ as compared to the cells exposed to C_2-ceramide alone.

3.4.4 PAR-1 activation prevents C_2-ceramide-induced cytochrome c release from mitochondria via regulating the secretion of GRO/CINC-1

Generally, cytochrome c release from mitochondria is a main hallmark of cell death. Thus, here we tested the release of cytochrome c from mitochondria into the cytoplasm to further clarify the mechanism by which PAR-1 and recombinant GRO/CINC-1 rescue astrocytes from C_2-ceramide-induced cell death. As shown in Fig. 3.33, C_2-ceramide significantly induced cytochrome c release from mitochondria, which was markedly reduced by treatment with the recombinant GRO/CINC-1 (100 ng/ml). Meanwhile, our results also show that thrombin- or TRag-induced PAR-1 activation concentration-dependently suppressed C_2-ceramide-induced cytochrome c release from mitochondria (Fig. 3.33). Notably, even treatment with the very low concentration of thrombin (0.1 U/ml) and TRag (1 μM) was sufficient to inhibit C_2-ceramide-induced cytochrome c release. The immunoblot with the mitochondrial marker protein porin demonstrated that all samples used for cytochrome c determination were devoid of any mitochondrial contamination (data not shown). These results confirm that GRO/CINC-1

and PAR-1 activation caused by low concentrations of thrombin or TRag can prevent cytochrome c release from mitochondria and thus protect astrocytes from apoptosis.

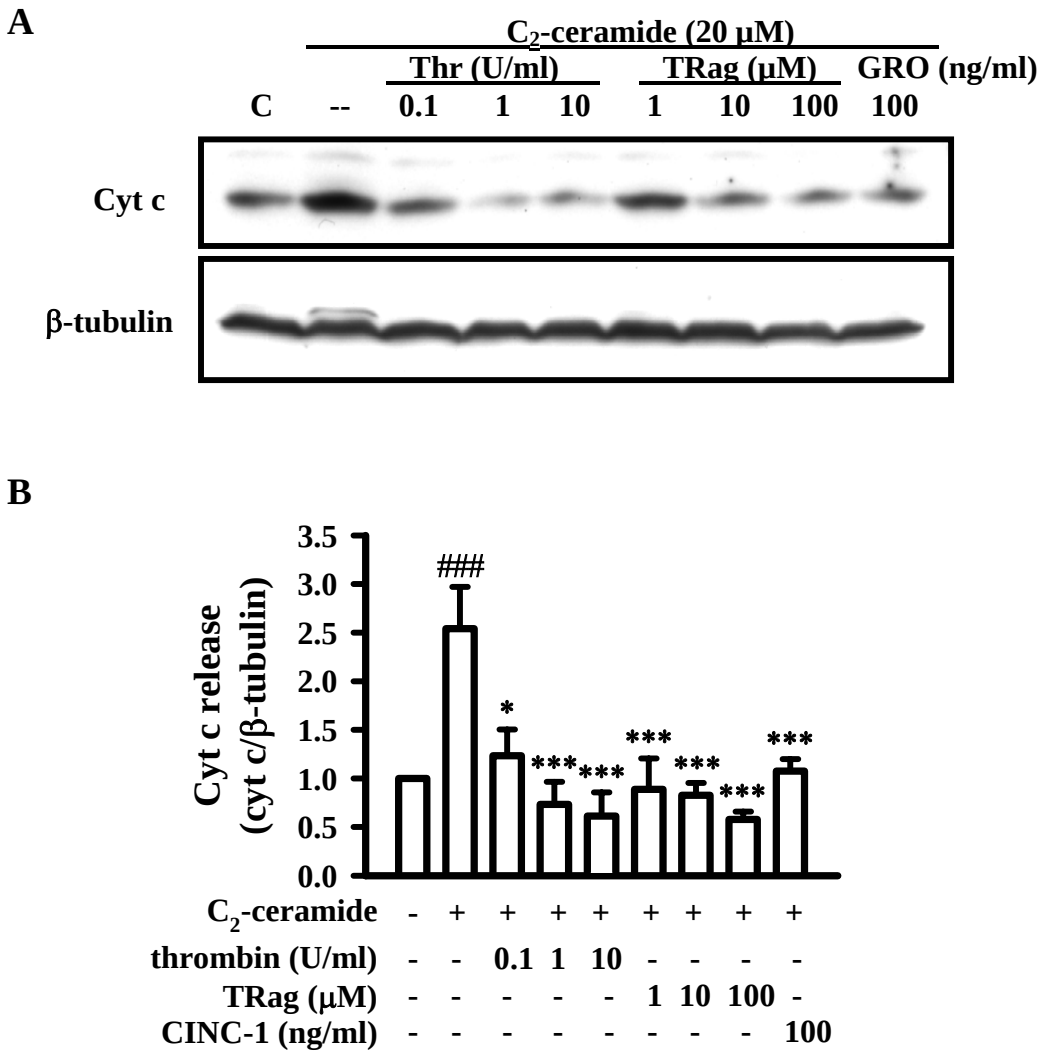


Fig. 3.33. PAR-1 activation and GRO/CINC-1 treatment prevent C_2-ceramide-induced cytochrome c release from mitochondria. Serum-starved cells were treated with 20 μM C_2-ceramide in the absence or presence of thrombin, TRag or GRO/CINC-1 at the concentrations indicated for 7 h. Then, the cell cytoplasm fraction was isolated for determining cytochrome c (cyt c) release. Representative blots from one experiment (A) and quantification (B). The amounts of cytochrome c were normalized relative to β-tubulin. Data shown in B represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, * $p < 0.05$ as compared with the cells exposed to C_2-ceramide alone; ### $p < 0.001$ as compared to the untreated cells.

To definitely prove that PAR-1 could rescue astrocytes from C_2-ceramide-induced apoptosis through regulating GRO/CINC-1 release, we applied SB-332235, a specific antagonist of the unique receptor CXCR2 for GRO/CINC-1. The prevention of C_2-ceramide-induced cytochrome c release from mitochondria by GRO/CINC-1 was significantly reduced by the specific CXCR2 antagonist (Fig. 3.34). Importantly, the application of the CXCR2 antagonist was also able to largely eliminate the effects of thrombin and TRag on the prevention of cytochrome c release (Fig. 3.34). These results clearly demonstrate that PAR-1 could rescue primary astrocytes from cell death through regulating the release of the chemokine GRO/CINC-1.

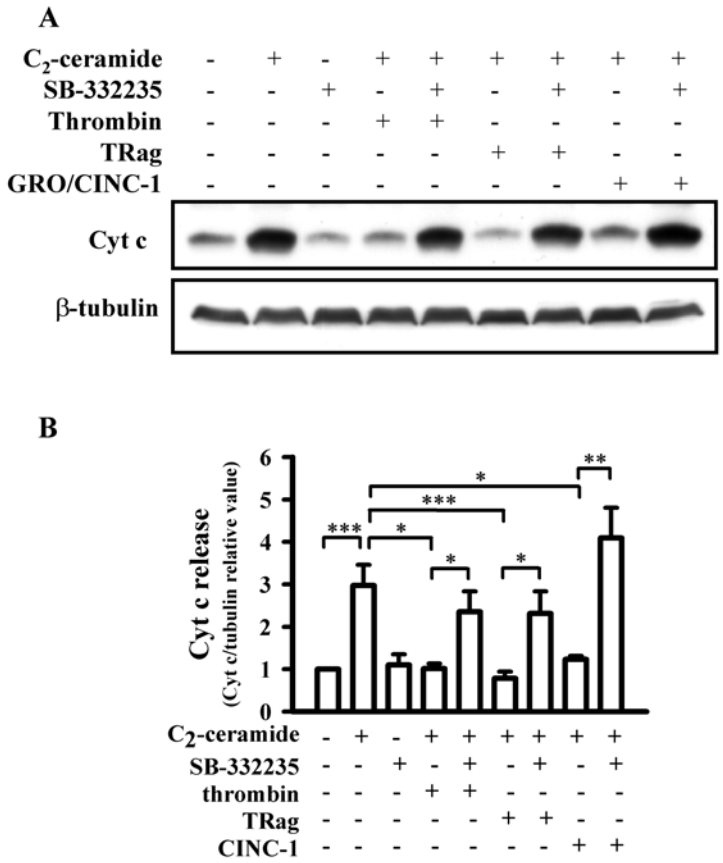


Fig. 3.34. PAR-1 activation prevents C_2-ceramide-induced cytochrome c release from mitochondria by regulating GRO/CINC-1 release. Serum-starved cells were treated with 20 μM C_2-ceramide in the absence or presence of 10 μM CXCR2 antagonist SB-332235 together with thrombin (1 U/ml), TRag (10 μM) or GRO/CINC-1 (100 ng/ml) for 7 h. The cell cytoplasm fraction was isolated for measuring cytochrome c (cyt c) release. Representative blots (A) and quantification (B). The amounts of cyt c were normalized relative to β-tubulin. Data shown in B represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

3.4.5 JNK inhibition abolishes the protection mediated by PAR-1 in primary astrocytes

JNK is a main contributor for PAR-1-induced GRO/CINC-1 release, as our data above show. Therefore, we further investigated whether JNK was involved in the protective action of PAR-1 to suppress C_2-ceramide-induced cytochrome c release. As shown in Fig. 3.35 A & B, the specific JNK inhibitor SP600125 concentration-dependently eliminated the protective effects of PAR-1. The protection was quantified by measuring the prevention of C_2-ceramide-induced cytochrome c release. These results suggest that JNK apparently contributes to the protection of PAR-1, that is suppressing cytochrome c release from mitochondria via regulating the release of the chemokine GRO/CINC-1.

