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Signaling From P2 Nucleotide Receptors to Protein Kinase Cascades Induced by CNS Injury

Implications for Reactive Gliosis and Neurodegeneration

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Abstract

Gliosis is a hypertrophic and hyperplastic response to many types of central nervous system injury, including trauma, stroke, seizure, as well as neurodegenerative and demyelinating disorders. Reactive astrocytes, a major component of the glial scar, express molecules that can both inhibit and promote axonal regeneration. ATP, which is released upon traumatic injury, hypoxia, and cell death, contributes to the gliotic response by binding to specific cell surface astrocytic P2 nucleotide receptors and evoking characteristic features of gliosis such as increased expression of glial fibrillary acidic protein (GFAP), generation and elongation of astrocytic processes, and cellular proliferation. Here, we review recent studies that demonstrate that (1) metabotropic, P2Y, and ionotropic, P2X, receptors expressed in astrocytes are coupled to protein kinase signaling pathways that regulate cellular proliferation, differentiation, and survival such as ERK and protein kinase B/Akt and (2) these P2 receptor/protein kinase cascades are activated after trauma induced by mechanical strain. We suggest that P2 receptor/protein kinase signaling pathways might provide novel therapeutic targets to regulate the formation of reactive astrocytes and the production of molecules that affect axonal regeneration and neurodegeneration.

Index Entries: Purinergic receptors; ATP; protein kinases; gliosis; trauma; ERK; Akt.

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Reactive Gliosis and P2 Nucleotide Receptors

Following central nervous system (CNS) injury, astrocytes react in a hypertrophic and hyperplastic manner that contributes to the formation of the glial scar *(1–3).* Reactive gliosis is associated with trauma, stroke, and seizure, as well as neurodegenerative and demyelinating disorders such as Alzheimer's disease and multiple sclerosis. Reactive astrocytes induced by CNS injury express molecules that can impede (e.g., proteoglycans) or promote (e.g., growth factors, cell adhesion molecules) axonal regeneration *(4).* Thus, whereas reactive astrocytes can inhibit axonal regeneration, they also exhibit neuroprotective qualities. A better understanding of the molecular mechanisms underlying the formation of reactive astrocytes could provide new approaches to restore deficits in motor skills and cognitive function caused by CNS injury.

Extracellular nucleotides and nucleosides can contribute to the development of gliosis. Purines, including ATP, are released from damaged or dying cells following injury such as hypoxia *(5–7)* and trauma *(8).* Studies from our laboratory and others have demonstrated that extracellular nucleotides and nucleosides have trophic actions on glial and neuronal cells (reviewed in ref. *9*). For example, extracellular ATP stimulates stellation and proliferation of cultured astrocytes as well as increases in glial fibrillary acidic protein (GFAP) and GFAP messenger RNA (mRNA) *(10–14).* In human astrocytes, mitogenesis is stimulated by ATP and adenosine analogs *(15).* In vivo, GFAP and astrocyte proliferation are increased following infusion of ATP analogs into the rat brain *(16,17).* These effects of ATP are blocked by P2 receptor antagonists, thereby indicating that P2 receptors mediate the trophic actions of extracellular ATP in vivo.

P2 Receptor Signaling to Protein Kinase Pathways in Astrocytes

To determine the molecular mechanisms that mediate trophic actions of P2 nucleotide receptors in astrocytes, recent studies have focused on protein kinase cascades. Several protein kinase signaling pathways have been implicated in cell growth and survival *(18–20).* These include extracellular signal-regulated protein kinase (ERK), a key regulator of cellular proliferation and differentiation, and protein kinase B/Akt, an important regulator of cell survival. As reviewed in this issue by Weisman et al., astrocytes express many subtypes of the two major types of P2 nucleotide receptors, P2Y (metabotropic, G protein-coupled receptors) and P2X (ionotropic, ligand-gated ion channels). P2Y receptors in astrocytes, including P2Y2 receptors *(21),* are coupled to ERK *(22–25).* Astrocytic P2X7 receptors are also coupled to ERK *(21,26).* The ERK cascade consists of three protein kinases: Raf, MEK, and ERK. We found that when MEK, the upstream activator of ERK, was inhibited in human fetal and rat cortical astrocytes, ATP-induced astrocyte mitogenesis was blocked, thereby demonstrating that the ERK cascade mediates mitogenic signaling by P2 receptors *(15,24).* Similarly, inhibition of MEK blocked P2 receptor-mediated process elongation in astrocytes *(25).*

