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The APCs of neuroprotection

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Mutations in the enzyme superoxide dismutase 1 (SOD1) have been linked to the neurodegenerative disease amyotrophic lateral sclerosis (ALS). In this issue of the *JCI*, Zhong et al. report that the endogenous anticoagulant activated protein C (APC) is able to cross the blood–spinal cord barrier in mice and signal to both neuronal and non-neuronal cells (see the related article beginning on page 3437). This signaling resulted in the suppression of mutant SOD1 synthesis and retarded disease progression in a murine model of ALS. Here we discuss the potential importance of these data and possible relevance to human neurodegenerative diseases.

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease that strikes in midlife, causing progressive weakness, disability, and death. Unfortunately, the cause of ALS is unknown, and the treatment is largely palliative. Research into the pathogenesis of and potential treatments for ALS focuses heavily on experimental models that use human genetic mutations in transgenic animals or on cellular models that express mutant proteins known to impart a high risk of developing ALS in people who carry these mutations. Mutant superoxide dismutase 1 (SOD1) is the most common protein known to cause ALS in humans; however, the mechanisms underlying mutant SOD1–related ALS are unknown. People with mutant SOD1–related ALS represent only about 20% of inherited (i.e., familial) ALS cases and about 2% of all patients with ALS (1). Nevertheless, the animal models of mutant SOD1–related ALS develop a neurological disorder that mimics the human disease, and investigations using these models have taught us a lot about motor neuron biology as well as the potential interactions between motor
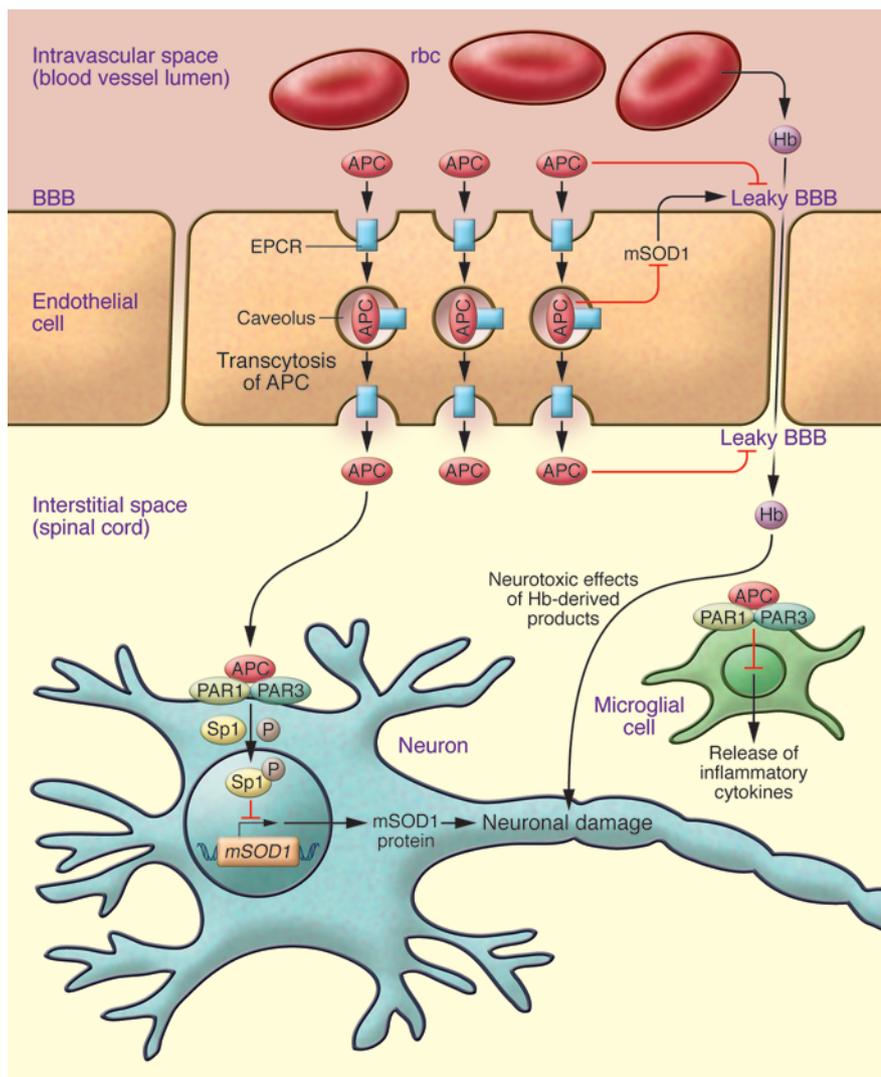
Figure 1
Potential mechanism for the protection of neural tissue in ALS. APC binds to the EPCR located in caveolae. Caveolae translocate the endothelium and allow deposition of APC across the BBB, consistent with the results reported in this issue by Zhong et al. (4). Once across the BBB, APC can dissociate from EPCR and signal neuronal cells through activation of PAR1 in a process dependent on PAR3 (4), and this in turn increases the phosphorylation of the nuclear transcription factor Sp1, thereby downregulating the production of mutant SOD1 (mSOD1), a mediator of neuronal damage in this animal model of familial ALS. As described by Zhong et al. (4), APC can also provide neuroprotective effects by reducing leakage through the BBB, inhibiting release of inflammatory cytokines from microglia as well as lessening the oxidative damage to neurons by Hb products entering the extravascular space through the endothelium.

neurons and their environment that support or destabilize their normal function and survival.