On the other side, by using SP600125, we investigated the role of JNK activation in the protective action of PAR-1 to rescue primary astrocytes from C_2-ceramide-induced cell death. Also the influence of JNK activation on cell death following exposure to C_2-ceramide alone was studied here. Cells treated with SP600125 alone served as a negative control. As expected, C_2-ceramide significantly increased astrocytes death (Fig. 3.35 C). This effect was apparently not affected by treatment with SP600125 alone following C_2-ceramide exposure, indicating that JNK does not directly protect astrocytes from C_2-ceramide-induced cell death. However, JNK inhibition caused by SP600125 significantly reduced the protective action of TRag treatment after C_2-ceramide challenge (Fig. 3.35 C). These data suggest that activation of JNK apparently contributes to the protective action of PAR-1 to rescue primary astrocytes from cell death. Importantly, the protective role of JNK activation is specifically dependent on PAR-1 activation.

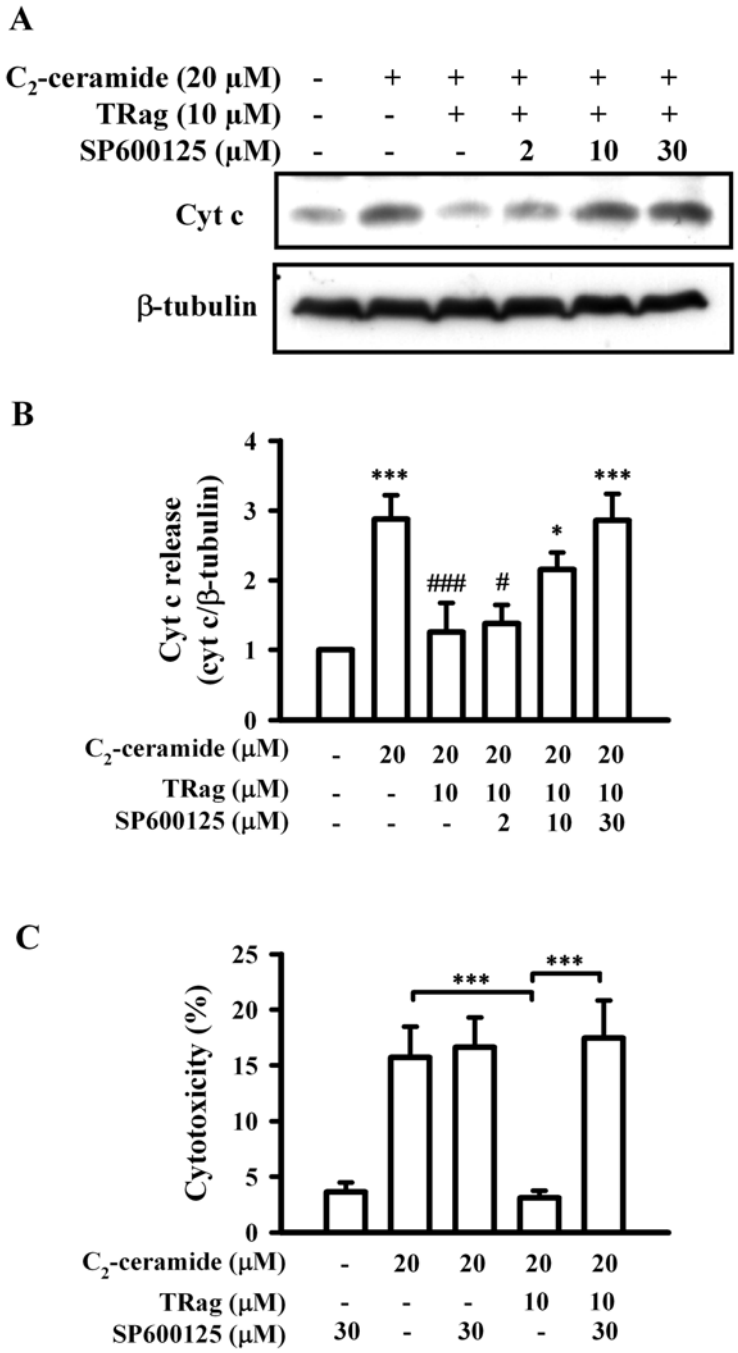


Fig. 3.35. JNK inhibition eliminates the protective action of PAR-1 which results in prevention of C_2-ceramide-induced cell death. Serum-starved cells were treated with 20 μM C_2-ceramide in the absence or presence of JNK specific inhibitor SP600125 (2 μM, 10 μM or 30 μM) together with TRag (10 μM) for 7 h. Representative blots for cyt c and β-tubulin from one experiment (A) and quantification (B). The amounts of cyt c were normalized relative to β-tubulin. Cytotoxicity assessment by measuring LDH release is presented in C. Data shown in B & C represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$, * $p < 0.05$ as compared to the untreated cells; $^{###}p < 0.001$, $^{\#}p < 0.05$ as compared to the cells exposed to C_2-ceramide alone.

3.4.6 Activation of PAR-2 suppresses C_2-ceramide-induced cytochrome c release and rescues astrocytes from cell death via regulating GRO/CINC-1 release

Our data above have showed that release of the chemokine GRO/CINC-1 could give a feedback to prevent C_2-ceramide-induced cytochrome c release from mitochondria and rescue astrocytes from cell death. Moreover, our results indicated that PAR-1 activation by TRag significantly reduced cytochrome c release from mitochondria via regulating cheomkine GRO/CINC-1 secretion. Finally here, we investigated whether PAR-2AP could exert an effect similar to that of TRag. As shown in Fig. 3.36 A & B, PAR-2 activation by PAR-2AP concentration-dependently prevented C_2-ceramide-induced cytochrome c release from mitochondria. Furthermore, the protective effect of PAR-2 on suppressing C_2-ceramide-induced cytochrome c release from mitochondria was remarkably abolished by treatment with the specific antagonist of the unique GRO/CINC-1 receptor CXCR2, SB332235 (Fig. 3.36 A & B). These results suggest that PAR-2 activation by PAR-2AP likewise prevented cytochrome c release from mitochondria via regulating the secretion of the chemokine GRO/CINC-1.

Our data above show that PAR-1 activation prevented C_2-ceramide-induced LDH release. Therefore, here we investigated whether PAR-2 has a similar protective effect on rescuing astrocytes, and further compared the protective capacity of PAR-1 and PAR-2. We stimulated cells with pro-apoptotic lipid C_2-ceramide in the presence or absence of TRag or PAR-2AP, and then tested the LDH activity in the culture supernatant. As Fig. 3.36 C shows, C_2-ceramide treatment significantly increased the LDH release. The increase in LDH release caused by C_2-ceramide was apparently reduced by both TRag-induced PAR-1 activation and PAR-2AP-induced PAR-2 activation (Fig. 3.36 C). The protective capacity of PAR-2 is comparable to that of PAR-1 (Fig. 3.36 C). These results demonstrate that PAR-2 activation, like PAR-1 activation, is able to protect neural cells from toxic insults by regulating chemokine GRO/CINC-1 release.