Activated ERK can target proteins in the cytoplasm, membrane, or cytoskeleton, or it can translocate to the nucleus, where ERK can activate or induce transcription factors, thereby leading to the expression of genes important in cell growth. Transcription factors such as CREB and c-fos can be regulated by ERK, and both of these factors are increased after traumatic brain injury *(27–31).* In astrocytes, stimulation of P2 receptors leads to upregulation of early response genes such as c-fos *(32)* and the formation of activator protein (AP)-1 transcriptional complexes *(33).* ERK mediates, at least partially, AP-1 complex formation because when MEK was inhibited, P2 receptor stimulation of AP-1 activation was partially attenuated *(34).* Levels of AP-1 are increased after brain injury *(28,29),* and GFAP contains in its promoter a binding site for AP-1 *(35).* Collectively, these observations suggest a key role for extracellular ATP, P2 nucleotide receptors, and ERK in the upregulation of GFAP and the formation of reactive astrocytes after brain injury.

Protein kinase B/Akt is a member of another interesting signaling pathway because of its important role in promoting cell survival and inhibiting apoptosis through the phosphoinositide 3-kinase (PI3-K) pathway *(20).* Akt activation is a multistep process involving phosphorylation of Ser473 and Thr308 residues, and the phosphorylation of these sites closely correlates with the activity of Akt *(36).* Studies in primary cultures of rat cortical astrocytes indicated that P2Y receptor agonists such as UTP and 2-methylthio ADP and a P2X receptor agonist, 2′,3′-*O*-(4-benzoyl)-benzoyl ATP (BzATP), stimulated Akt phosphorylation *(37).* In addition, pertussis toxin, an inactivator of G_i/G_o proteins, effectively reduced ATP- and UTP-induced Akt phosphorylation but was unable to inhibit BzATP-induced Akt phosphorylation. These studies indicate that astrocytic P2Y and P2X receptors are coupled to Akt.

Glycogen synthase kinase (GSK)-3β is another protein kinase that is linked to P2Y and P2X nucleotide receptors in astrocytes *(38).* This protein kinase was initially identified as an enzyme that regulates glycogen synthesis in response to insulin, but more recent studies indicate that it has neuroprotective effects and is involved in cell survival *(39).* Phosphorylation on serine 9 inactivates GSK-3β, thereby leading to the activation of transcription factors such as myc that are involved in cell survival and proliferation. GSK-3β can be phosphorylated by Akt, but studies with primary cultures of rat cortical astrocytes suggest that P2 nucleotide receptors can signal independently to GSK-3β because inhibition of Akt signaling did not block phosphorylation of GSK-3β. This suggests that another protein kinase, perhaps protein kinase C, links P2 receptors to GSK-3β. Because there is information flow among key elements of protein kinase signaling networks *(40),* we investigated whether there might be crosstalk among ERK, Akt, and GSK-3β stimulated by P2 nucleotide receptors. Our initial studies indicate that Akt and GSK-3β can still be phosphorylated when P2 receptor/ERK signaling is blocked; similarly, ERK and GSK-3β can still be phosphorylated when PI3-K/Akt signaling is blocked *(38).* These findings suggest that P2 nucleotide receptors are coupled independently to several key protein kinase signaling pathways.