Recent publications by Garbuzova-Davis et al. (2) and Zhong et al. (3) reported evidence of abnormalities in blood-brain barrier (BBB) function in mutant SOD1 animal models of ALS. These investigators hypothesized that compromise of the BBB allows exposure of motor neurons to potentially neurotoxic proteins from the blood, such as hemoglobin (Hb), that could either initiate or accelerate the process of motor neuron degeneration. In the current issue of the *JCI*, Zhong and colleagues (4) have followed up on the findings of these previous studies by carefully investigating BBB leakage in a mouse model of ALS. The authors introduced a therapeutic intervention, the anticoagulant protease activated protein C (APC),

which reduced the vascular leak of Hb-derived products into the spinal cords of transgenic mice expressing ALS-linked mutant SOD1 ($SOD1^{G93A}$) and had the added effect of downregulating production of the mutant SOD1 protein in both neuronal and non-neuronal cells (Figure 1), resulting in slower disease progression and extension of survival time in this animal model.

APC biology
APC is a serine protease generated from the inactive plasma zymogen, protein C, via proteolysis mediated by the complex of thrombin, thrombomodulin, and endothelial protein C receptor (EPCR) primarily on the surface of the endothelium. Originally identified primarily as an anticoagulant, both because of its potent in vitro anticoagulant activity and because

of the dramatic thrombotic complications that arise in infants deficient in protein C (5), APC has recently drawn attention for its ability to protectively modulate a variety of disease processes, including human sepsis (6) and rodent models of Crohn disease (7), diabetic nephropathy (8), stroke (9), tumor metastasis (10), multiple sclerosis (11), and reperfusion injury (12). Most of these protective effects are primarily or potentially independent of the anticoagulant activity of APC. The common feature of most of these protective effects is dependency on the EPCR, found primarily on endothelial cells (13), and on protease-activated receptor 1 (PAR1), a 7-transmembrane G protein–coupled receptor expressed by most cell types (14). Thrombin is the prototypical activator of PAR1, and when it proteolytically activates PAR1, it elicits a variety of

proinflammatory responses leading to expression of adhesion molecules on the cell surface and loss of endothelial cell barrier function. APC can also activate PAR1 (15), but APC-mediated activation gives rise to a different response, which protects against loss of barrier function and decreases proinflammatory responses through downregulation of NF-κB. The mechanisms underlying the pro- versus antiinflammatory effects of PAR1 activation remain the subject of investigation. One model from the literature (16) suggests that the location of APC bound to EPCR within caveolae (small invaginations of the plasma membrane) of luminal endothelial cells may help to dictate which G protein is linked to PAR1 and hence the nature of the downstream signaling. The ability of APC to elicit cellular responses that are cytoprotective and antiinflammatory through PAR1 activation may explain why APC is effective in preventing disease progression in many of the disease models mentioned above (6–12). Indeed, variants of APC with low anticoagulant activity have been generated that retain cytoprotective activity and remain protective in many animal models of disease, including sepsis (17). One of these variants was used in the present study by Zhong et al. (4). In most of these disease models, the target cells are located within the intravascular space, but several of the diseases cited above, including ALS, involve extravascular cells. Key to extravascular signaling is the fact that APC needs to reach the extravascular tissue from the intravascular space. Because EPCR binds APC reversibly and can be observed in caveolae (16), and because these organelles are known for their ability to transcytose the cell (18), it is likely that APC bound to EPCR located on the luminal surface of the endothelium is carried across the endothelium during transcytosis of the caveolae, resulting in the delivery of proteins either present in the caveolae or bound to other proteins present therein, resulting in the delivery of proteins that potentially activate PAR1 to the interstitial space (i.e., spinal cord; Figure 1), as reported by Zhong et al. in their current study (4). Then, signaling through PAR1— with the participation of PAR3 — would elicit cellular responses (4), including downregulation of mutant SOD1 through decreases in the levels of the nuclear transcription factor Sp1, manifested at least in part through increased phosphorylation of Sp1.