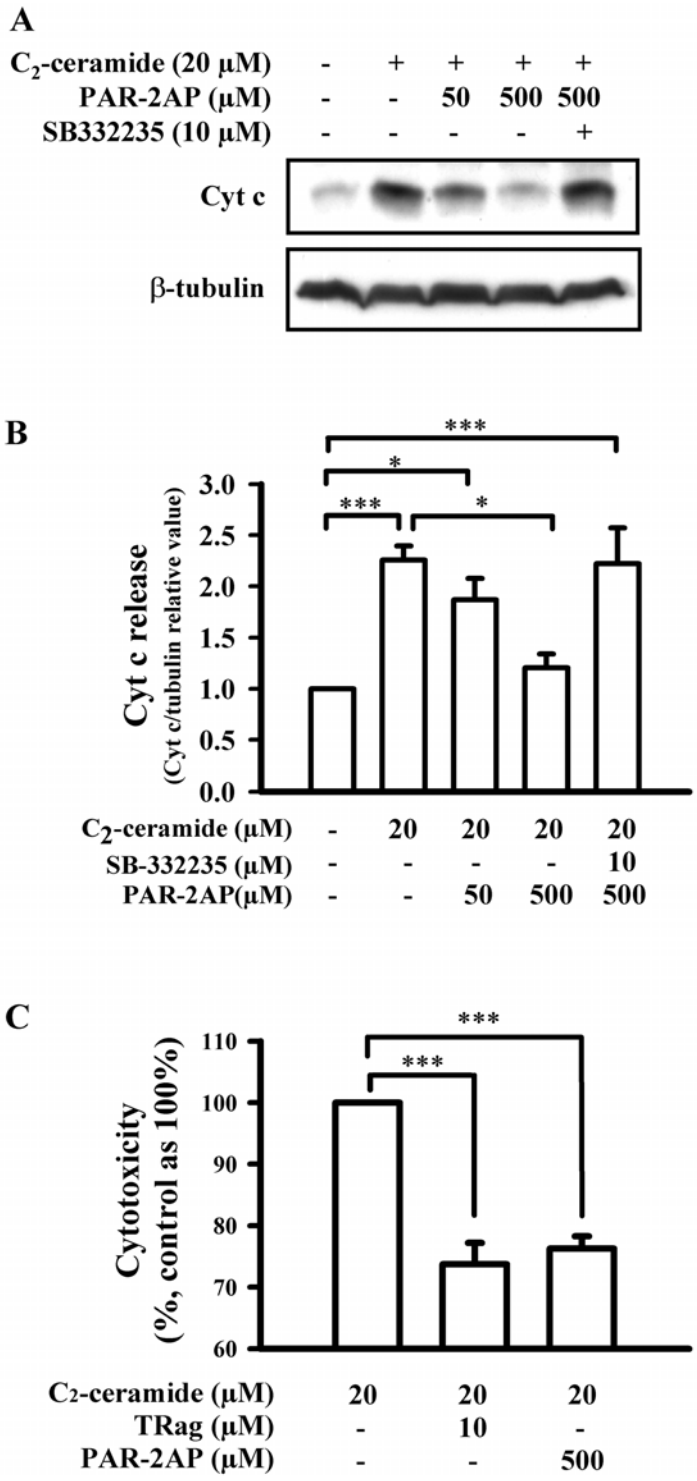


Fig. 3.36. PAR-2 has a similar capacity to prevent C_2-ceramide-induced cytochrome c release from mitochondria and to rescue astrocytes from cell death by regulating GRO/CINC-1 release. Serum-starved cells were treated with 20 μM C_2-ceramide in the absence or presence of PAR-2AP (50 μM or 500 μM) together with CXCR2 antagonist SB-332235 (10 μM) for 7 h. Representative blots for cytochrome c (Cyt c, upper panel) and β-tubulin (lower panel) (A) and quantification (B). The amounts of cytochrome c were normalized relative to β-tubulin. Cytotoxicity assessment by measuring LDH release is presented in C. The protective effects of TRag and PAR-2AP treatment are shown in relative values as compared to C_2-ceramide-induced LDH release (100%). Data shown in B & C represent the mean ± SEM of at least three independent experiments. *** $p < 0.001$.

4 Discussion

Thrombin and tryptase, two multifunctional serine proteases, are early phase modulators at sites of tissue injury. Previous data already showed that thrombin at low concentrations is protective in ischemic and hemorrhagic models of stroke mainly via PAR-1 (Masada et al., 2000; Riek-Burchardt et al., 2002). In line with those observations in vivo, the present study provides novel insights, revealing that thrombin via PAR-1 protects astrocytes from ceramide-induced cell death by regulating release of the chemokine GRO/CINC-1. Importantly, here we show that PAR-2 activation could be another protective pathway in astrocytes. Traditionally, astrocytes have been considered as secondary players in the CNS. However, recently accumulating evidence suggests that glial cells, in particular astrocytes, play an active and important role in the demise of brain tissue after injury, such as cerebral ischemia (Nedergaard and Dirnagl, 2005; Trendelenburg and Dirnagl, 2005). Astrocytes, the most abundant cell type within the CNS, are essential for neural survival and function, neurogenesis and neural repair. Thus, the present study raises the suggestive idea that astrocytes could be a promising target for novel therapeutic approaches, to induce in brain protection via regulating immune responses.

4.1 PAR activation differentially regulates levels of the chemokine GRO/CINC-1 and other proinflammatory cytokines in astrocytes

The significance of PAR in inflammation has been well addressed in the respiratory system and peripheral nervous systems (Asokananthan et al., 2002; Vergnolle, 2003). PAR activation regulates multiple proinflammatory cytokines (i.e. IL-6, TNF-α, IL-1β) and chemokines (IL-8) release from the epithelial cells and endothelial cells (Asokananthan et al., 2002; Namazi, 2005). Although the distribution of the four PAR types in the CNS has been studied, the role of PAR in regulating proinflammatory chemokines or cytokines expression in the CNS still remains largely unknown. In the present study, our results clearly indicate that both PAR-1 and PAR-2 activation, but not PAR-3 or PAR-4, induced chemokine GRO/CINC-1 release from astrocytes. Similarly, the mRNA levels of proinflammatory cytokines TNF-α and IL-1β were concentration-dependently upregulated by both PAR-1 and PAR-2 activation. However, PAR activation failed to increase the protein secretion of TNF-α and IL-1β. IL-6 expression at both mRNA and protein levels was not affected by PAR activation. Taken together, our results indicate

that PAR-1 and PAR-2 activation are specifically involved in chemokine GRO/CINC-1 release, but not IL-6, TNF-α or IL-1β secretion. The mechanisms involved in upregulating IL-6, TNF-α and IL-1β mRNA levels caused by thrombin or agonists of PAR-1 and PAR-2, without inducing the protein release, remain an interesting issue for future studies.

4.2 JNK is a central mediator for PAR-induced GRO/CINC-1 secretion in astrocytes

Following brain injury, the levels of serine proteases, such as thrombin, tryptase and p22, can be increased in the CNS (Roznieckі et al., 1995; Sawada et al., 2000; Xi et al., 2003; Lozada et al., 2005; Arai et al., 2006). Consequently, the thrombin receptor PAR-1 or tryptase receptor PAR-2 could be activated in astrocytes, separately or simultaneously. Activation of PAR-1 and PAR-2 both resulted in significant increase of the chemokine GRO/CINC-1 secretion, as our data show. Here, we investigated whether these two similar receptor subtypes could signal separately. The signal transduction mechanisms underlying PAR-induced GRO/CINC-1 release were investigated by focusing on MAPKs, which are known to stimulate the production of inflammatory mediators (Luo et al., 2004; Park et al., 2004; Zhu et al., 2005).

4.2.1 JNK plays a pivotal role in both PAR-1 and PAR-2 signaling pathways

The immunoblot analysis indicates that JNK and ERK1/2 activation, but not p38 MAPK, are apparently involved in the PAR-1 signaling pathway. In parallel, ELISA data demonstrate that both the JNK inhibitor SP600125 and the MEK1/2 specific inhibitor U0126 significantly reduced PAR-1-induced GRO/CINC-1 release indicating that JNK and MEK/ERK both might contribute to PAR-1-induced GRO/CINC-1 release. Interestingly, here we found that U0126 almost completely abolished PAR-1-induced JNK phosphorylation, which is in line with a recent report (Waetzig and Herdegen, 2005). These results imply that the reduction of GRO/CINC-1 release induced by U0126 treatment might result from JNK inhibition as well. To ascertain the role of ERK1/2 in GRO/CINC-1 release, a specific ERK1/2 inhibitor is needed, which will not disturb the activities of MEK1/2 and JNK. Unfortunately, this kind of ERK1/2 inhibitor is not available so far. Here, through comparing the effects of JNK and MEK1/2 inhibitors on GRO/CINC-1 release, we found that the JNK inhibitor remarkably reduced GRO/CINC-1 release (by about 76%), and the MEK1/2 inhibitor completely blocked PAR-1-induced GRO/CINC-1 release (Figs. 3.4 and 3.7). These results indicate that JNK activation mainly contributes to

GRO/CINC-1 secretion in astrocytes, and ERK1/2 is likely to be partially involved in PAR-1-induced GRO/CINC-1 release (Fig. 4.1 A).

Similarly, both ELISA and western blot data reveal that JNK is also a main contributor for PAR-2-induced GRO/CINC-1 secretion (Fig. 4.1 B). However, MEK1/2 and p38 inhibition did not remarkably affect PAR-2-induced GRO/CINC-1 secretion, suggesting that p38 and MEK/ERK are apparently not involved in PAR-2-induced GRO/CINC-1 secretion. Therefore, our data clarify that JNK plays a pivotal role in both PAR-1 and PAR-2 signaling pathways.

4.2.2 PAR-1 and PAR-2 activate different JNK isoforms

Three different JNK genes (*jnk1*, *jnk2* and *jnk3*) have been identified. Splicing at the C-terminus of the JNK isoforms yields 46-kDa and 54-kDa polypeptides of JNK1, JNK2 and JNK3 (Waetzig and Herdegen, 2005). The treatment with the PAR-1 specific agonist TRag could strongly induce the phosphorylation of both 46-kDa and 43-kDa JNK isoforms. However, PAR-2AP could only cause the phosphorylation of the 43-kDa JNK isoform (Fig. 3.10 D). This indicates a clear difference between PAR-1- and PAR-2-induced JNK activation, although JNK plays a pivotal role in both PAR-1 and PAR-2 signaling pathways.

4.2.3 Different JNK upstream factors contribute to PAR-1- and PAR-2-induced GRO/CINC-1 release

Pharmacological blockade of PKC or PI3K not only significantly reduced PAR-1-induced JNK activation (Fig. 3.11-3.12), but also remarkably attenuated PAR-1-induced GRO/CINC-1 secretion (Fig. 3.13). These results suggest that PKC- and PI3K-mediated JNK activation mainly contributes to PAR-1-induced GRO/CINC-1 release (Fig. 4.1 A). However, PAR-2-mediated JNK activation was not suppressed by the PKC inhibitor, only partially reduced by the PI3K inhibitor (Fig. 3.14). This indicates that different upstream activators mediate JNK activation either in the PAR-1 or the PAR-2 signaling pathways. Importantly, PAR-2-induced GRO/CINC-1 release, unlike PAR-1-induced GRO/CINC-1 secretion, was independent of either PKC or PI3K activation (Fig. 3.15). Therefore, PI3K-mediated JNK activation should not be essential for PAR-2-induced GRO/CINC-1 release (Fig. 4.1 B). Taken together, another important clear difference between PAR-1 and PAR-2 signaling pathways clarified here is that PKC and PI3K are only involved in PAR-1-

induced GRO/CINC-1 release, but not in PAR-2-induced GRO/CINC-1 secretion, which is also clearly depicted in Fig. 4.1.