Activation of Protein Kinase Pathways by Traumatic Injury

The studies described thus far demonstrate that P2 nucleotide receptors in astrocytes are coupled to several protein kinase signaling pathways. Because ATP is released by damaged or dying cells, this raised the question of whether P2 receptor/protein kinase signaling is activated after CNS injury. To address this question, we have conducted studies with an in vitro model of trauma to determine whether protein kinase cascades in astrocytes are activated by rapid and reversible mechanical strain, and if so, whether such activation is the result of P2 nucleotide receptor signaling. Traumatic brain injury (TBI) imparts a mechanical force to the brain, causing a rapid deformation of brain tissue. Several in vivo and in vitro models have been developed to study the mechanisms of TBI. Both types of model have their advantages and disadvantages *(41).* Some of the advantages of the in vivo models are that they allow for measurement of biochemical responses of the intact brain to mechanical force as well as for behavioral function and recovery studies. Activation of ERK *(31,42–45)* has been reported after in vivo TBI and stab lesion *(46).* Akt is also activated after in vivo TBI *(47,48),* and dominant negative Akt hastened nerve transection-induced neuronal death *(49).* However, a disadvantage of in vivo models is that because of the complexity of the brain, it is difficult to study molecular mechanisms of TBI at the cellular level. To address this issue, in vitro models have been developed. For example, "scratch-wounding" of cultured astrocytes resulted in hypertrophy and proliferation, responses resembling brain injury in vivo *(50),* and ERK activation was observed in this

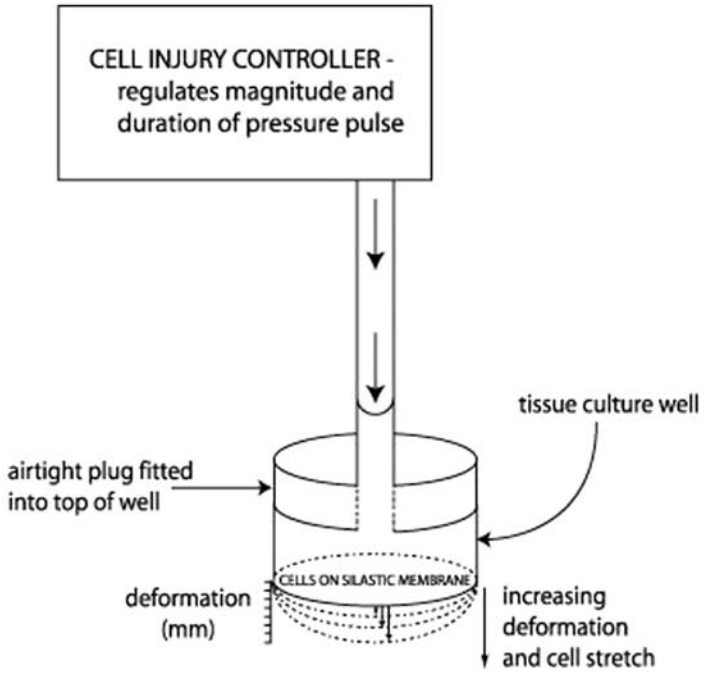

Fig. 1. Model for in vitro traumatic injury studies. The diagram depicting the system for subjecting cells to mechanical strain was adapted from Ellis et al. *(52).*

model *(51).* Ellis and colleagues have developed a model of strain, an important component of brain trauma. In this model, cells are grown on a silastic membrane that deforms rapidly and reversibly when subjected to pressure *(52);* the extent and duration of injury is precisely controlled by a pressure regulator and timer (Fig. 1). The range of membrane displacements generated in this model correspond to biaxial strains, or stretch, that are relevant to those that occur in humans after rotational acceleration–deceleration injury, as indicated by studies with gel-filled human skulls *(53).* As verification of this model, many of the posttraumatic responses observed in vivo are replicated in vitro with this model, including transient increases in intracellular calcium *(54),* activation of phospholipases *(55,56),* free-radical formation *(57),* and depletion and release of intracellular ATP *(8).* In addition, GFAP immunofluorescence is also increased *(58).* Although an increase in GFAP immunofluorescence does not always correspond to an increase GFAP content, results of preliminary experiments using reverse transcription–polymerase chain reaction and enzyme-linked immunoserbent assay suggest that GFAP mRNA and protein levels are elevated 1 d after in vitro trauma (Neary J.T., Wanner T.B., Kang Y., unpublished observations).