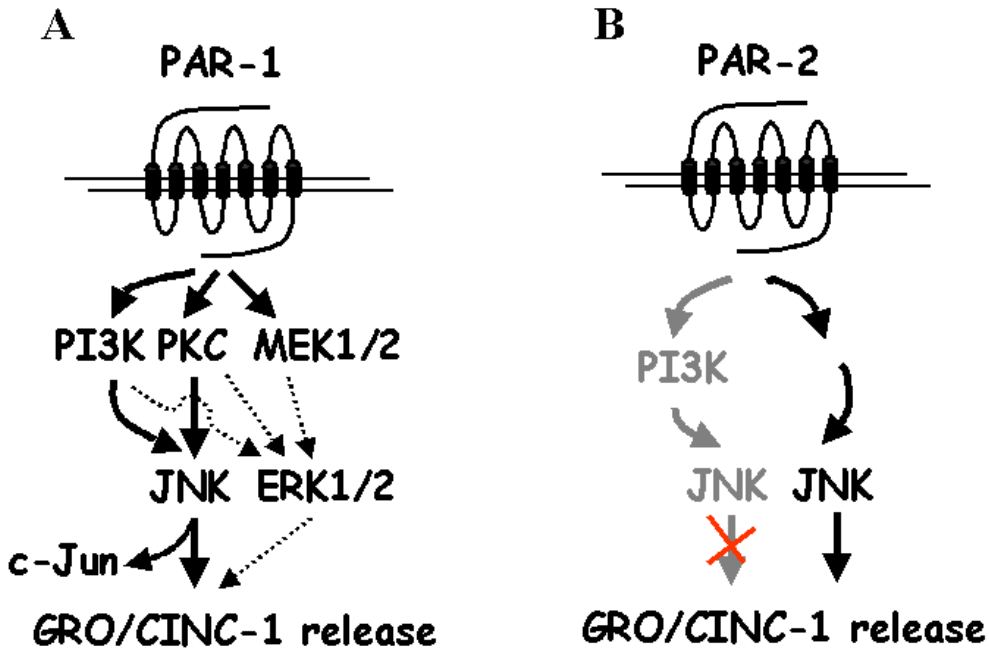


Fig. 4.1. JNK plays a central role in both PAR-1- and PAR-2-induced release of the chemokine GRO/CINC-1 from rat astrocytes. **A**. PAR-1-induced GRO/CINC-1 release is mainly mediated by PI3K- and PKC-dependent JNK activation. In addition, PI3K-, PKC- and MEK1/2-mediated ERK1/2 activation might also partially contribute to PAR-1-induced GRO/CINC-1 release (broken line). PAR-1-induced JNK activation might further phosphorylate c-Jun. **B**. PAR-2-induced GRO/CINC-1 release is mainly mediated by JNK activation. Although PI3K is also involved in PAR-2-induced JNK phosphorylation (in gray), the secretion of GRO/CINC-1 is independent of PI3K or PKC activation, which differs from the PAR-1 signaling pathway.

4.2.4 PAR-1- and PAR-2-induced JNK activation have different effects on c-Jun phosphorylation

c-Jun, a downstream transcription factor of JNK, was strongly phosphorylated and upregulated by thrombin or TRag stimulation (Fig. 3.16, 3.19). The phosphorylation of c-Jun was mediated by PAR-1-dependent JNK activation (Fig. 3.17). Unlike the observation from several previous reports that c-Jun activation could further regulate its own gene expression (Yin et al., 1997; Sugden and Clerk, 1998; Herdegen and Waetzig, 2001), our results imply that the upregulation of total c-Jun is likely to be regulated by other transcription factors caused by PAR-1 activation, since JNK blocker did not inhibit the increase of c-Jun.

Interestingly, we found that the time course of JNK activation did not match that of c-Jun phosphorylation. The maximum JNK activation was obtained at 10 min and it persisted for 2 h (Fig. 3.5), whereas the strongest phosphorylation of c-Jun was detected at 60 min (Fig. 3.16). The not-synchronous time course in activation of JNK and c-Jun might be due to the fact that JNK phosphorylation might be the overall activation of the three JNK isoforms. The individual isoforms could have different specificities of activation by PAR-1. This was confirmed later by the loss-of-function studies.

Unlike PAR-1-induced JNK activation, JNK activation induced by PAR-2 failed to further phosphorylate c-Jun (Fig. 3.18-3.19). This indicates that JNK activity concerning its downstream transcription factor c-Jun phosphorylation is completely different in the PAR-1 and PAR-2 signaling pathways.

Taken this part together, our results clearly indicate that JNK plays a central role in both PAR-1 and PAR-2 signaling pathways (Fig. 4.1). However, the analysis, by comparing the JNK upstream activators, the phosphorylation of JNK isoforms and JNK effects on its downstream transcription factor c-Jun, evidently demonstrate that different JNK isoforms with distinct JNK properties are involved in the PAR-1 and PAR-2 signaling pathways. They all might contribute to the chemokine GRO/CINC-1 secretion.

4.3 Roles of three JNK isoforms in PAR-induced GRO/CINC-1 release and c-Jun activation

4.3.1 Distinct roles of three JNK isoforms in PAR-induced GRO/CINC-1 release

Here, we established that all three JNK isoforms (JNK1, JNK2 and JNK3) are expressed in astrocytes. PAR-1 and PAR-2 activation both resulted in JNK1

phosphorylation (Fig. 3.24-3.25). The loss-of-function studies further clearly demonstrate that PAR-1-induced JNK1 activation was not involved in chemokine GRO/CINC-1 release. However, PAR-2-mediated JNK1 activation was essential for GRO/CINC-1 release (Fig. 3.27). Therefore, our results suggest that JNK1 exerts completely different functions in the PAR-1 and PAR-2 signaling pathways.

JNK3 is likewise activated by both PAR-1 and PAR-2, but exerts different functions in these two signaling pathways. Our finding here that PAR-1-induced JNK3 activation prominently contributes to GRO/CINC-1 release. However, PAR-2-induced GRO/CINC-1 release is independent of JNK3 activation (Fig. 3.27).

Unlike JNK1 and JNK3, the JNK2 isoform has been identified to be activated only by PAR-1, but not by PAR-2 (Fig. 3.24-3.25). Furthermore, the loss-of-function studies clearly demonstrate that JNK2 activation contributes to PAR-1-induced GRO/CINC-1 release (Fig. 3.27). In contrast, JNK2-knockdown apparently did not interfere with the secretion of GRO/CINC-1 induced by PAR-2, which further supports our data that JNK2 activation is not involved in the PAR-2 signaling pathway (Fig. 3.25).

Therefore, our results show that the three JNK isoforms function differently in PAR-1- and PAR-2-induced GRO/CINC-1 release. Even the same JNK isoform, depending on PAR-1 or PAR-2 activation, exerts different functions. This finding contributes to the explanation of the physiological role of the different JNK isoforms.

4.3.2 JNKs differentially regulate c-Jun phosphorylation and the role of c-Jun activation in PAR-1-induced GRO/CINC-1 release

It is well known that all three JNK isoforms are able to phosphorylate the transcription factor c-Jun and to play distinct biological functions in different systems (Kallunki et al., 1994; Gupta et al., 1996; Yang et al., 1997; Fuchs et al., 1998; Morishima et al., 2001; Liu et al., 2004; Sabapathy and Wagner, 2004; Keramaris et al., 2005). In the present study, we establish that PAR-1-mediated JNK1 activation, but not JNK2, is mainly responsible for c-Jun phosphorylation in rat astrocytes (Fig. 3.26). In contrast, PAR-2-induced JNK1 activation failed to activate c-Jun (Fig. 3.18-3.19).

Although PAR-1-mediated JNK3 activation failed to further phosphorylate c-Jun, JNK3 might be a negative regulator for JNK1-mediated c-Jun phosphorylation. Because our data showed that c-Jun phosphorylation was pronouncedly increased in JNK3-deficient cells even without TRag stimulation (Fig. 3.26). We have already seen that PAR-1-induced c-Jun phosphorylation was significantly upregulated after the pretreatment with the

inhibitor of PI3K which is a JNK upstream factor (Fig. 3.17). From these data, we hypothesize that PI3K is likely to activate JNK3, which further negatively regulates JNK1-induced c-Jun phosphorylation by competing for the common substrate c-Jun.

It has been reported that the inactive JNKs caused degradation of transcription factors such as c-Jun, ATF2 and p53 (Fuchs et al., 1997), while phosphorylated JNK stabilized c-Jun in vivo by suppressing multi-ubiquitination processes (Musti et al., 1997). Therefore, our data suggest the implication that JNK3 is likely to have higher affinity to bind to c-Jun at the resting state and to cause c-Jun degradation in our system, so as to keep low levels of c-Jun phosphorylation (Fig. 4.2). In JNK3-deficient cells, however, the phosphorylation of c-Jun is significantly increased, which is likely resulting from the basal activation of JNK1 in brain (Kuan et al., 2003). On the other hand, upon stimulation with TRag, PAR-1 activation increases the phosphorylation of both JNK1 and JNK3 isoforms. Although PI3K-mediated JNK3 activation is unable to phosphorylate c-Jun, it can compete with JNK1 to bind to c-Jun and negatively regulate JNK1-mediated c-Jun activation. Future studies are needed to test this model.

Although treatment with thrombin and TRag both strongly activate c-Jun via JNK1, c-Jun is apparently not involved in GRO/CINC-1 release (Fig. 3.27), which is in line with the fact that the potential AP-1 binding site does not exist in the promoter region of GRO/CINC-1 (Shibata et al., 1998). It has been known that JNKs could regulate the activity of several transcription factors, including c-Jun, ATF-2, Elk-1, p53 and c-Myc (Karin, 1995; Yu et al., 2004). Thus, it still needs to be investigated further which transcription factor regulates GRO/CINC-1 gene expression.

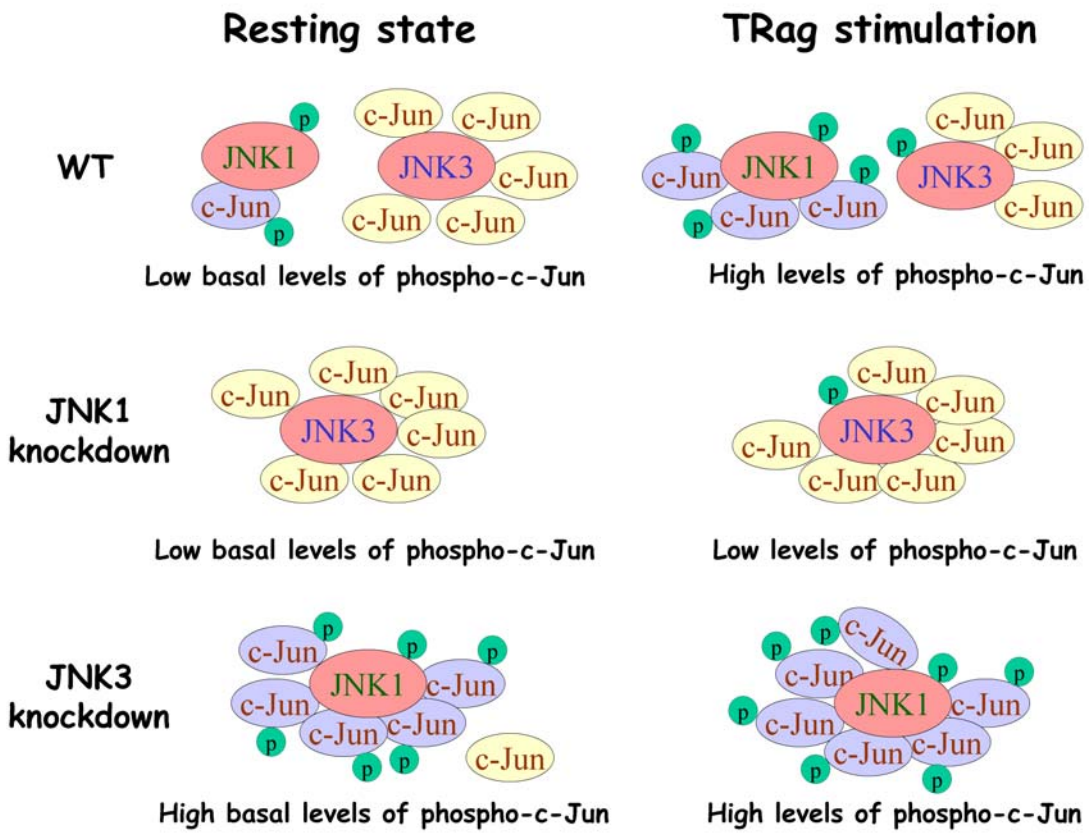


Fig. 4.2. Proposed model of JNK1 and JNK3 actions in primary astrocytes. When WT cells are in a quiescent state, c-Jun is predominantly bound to inactive JNK3. Only a low proportion of c-Jun binds to the phospho-JNK1, which is primarily responsible for the basal JNK phosphorylation in brain (Kuan et al., 2003). Therefore, there are only low basal levels of c-Jun phosphorylation. Upon stimulation with TRag, PAR-1 activation results in the phosphorylation of both JNK1 and JNK3 isoforms. Consequently, the phosphorylation of c-Jun is increased by JNK1, whereas phospho-JNK3 can also bind to c-Jun but fail to activate it. Therefore, absence of JNK1 leads to low levels of phospho-c-Jun in JNK1 knockdown cells either in a quiescent or active state. In constrast, the phosphorylation of c-Jun is dramatically increased by the basal phospho-JNK1 in the resting JNK3-deficient cells. Increase of JNK1 phosphorylation caused by TRag stimulation might further slightly elevate the c-Jun phosphorylation in the JNK3-deficient cells.

4.3.3 PI3K and PKC selectively activate different JNK isoforms to mediate PAR-induced GRO/CINC-1 release

In the present study, PKC and PI3K were identified as two important upstream factors of PAR-1-dependent JNK activation, which were also required for PAR-1-induced release of GRO/CINC-1, as shown in Fig. 4.1. Our further data showed that c-Jun phosphorylation was not suppressed after blocking the activation of either PI3K or PKC (Fig. 3.17), suggesting that PAR-1-mediated JNK1/c-Jun phosphorylation is independent of PI3K and PKC activation. Our data, which show that PI3K and JNK3 both negatively regulate PAR-1-induced c-Jun phosphorylation, suggest that PI3K is likely to regulate the activation of JNK3 and further contributes to PAR-1-induced GRO/CINC-1 release. On the other hand, our results show that JNK3 is activated by PAR-2, but is not essential for PAR-2-induced GRO/CINC-1 release. This is consistent with the fact that PI3K contributes to PAR-2-induced JNK3 phosphorylation, but is not involved in the secretion of GRO/CINC-1 (Fig. 3.14-3.15).

Therefore, PKC is likely to mediate JNK2 activation in the PAR-1 signaling pathway. In the PAR-2 signaling pathway, PKC was identified to be not involved in either JNK activation or GRO/CINC-1 secretion (Fig. 3.14-3.15). These results are identical to our other data showing that the JNK2 isoform, the downstream factor of PKC, was not activated by PAR-2 (Fig. 3.25) and thereby did not interfere with the secretion of GRO/CINC-1 induced by PAR-2 (Fig. 3.27).

Taken together, our results so far show that activation of PAR-1 and PAR-2 both result in increase of the chemokine GRO/CINC-1 release and three JNK isoforms play different but pivotal roles in these processes. As summarized in Fig. 4.3, PAR-1-induced GRO/CINC-1 release is mainly mediated by PKC/JNK2 and PI3K/JNK3. However, PAR-2-induced GRO/CINC-1 release is mainly mediated by JNK1 activation. Therefore, our results for the first time indicate that JNK mediates GRO/CINC-1 release in a JNK isoform-dependent fashion in astrocytes and evokes PAR subtype-dependent mechanisms. These data provide novel functional insights into the physiological role of different JNK isoforms in astrocytes.

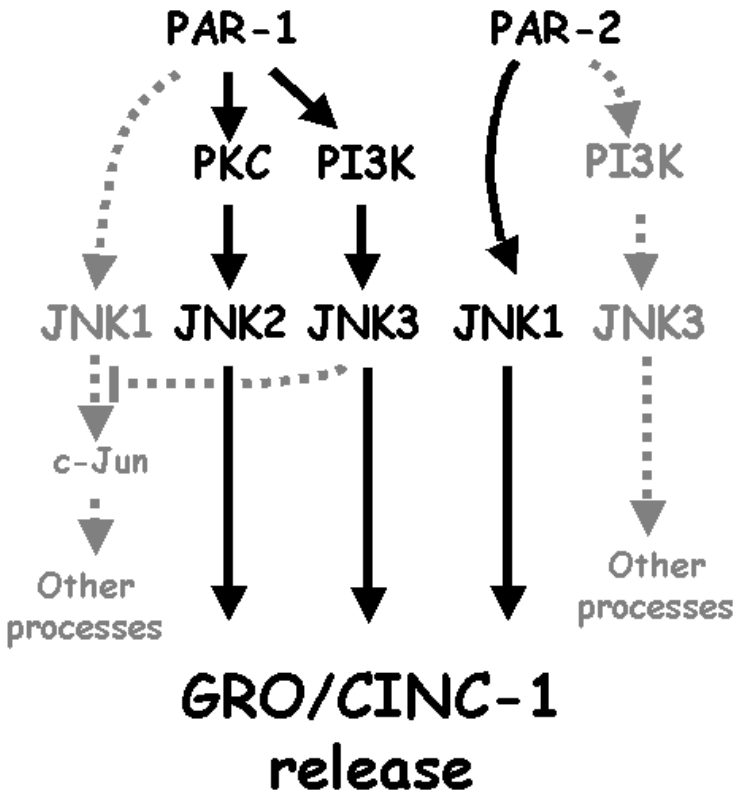


Fig. 4.3. Three JNK isoforms differentially regulate the release of the chemokine GRO/CINC-1 induced by PAR-1 and PAR-2 activation. Activation of PAR-1 and PAR-2 both induce chemokine GRO/CINC-1 release from rat astrocytes. However, the mechanisms of GRO/CINC-1 release in both signaling pathways are strictly regulated by different JNK isoforms. PAR-1-induced GRO/CINC-1 release is mainly mediated by PKC/JNK2 and PI3K/JNK3. Although PAR-1 activation also results in JNK1 phosphorylation, which further activates c-Jun, JNK1 is not essential for GRO/CINC-1 release (broken line, in gray). Moreover, JNK3 can compete with JNK1 for c-Jun phosphorylation. In the PAR-2 signaling pathway, JNK1 activation mainly contributes to secretion of the chemokine GRO/CINC-1. In contrast, PI3K-mediated JNK3 activation is not involved in PAR-2-induced GRO/CINC-1 release (broken line, in gray).