We have utilized the model diagrammed in Fig. 1 to study the activation of protein kinase cascades in primary cultures of rat cortical astrocytes subjected to rapid and reversible mechanical strain. Our studies indicated that mechanical strain rapidly activates ERK *(59).* Trauma-induced ERK activity peaked at 10 to 30 min and remained elevated over baseline for at least 3 h. ERK activity was increased in a graded manner corresponding to mild, moderate, and severe strain. ERK activation was more intense with rapid stretch (20–50 ms) as compared to slower stretch (99 ms). Strain-induced ERK activation was blocked by

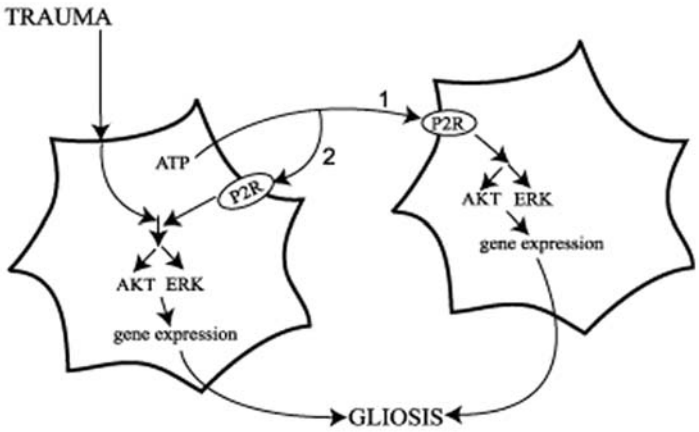

Fig. 2. Scenarios for trauma-induced activation of ERK and Akt in astrocytes. In the model proposed here, traumatic injury induces the release of ATP, which can stimulate P2 nucleotide receptors in a paracrine or autocrine manner. Trauma-induced stimulation of these receptors activates protein kinases such as ERK and Akt. Acitvation of these kinases leads to the activation of transcription factors, resulting in expression of gene products that contribute to the gliotic response following trauma.

inhibiting MEK, the upstream activator of ERK. Calcium influx plays an important role in ERK activation by mechanical strain because chelation of extracellular calcium with EGTA prior to trauma reduced ERK activation by about 85%. To determine whether reactive oxygen species (ROS) are also involved in ERK activation induced by mechanical strain, astrocytes were treated with the free-radical scavenger *N*-acetyl cysteine prior to trauma. ERK activity was measured 5 min postinjury using a highly selective peptide as a substrate *(59).* ERK activity was reduced by 62%, thereby indicating that ROS formed after mechanical strain can participate in ERK signaling.

Akt was also activated in mechanically strained astrocytes *(60).* Akt phosphorylation peaked earlier and declined more rapidly than ERK. Similar to ERK, Akt phosphorylation increased in a graded manner with increasing degrees of mechanical strain. Stretch-induced Akt activation was blocked by wortmannin, thereby indicating that PI3-K is upstream of Akt in trauma-induced signaling. Calcium influx is also involved in Akt activation by mechanical strain because chelation of extracellular calcium with EGTA prior to trauma significantly reduced Akt phosphorylation.