4.4 Physiological significance of PAR activation in brain

In addition to unravelling the biochemical mechanism of PAR subtype-specific JNK isoform activation and the signaling pathways leading to chemokine release, we further aimed to shed light on the (patho)physiological significance of these results. Our previous data have shown that PAR-1 activation resulted in astrocyte proliferation (Wang et al., 2002b). In the present study, we found that GRO/CINC-1 is apparently not involved in proliferation, although its release caused by thrombin or TRag stimulation is an event occurring early before proliferation.

It has been known that brain injury like ischemia may also lead to accumulation of the endogenous apoptotic mediator ceramide in neural astrocytes and thus cause apoptosis (Ariga et al., 1998; Blazquez et al., 2000; Blazquez et al., 2001; Barrier et al., 2005). Ceramide has been reported to induce apoptosis in many cell types by activating a variety of signaling cascades (Andrieu-Abadie et al., 2001; Pettus et al., 2002). It was also shown that ceramides cause apoptosis during early neural differentiation (Herget et al., 2000) and signal apoptosis in astrocytes via ERK (Blazquez et al., 2000). On the other hand, brain injury, such as trauma, AD, PD and ischemia/stroke, increases the levels of serine proteases thrombin, tryptase or P22 in the brain tissue (Roznieckі et al., 1995; Sawada et al., 2000; Riek-Burchardt et al., 2002; Xi et al., 2003; Lozada et al., 2005; Arai et al., 2006). Subsequently, PAR-1 and/or PAR-2 activation resulted in chemokine GRO/CINC-1 release.

Considering that most chemokines are double-edged swords, we stimulated astrocytes with GRO/CINC-1 at different concentrations together with exogenous C_2-ceramide to investigate whether GRO/CINC-1 could exert protective effects on astrocytes. Our results clearly showed that GRO/CINC-1 concentration-dependently rescued astrocytes from the C_2-ceramide-induced cell death (Fig. 3.31). It is well known that cytochrome c represents a crucial factor in the process of apoptosis. Here, we found that GRO/CINC-1 significantly inhibited C_2-ceramide-induced cytochrome c release from mitochondria (Fig. 3.33). This inhibition was abolished by the CXCR2 antagonist SB-332235 (Fig. 3.34). Noteworthy, both PAR-1 activation induced by low concentrations of thrombin or TRag and PAR-2 activation induced by PAR-2AP also significantly suppressed C_2-ceramide-induced cytochrome c release from mitochondria, which were likewise inhibited by the CXCR2 antagonist (Fig. 3.33, Fig. 3.34, Fig. 3.36). Moreover, activation of PAR-1 and PAR-2 both rescued astrocytes from C_2-ceramide-induced cell death (Fig. 3.32, Fig. 3.36). These results clearly suggest that PAR-1 and PAR-2 activation

under certain physiological or pathological conditions is able to protect neural cells from apoptosis via regulating release of the chemokine GRO/CINC-1.

JNKs are involved in numerous physiological or pathological processes. It has been shown previously that JNKs mediate cytochrome c release (Eminel et al., 2004). However, the role of JNKs is varied and likely depends upon the biological context. Here, we found that PAR-1-dependent JNK activation rescued astrocytes from ceramide-induced cell death. Thus, the activation of JNK seems to be one main contributor for PAR-1 protective action, which provides a novel insight into JNK functions.

Astrocytes are known to produce multiple cytokines and chemokines after brain injury (Chen and Swanson, 2003; Trendelenburg and Dirnagl, 2005). GRO/CINC-1 is probably only one of several important mediators, which are involved in thrombin-induced astrocytic protection. Additional factors, such as macrophage inflammatory protein-2 (MIP-2), which also acts as a ligand for CXCR2 and can be released from astrocytes under certain conditions (Otto et al., 2002), cannot be excluded from this process. Thus, it still remains an interesting issue to find out whether thrombin and tryptase/P22 can rescue astrocytes through regulating the release of some other chemokines. In addition, it still needs to be further investigated whether PAR activation could protect astrocytes in vivo via regulating chemokine GRO/CINC-1 release.

Thrombin at low concentrations has been reported to prevent neural cell death in ischemic and traumatic brain injury via activating its receptor PAR-1 (Xi et al., 2003). Our data give the evidence that PAR-1 protects neural cells from apoptotic cell death via regulting JNK-mediated GRO/CINC-1 release. Therefore, here we provide a new mechanism which is likely to explain the protective action of low concentrations of thrombin after brain injury.

The protective effect of PAR-2 in brain has not received great attention. Only recently appeared a few reports showing that PAR-2 might prevent neural cell death during HIV infection and acute focal ischemic brain injury (Jin et al., 2005; Noorbakhsh et al., 2005). However, the protective action of PAR-2 is still poorly understood. Our results here in this context are of high significance showing that activation of PAR-2, similar to PAR-1, rescued neural cells from ceramide-induced cell death via regulating chemokine GRO/CINC-1 release. Possibly, these protective actions only occur at the early state after mild brain injury, since we found that the protection of PAR-1 and PAR-2 after C_2-ceramide treatment was decreased after 24 h compared to that at 5-10 h (data not shown). Therefore, our data elucidate that the PAR-2 signaling pathway could be another additional

protective pathway in brain. These findings provide important implications for understanding the protective role of PARs in brain. A diagram of intracellular consequences of PAR activation after brain injury is given in Fig. 4.4. Since neurons also express the CXCR2 receptor (Horuk et al., 1997), a similar beneficial effect might exist for neurons.

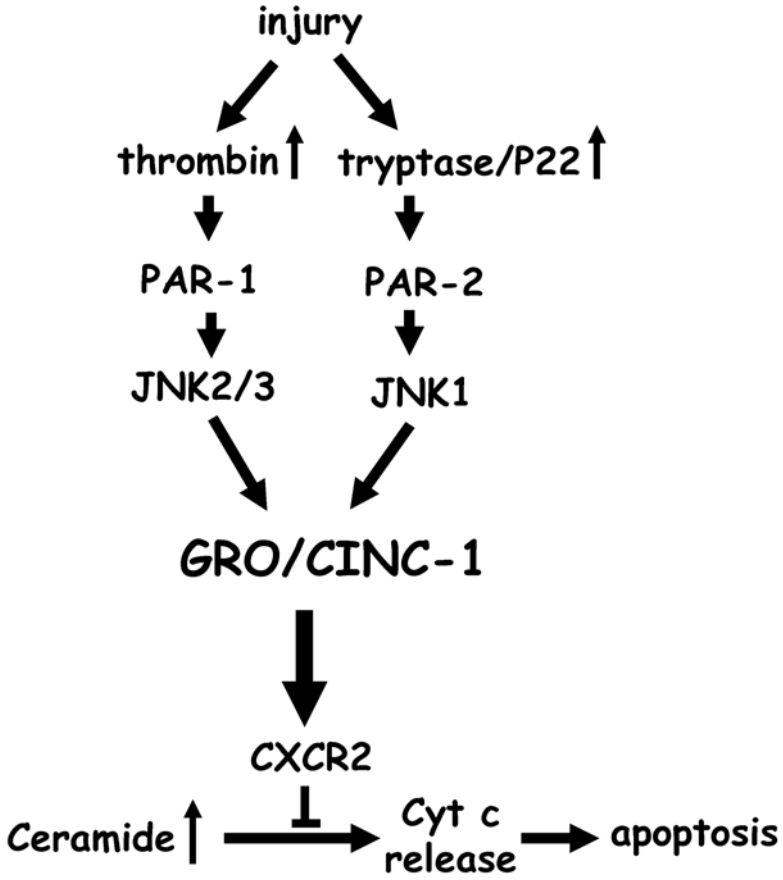


Fig. 4.4. Activation of PAR-1 and PAR-2 protects astrocytes from toxic insults via regulating the release of the chemokine GRO/CINC-1. Under pathophysiological conditions, such as ischemia, trauma, AD, MS, the accumulation of ceramide causes apoptosis in neural astrocytes. On the other hand, the levels of some serine proteases, like thrombin, tryptase or P22, are also increased in brain. Subsequently, these proteases will activate PAR-1 or PAR-2, or both receptors. Activation of PAR-1 and PAR-2 both induce chemokine GRO/CINC-1 release from astrocytes via different JNK isoforms. GRO/CINC-1 can give a feedback through CXCR2 to rescue neural cells from ceramide-induced cell death.

5 Abstract

Protease-activated receptors (PARs), a subfamily of G protein-coupled receptors, are abundantly expressed in rat astrocytes. PARs are activated by certain proteases, like thrombin, trypsin and tryptase, through a unique mechanism that involves irreversible cleavage of the receptor and exposure of a new N-terminal domain acting as a tethered ligand. PAR-1, PAR-3 and PAR-4 are thrombin receptors, while PAR-2 is activated by serine proteases trypsin and mast cell tryptase, etc. In addition to the mitogenic role of PAR-1 on astrocytes established by our group previously, here we investigate whether different PARs, especially PAR-1 and PAR-2, play a role in regulating chemokine or cytokine release and whether they can exert a protective role under the ceramide-mimiced cell pathological condition.

In the present study, we report that thrombin, thrombin receptor agonist peptide (TRag) and PAR-2 activating peptide (PAR-2AP, SLIGRL) concentration-dependently upregulated the secretion of the chemokine growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1), a functional counterpart of human interleukin-8, from primary rat astrocytes. However, treatment with either PAR-3AP (TFRGAP) or PAR-4AP (GYPGKF) failed to increase GRO/CINC-1 mRNA level.

Because activation of PAR-1 and PAR-2 both resulted in the release of the chemokine GRO/CINC-1 from rat astrocytes, we investigate whether the two PAR receptor subtypes can signal separately. By both ELISA and immunoblotting detection, it was found that PAR-1-induced GRO/CINC-1 release was mediated by protein kinase C (PKC), phosphatidylinositol 3 kinase (PI3K) and mitogen-activated protein kinase kinase 1/2 activation, whereas these three kinases were not involved in PAR-2-induced GRO/CINC-1 release. Extracellular signal-regulated kinase 1/2 seemed to be only partially involved in the GRO/CINC-1 secretion induced by PAR-1, but not by PAR-2. Despite such clear differences between PAR-1 and PAR-2 signaling pathways, c-Jun N-terminal kinase (JNK) was identified in both signaling pathways to play a pivotal role. In contrast, p38 mitogen-activated protein kinase was not involved in either PAR-1 or PAR-2 signaling pathway.

Although JNK played a pivotal role in both PAR-1 and PAR-2 signaling pathways, PAR-1 and PAR-2 activated different JNK isoforms. PAR-1 induced the phosphorylation of both 46 kDa and 43 kDa JNK isoforms, whereas PAR-2 caused only 43 kDa JNK phosphorylation. Unlike PAR-1-induced JNK activation, which was mediated by PKC and PI3K, PAR-2-induced JNK phosphorylation was apparently independent of PKC. Moreover, only activation of PAR-1, but not PAR-2, led to c-Jun phosphorylation via JNK.

All differences between the isoforms of JNK phosphorylation, JNK upstream activators and JNK effects on its downstream transcription factor c-Jun clearly indicate that different JNK isoforms with distinct properties might be involved in the PAR-1 and PAR-2 signaling pathways.

By JNK isoform-specific loss-of-function studies using small interfering RNA, we demonstrate that PKC-mediated JNK2 activation and PI3K-mediated JNK3 activation were essential for PAR-1-induced the chemokine GRO/CINC-1 secretion, whereas PAR-1-mediated JNK1 activation was mainly responsible for c-Jun phosphorylation, which was not involved in GRO/CINC-1 release. In contrast, PAR-2-induced JNK1 activation, which failed to phosphorylate c-Jun, uniquely contributed to GRO/CINC-1 release. Therefore, our results for the first time show that JNK-mediated chemokine GRO/CINC-1 release occurs in a JNK isoform-dependent fashion and invokes PAR subtype-specific mechanisms.

Further studies demonstrate that activation of PAR-1 and PAR-2 as well as application of the recombinant GRO/CINC-1 protected astrocytes from C_2-ceramide-induced cell death. Protection occurred with suppression of cytochrome c release from mitochondria. The inhibition of cytochrome c release was largely reduced by the antagonist of chemokine receptor CXCR2, SB-332235. Importantly, the specific JNK inhibitor SP600125 significantly abolished the protective action of PAR-1. These results for the first time demonstrate that PAR-1 plays an important anti-apoptotic role in brain by regulating the release of the chemokine GRO/CINC-1, which gives a feedback through its receptor CXCR2 to preserve astrocytes from toxic insults. This novel mechanism is likely to explain the protective action of thrombin at low concentrations after brain injury. So far, the protective effect of PAR-2 in brain has not received great attention. Our results here in this context are of high significance showing that PAR-2 signaling pathway could be another additional protective pathway in brain via regulating the release of the chemokine GRO/CINC-1.

Our results suggest that PAR-1 and PAR-2 have overlapping functions, but can activate separate pathways under certain pathological conditions, to rescue neural cells from cell death. Different JNK isoforms play pivotal roles in the PAR-induced cell protection via regulating the chemokine GRO/CINC-1 secretion. This provides new functional insights into PAR-JNK signaling and the protective actions of PARs in brain.

6 Zusammenfassung

Protease-aktivierte Rezeptoren (PAR), eine Unterfamilie der G-Protein-gekoppelten Rezeptoren, werden in Astrozyten aus Rattenhirn stark exprimiert. Proteasen, wie Thrombin, Trypsin und Tryptase, aktivieren PARs durch einen einzigartigen Mechanismus, nämlich irreversible Spaltung des Rezeptors und Exposition einer neuen N-terminalen Domäne, welche als intramolekularer Ligand ('tethered ligand') wirkt. Thrombin aktiviert PAR-1, PAR-3 und PAR-4, während PAR-2 durch Trypsin und Mastzell-Tryptase, aktiviert wird. Hier wurde die durch PAR-1 und PAR-2 regulierte Chemokin- und Cytokinfreisetzung untersucht, und studiert, ob diese Chemokine eine protektive Funktion bei neuronalem Zelltod haben.

Hier wird beschrieben, dass PAR-1- und PAR-2-Agonisten konzentrationsabhängig die Sekretion des Chemokins GRO/CINC-1 aus Rattenhirnastrozyten in Primärkultur hochregulieren. GRO/CINC-1 (growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1) ist ein funktionelles Gegenstück zum humanen Interleukin (IL)-8. Die Behandlung sowohl mit PAR-3-aktivierendem Peptid (AP) als auch mit PAR-4-AP führt nicht zu einem Anstieg des GRO/CINC-1 mRNA-Spiegels. Da sowohl PAR-1 als auch PAR-2 die Freisetzung des Chemokins GRO/CINC-1 aus Astrozyten bewirkte, untersuchten wir im Weiteren, ob die beiden PAR-Rezeptorsubtypen ihre Signale über getrennte Transduktionswege weiterleiten. Mittels ELISA- und Immunoblot-Techniken fanden wir, dass die PAR-1-induzierte GRO/CINC-1-Freisetzung durch Proteinkinase C (PKC), Phosphatidylinositol 3-Kinase (PI3K) und Mitogen-aktivierte Proteinkinase-Kinase (MEK1/2) vermittelt wurde. Diese drei Kinasen sind nicht an der PAR-2-induzierten GRO/CINC-1-Freisetzung beteiligt. Trotz dieser klaren Unterschiede zwischen den PAR-1- und PAR-2-Signaltransduktionswegen fanden wir, dass die c-Jun N-terminale Kinase (JNK) in beiden Signaltransduktionswegen eine herausragende Rolle spielt.

JNK ist sowohl bei der PAR-1-, als auch bei der PAR-2 Signaltransduktion wichtig. Aber durch PAR-1 und PAR-2 werden unterschiedliche JNK-Isoformen aktiviert. PAR-1 induzierte die Phosphorylierung der 46 kDa und 43 kDa JNK Isoformen, während PAR-2 nur die Phosphorylierung der 43 kDa JNK bewirkte. Im Gegensatz zur PAR-1-induzierten JNK-Aktivierung, die durch PKC und PI3K vermittelt wurde, war die PAR-2-induzierte JNK-Phosphorylierung unabhängig von der PKC. Die Aktivierung von PAR-1, nicht aber von PAR-2, führte zur Phosphorylierung von c-Jun durch die JNK. Alle diese Unterschiede zeigen deutlich, dass verschiedene JNK-Isoformen mit unterschiedlichen Eigenschaften an den PAR-1- und PAR-2-Signaltransduktionswegen beteiligt sind.

“Loss-of-function-Studien” unter Einsatz von siRNA, die die Expression der verschiedenen JNK-Isoformen unterdrückten, zeigten, dass für die PAR-1-induzierte Freisetzung von GRO/CINC-1 die PKC-vermittelte Aktivierung von JNK2 und die PI3K-vermittelte Aktivierung von JNK3 essentiell waren. Die durch PAR-1 induzierte JNK1-Aktivierung bewirkte die Phosphorylierung von c-Jun, welche aber nicht an der GRO/CINC-1-Freisetzung beteiligt war. Im Gegensatz dazu bewirkte die PAR-2-induzierte Aktivierung von JNK1, welche nicht zur Phosphorylierung von c-Jun führte, die Freisetzung von GRO/CINC-1. Diese Ergebnisse zeigen zum ersten Mal, dass die JNK-vermittelte GRO/CINC-1-Freisetzung in einer JNK-Isoform-spezifischen Weise erfolgt und PAR-Subtypen-spezifische Mechanismen beinhaltet.

Weitere Untersuchungen ergaben, dass erstens die Aktivierung von PAR-1 und PAR-2 und zweitens die Zugabe von rekombinantem GRO/CINC-1 den durch C_2-Ceramid induzierten Zelltod in Astrozyten unterdrückten. Diese Protektion ging mit einer Verhinderung der Freisetzung von Cytochrom C aus den Mitochondrien einher. Die Hemmung der Cytochrom C-Freisetzung wurde durch einen Antagonisten für den Chemokinrezeptor CXCR2, (SB-332235), aufgehoben. Ein weiterer wichtiger Befund ist, dass der spezifische JNK-Inhibitor SP600125 die protektive Wirkung von PAR-1 signifikant unterdrückt. Diese Ergebnisse zeigen zum ersten Mal, dass PAR-1 eine wichtige antiapoptotische Rolle im Gehirn spielt. PAR-1 schützt über die Regulation der Freisetzung des Chemokins GRO/CINC-1, welches über den Chemokinrezeptor CXCR2 wirkt, vor toxischen Insulten. Dieser neuartige Mechanismus erklärt teilweise die seit längerem bekannte protektive Wirkung von niedrigen Thrombinkonzentrationen bei Hirnschädigungen. Über den protektiven Effekt von PAR-2 im Gehirn war bisher nichts bekannt. Daher sind unsere Ergebnisse in diesem Kontext besonders interessant, da sie zeigen, dass der PAR-2-Signaltransduktionsweg über die Regulation der Freisetzung des Chemokins GRO/CINC-1 einen weiteren protektiven Mechanismus im Gehirn darstellt.