Role of P2 Nucleotide Receptors in Trauma-Induced Signaling

To address the question of whether the activation of ERK and Akt by mechanical strain involves P2 nucleotide receptor signaling, two approaches were used. The first approach utilized an ATP diphosphohydrolase, apyrase, to hydrolyze released ATP to AMP. When apyrase was added to primary cultures of rat cortical astrocytes before trauma, ERK activation was reduced by as much as 75% *(59).* The second approach involved the use of P2 nucleotide receptor antagonists, such as suramin, a broad-spectrum antagonist of numerous subtypes of P2Y and P2X receptors *(61).* When suramin was added to astrocytes before mechanical strain, ERK activation was again reduced about 75% *(59).* Additional antagonist studies indicated that trauma-induced ERK activation is stimulated by selected P2 nucleotide receptor subtypes. For example, iso-

pyridoxal-5′phosphate-6-azophenyl-2′,5′ disulfonate (iso-PPADS), reactive blue-2, and N^6-methyl 2′-deoxyadenosine 3′,5′-bisphosphonate (MRS-2179) significantly inhibited trauma-induced ERK activation, whereas brilliant blue G and trinitrophenyl-ATP were ineffective. These results suggest that P2X2 and P2Y1 receptors, but not P2X1, P2X3, and P2X7 receptors, signal to the ERK cascade after trauma. The potential involvement of other P2 receptors expressed in astrocytes, such as P2Y2, P2Y2, P2X4, and P2X6, remains to be determined.

P2 nucleotide receptors are also involved in trauma-induced Akt activation because P2 receptor antagonists significantly reduced Akt activation after mechanical strain *(62)* (*see* Fig. 2). However, a different pattern of P2 receptors stimulated by mechanical trauma might be involved because antagonist profile studies suggest that not all of the antagonists that effectively inhibited ERK activation also inhibited Akt activation. GSK-3β is also phosphorylated after in vivo TBI *(48)* and in mechanically strained astrocytes *(63),* but further studies are needed to determine whether trauma-induced GSK-3β phosphorylation is mediated by release of ATP and P2 nucleotide receptor signaling.

Many factors are increased after CNS injuries, some of which might act in a synergistic or combinatorial manner *(4).* The large but incomplete blockade of trauma-induced ERK and Akt activation that resulted from interfering with P2 receptor signaling suggests that, in addition to ATP, other molecules participate in the activation of these kinases. One potential candidate is tumor necrosis factor-α (TNF-α), which can be rapidly released from astrocytes via activation of the CXCR4 receptor *(64)* and can lead to ERK activation *(65).* However, we were unable to detect TNF-α release from astrocytes within minutes after mechanical strain (Kucher et al., unpublished observations). Glutamate is another interesting candidate because glutamate receptors have been implicated in CNS injury *(66,67).* We found that antagonists of ionotropic and metabotropic glutamate receptors reduced trauma-induced ERK activation in astrocytes by about 10% (Neary and Kang, unpublished observations). For example, *(RS)*-1-aminoindan-15-decarboxylic acid (AIDA), an antagonist of group 1 metabotropic glutamate receptors that almost completely blocks trauma-induced inositol phosphate production in astrocytes *(58),* inhibited trauma-induced ERK by 11% 10 min postinjury. Another antagonist of metabotropic glutamate receptors, methyl-4-carboxyphenylglycine (MCPG), and an antagonist of ionotropic AMPA/kainate receptors, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), inhibited strain-induced ERK activation by about 10%. Additional experiments showed that glutamate receptors are weakly coupled to ERK in primary cultures of rat cortical astrocytes. For example, addition of glutamate, quisqualate, or AMPA to noninjured astrocytes stimulated ERK activity by 50–100%, whereas ERK activity was increased fivefold to sixfold by extracellular ATP. These findings suggest that glutamate receptor signaling plays a minor role in trauma-induced ERK activation in astrocytes, at least in the early postinjury response phase. Neurons are more susceptible to glutamate-induced excitotoxicity than astrocytes *(68)* and it could be that glutamate receptor/protein kinase signaling plays a more important role in neurons.

Although many factors can contribute to the development of reactive gliosis, the studies reviewed here point to a major role for P2 receptor/protein kinase signaling pathways in the astrocytic response to CNS injuries. Components of these pathways might serve as novel therapeutic targets for translational studies designed to minimize or prevent neurological deficits caused by CNS injuries.

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