PAR-1 und PAR-2 haben auch überlappende Funktionen, aktivieren aber unter bestimmten pathologischen Bedingungen unterschiedliche Signaltransduktionswege, um neurale Zellen vor dem Zelltod zu schützen. Bei dieser PAR-induzierten Zellprotektion über die Regulation der GRO/CINC-1-Sekretion spielen unterschiedliche JNK-Isoformen eine Rolle. Daraus ergeben sich neue funktionelle Einsichten in die PAR-JNK-Signaltransduktion und unser Verständnis der protektiven Wirkungen von PAR-Rezeptoren im Gehirn wird erweitert.

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8 Abbreviations

AD	Alzheimer's disease
AP	Activating peptide
AP-1	Activator protein-1
APC	Activator protein C
ATF-2	Activating transcription factor-2
BSA	Bovin serum albumin
CAPK	Ceramide activated protein kinase
CINC-1	Cytokine-induced neutrophil chemoattractant-1
CNS	Central nervous system
CREB	cAMP response element binding protein
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	Enzyme Linked-Immuno-Sorbent Assay
ELK-1	E-26-like protein 1
ER	Endoplasmic recticulum
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
GRO	Growth-regulated oncogene
GST	glutathione *S*-transferase
HIV	Human immunodeficiency virus
HSP	Heat shock protein
IL	Interleukin
iNOS	Inducible nitric oxide synthase
InsP3	Inositol 1, 4, 5-trisphosphate
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAPKAPK2, 3	MAP kinase-activated protein kinase 2, 3
MAPKKK	MAPK kinase kinase

MEK	Mitogen-activated protein kinase kinase
MEKK	MEK kinase
MLKs	Mixed lineage kinases
MP1	MEK partner 1
MS	Multiple sclerosis
NFAT	Nuclear factor of activated T cells
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
OGD	Oxygen-glucose deprivation
PAR	Protease-activated receptor
PD	Parkinson's disease
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PP1 (2A)	Protein phosphatase 1 (2A)
PRSS	Protease, serine
PTX	Pertussis toxin
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SAPK	Stress-activated protein kinase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SMases	Sphingomyelinases
TM	Transmembrane
TNF	Tumor necrosis factor
TRag	Thrombin Receptor agonist peptide
WT	wild-type

9 Appendix

I. Publications during Ph. D studies

1. **Wang Y.**, Richter-Landsberg C., Reiser G. (2004) Expression of protease-activated receptors (PARs) in OLN-93 oligodendroglial cells and mechanism of PAR-1-induced calcium signaling. ***Neuroscience***. 126:69-82.
2. Major D.T., Nahum V., **Wang Y.,** Reiser G., Fischer B. (2004) Molecular recognition in purinergic receptors. 2. Diastereoselectivity of the h-P2Y1-receptor. ***J Med Chem***. 47:4405-4416.
3. Luo W., **Wang Y.,** Reiser G. (2005) Two types of protease-activated receptors (PAR-1 and PAR-2) mediate calcium signaling in rat retinal ganglion cells RGC-5. ***Brain Res.*** 1047: 159-67.
4. Luo W., **Wang Y.,** Hanck T., Stricker R., Reiser G. (2006) Jab1, a novel protease-activated receptor-2 (PAR-2)-interacting protein, is involved in PAR-2-induced activation of activator protein-1. ***J Biol Chem*** 281: 7927-36.
5. **Wang Y.,** Luo W., Stricker R., Reiser G. (2006) Protease-activated receptor-1 protects rat astrocytes from apoptotic cell death via JNK-mediated release of the chemokine GRO/CINC-1. ***J Neurochem.*** 98: 1046-60.
6. **Wang Y.,** Luo W., Wartmann T. Halangk W., Sahin-Tóth M., Reiser G. (2006) Mesotrypsin, a brain trypsin, activates uniquely proteinase-activated receptor-1, but not proteinase-activated receptor -2, in rat astrocytes ***J Neurochem.*** 99:759-69.
7. **Wang Y.,** Luo W., Reiser G. Proteinase-activated receptor-1 and proteinase-activated receptor-2 induce the release of chemokine GRO/CINC-1 from rat astrocytes via differential activation of JNK isoforms, evoking multiple protective pathways in brain. ***Biochem J.*** 2006 Aug 30; [Epub ahead of print].
8. **Wang Y.,** Luo W., Reiser G. The role of calcium in proteinase-activated receptor-induced GRO/CINC-1 secretion (submitted).
9. **Wang Y.,** Luo W., Reiser G. etc. The neuroprotection of astrocyte chemokines release controlled by protease-activated receptors. (in preparation).

II. Oral Presentations

1. **Wang Y.** Protease-activated receptor (PARs)-induced IL-8-like chemokine GRO/CINC-1 release from rat astrocytes. Molecular Mechanisms of Neurodegeneration and Neuroprotection-Experimental Approaches and the Diseased Brain, Annual Meeting of the Study Group Neurochemistry in Leipzig,

Germany, Sep 9-11, 2004.

2. **Wang Y**. The mechanism of IL-8-like chemokine (GRO/CINC-1) release from rat astrocytes mediated by protease-activated receptor. Satellite Symposium V (symposium of the DFG Neuroscience Graduate Schools) of 6th Meeting of the German Neuroscience Society-30th Göttingen Neurobiology Conference in Göttingen, Germany, Feb. 16-20th, 2005.
3. **Wang Y.** Protease-activated receptor (PAR)-1 and -2 differently mediate release of the chemokine GRO/CINC-1 from rat astrocytes. Neural signal transduction in health and disease-cytokines, mitochondrial dysfunction and transport processes, annual Meeting of the Study Group Neurochemistry of the German Society of Biochemistry and Molecular Biology (GBM) in Leipzig, Germany, Oct. 6-8th, 2005.

III. Poster Presentations

1. **Wang YF**., Richter-Landsberg C. and Reiser G. Expression of protease-activated receptors (PARs) and PAR-induced calcium signaling in oligodendrocytes. The 6th European Meeting on Glial Cell Function in Health and Disease in Berlin, Germany, Sep 3-6, 2003. *Glia* 43, Supplement 2, p57, 2003.
2. **Wang Y**., Luo W., Stricker R. and Reiser G. Protease-activated receptors (PARs)-induced IL-8-like chemokine GRO/CINC-1 release from rat astrocytes. Molecular Mechanisms of Neurodegeneration and Neuroprotection-Experimental Approaches and the Diseased Brain, Annual Meeting of the Study Group Neurochemistry in Leipzig, Germany, Sep 9-11, 2004. *Int. J. Devl. Neurosci.* 22 (7), p598. 2004.
3. Reiser G., **Wang Y**., Luo W., and Stricker R. Protease-activated receptors (PAR-1 and -2)-induced interleukin-8-like chemokine GRO/CINC-1 release from rat astrocytes. The 34th Annual Meeting for American Society for neuroscience in San Diego, USA, Oct. 23-27, 2004.
4. **Wang Y**., Luo W., Stricker R. and Reiser G. The mechanism of IL-8-like chemokine GRO/CINC-1 release from rat astrocytes mediated by protease-activated receptor-1. 6th Meeting of the German Neuroscience Society-30th Göttingen Neurobiology Conference and Joint symposium of the DFG Neuroscience Graduate Schools in Göttingen, Germany, Feb. 16-20th, 2005.
5. **Wang Y**., Luo W., Stricker R. and Reiser G. Different mechanisms of GRO/CINC-1 release from rat astrocytes mediated by protease-activated receptor 1 and 2.

European Meeting on Glial Cell Function in Health and Disease in Amsterdam, Netherlands. May 17-20th, 2005.

6. Luo W., **Wang Y**., Stricker R., Hanck T. and Reiser G. Identification and characterization of human protease-activated receptor (PAR-2) interacting proteins. VII. European Meeting on Glial Cell Function in Health and Disease in Amsterdam, Netherlands. May 17-20th, 2005.
7. **Wang Y**., Luo W., Hanck T., Stricker R. and Reiser G. Protease-activated receptor (PAR)-1 and -2 differently mediate release of the chemokine GRO/CINC-1 from rat astrocytes. Neural signal transduction in health and disease-cytokines, mitochondrial dysfunction and transport processes, annual Meeting of the Study Group Neurochemistry of the German Society of Biochemistry and Molecular Biology (GBM) in Leipzig, Germany, Oct. 6-8th, 2005. Int. J. Devl Neuroscience 24, p218, 2006.
8. Luo W., **Wang Y**., Stricker R., Hanck T. and Reiser G. A proteasome subunit regulates a novel human protease-activated receptor-2 (PAR-2)-mediated inflammatory response pathway. Neural signal transduction in health and disease-cytokines, mitochondrial dysfunction and transport processes, annual Meeting of the Study Group Neurochemistry of the German Society of Biochemistry and Molecular Biology (GBM) in Leipzig, Germany, Oct. 6-8th, 2005. Int. J. Devl Neuroscience 24, p218, 2006.
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10. **Wang Y.,** Luo W. and Reiser G.. Activation of protease-activated receptors-1 and -2 on astrocytes protects from apoptotic cell death via JNK-mediated release of the chemokine GRO/CINC-1. 5th forum of European Neuroscience in Vennia, Austria, July 8-12, 2006.
11. **Wang Y**., Reiser G., Luo W. The role of JNK isoforms in protease-activated receptor (PAR)-1- and PAR-2-mediated cell protection via regulation of release of the chemokine GRO/CINC-1 in brain. Presented at: The 36th Annual Meeting for American Society for neuroscience in Atlanta, USA, Oct. 14-18, 2006.

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