Novel features of the proposed APC signaling mechanism in ALS

The features of the signaling mechanism reported by Zhong et al. (4) differ from those seen previously because the target neuronal cells appear to lack EPCR, raising the issue of how this signaling occurs mechanistically. Several proteases can activate PAR1, but the presence of EPCR is usually required for the protective signaling observed through PAR1 activation. The previously described function of PAR3 is to bind thrombin (but not signal directly) and facilitate PAR4 activation, leading to mouse platelet activation (14). Therefore, to our knowledge, the PAR1-PAR3 interaction observed by Zhong et al. (4) is new and of unknown mechanistic significance. Perhaps activation of the PAR1-PAR3 complex on neuronal cells leads to the altered signaling specificity normally observed with APC activation of PAR1 on EPCR-expressing cells. A second issue is that the binding of APC to EPCR will concentrate APC near the cell surface, thus facilitating activation of PAR1; thus, an unresolved question arises as to why APC is an efficient activator of neuronal cells lacking EPCR.

Limitations of the mouse model of ALS

The concept of BBB dysfunction in ALS is not new, and was suggested in a few human studies of spinal fluid characteristics that demonstrated an elevated CSF albumin/serum albumin ratio in a large percentage of ALS patients (19, 20). However, enthusiasm for treating ALS patients with APC might be dampened by several factors, including the disappointing experience of trying to translate therapeutic successes in mutant SOD1 animals to ALS patients (21). Clearly, mice that express multiple copies of the mutant human *SOD1* gene are not a faithful recapitulation of the genotype found in people carrying SOD1 mutations who express the mutant and wild-type SOD1 proteins at similar levels. Moreover, there is little evidence supporting the hypothesis that common mechanisms are involved in mutant SOD1–related ALS and sporadic ALS. Furthermore, pathological evidence of leakage of toxic substances through the BBB, as was demonstrated in mutant SOD1–ALS mice (2, 3), has not been reported in humans. Regardless, current treatments for ALS are woefully inadequate, and any therapeutic intervention that is well supported by a clear hypothesis and experimental evidence should be seri-

ously considered for a clinical trial. APC, with its ability to downregulate the production of mutant SOD1, should perhaps be tried first in ALS patients with known SOD1 mutations. However, clinical trials in this very rare population are hindered by the limited numbers of potential participants. The bonus of APC may be its ability to reduce BBB leakage in ALS patients, and a trial of APC therapy for ALS would not only address its possible therapeutic effect, but perhaps provide data on the role of BBB leakage in ALS progression.

Potential promise and complications of APC therapy for ALS

A trial of APC therapy in people with ALS would not be without risk. A potential complication of treating nonthrombotic diseases with wild-type APC is the increased risk of bleeding due to APC's anticoagulant functions, especially if the treatment requires prolonged infusion. Extensive structural and structure-function studies of APC led to the identification of APC variants (17) that retained signaling capacity but lost most of their anticoagulant function, thus reducing the risk of hemorrhage. These APC variants (17), used in the present study (4), provide a good example of the power of this approach. A concern with the use of mutant proteins in treating diseases, especially in the form of prolonged therapy, is the potential to elicit epitope spreading (22), in which antibodies to epitopes on the mutant proteins ultimately spread to react with the distinct epitopes on the native molecule, which in this case could lead to massive thrombotic complications.

The present study may have importance beyond its potential relevance to ALS. Zhong and colleagues illustrate that EPCR can be used to transport APC across the BBB (4). If their results from these mouse studies are reproducible in humans, either EPCR or derivatized APC could be used to transport therapeutic agents into the brain. Given that APC is a serine protease, it is relatively simple to design targeting agents to fill the active site reversibly and thus provide a delivery vehicle.

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Smad4: gatekeeper gene in head and neck squamous cell carcinoma

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Unchecked cell growth is a hallmark of cancer. During oncogenesis, cancer-ous cells become resistant to the TGF-β signaling pathway that usually keeps cell growth in check. The role of a critical mediator of this pathway, Smad4, in head and neck squamous cell carcinoma (HNSCC) remains unclear. In this issue of the *JCI*, Bornstein and colleagues report that Smad4 expression is decreased in malignant HNSCC and, surprisingly, also in normal-appear-ing buccal mucosa adjacent to HNSCC (see the related article beginning on page 3408). They also show that targeted conditional deletion of *Smad4* in the head and neck epithelium of mice is alone sufficient to initiate sponta-neous HNSCC, in conjunction with DNA repair gene dysregulation, genetic instability, and inflammation. These findings point to a novel function for *Smad4* as a guardian gene that maintains genomic stability.

The vast majority of head and neck cancers are squamous cell carcinomas (HNSCCs), all of which arise from a mucosal surface. HNSCCs can include cancers of the mouth, larynx, pharynx, tongue, lip, or nasal cavity but traditionally do not include cancers of the thyroid, esophagus, or skin. The malig-nancy is more prevalent in males and in individuals who smoke or chew tobacco and/or consume alcohol (1, 2). Certain viral agents, such as human papilloma virus types 16 and 18, increase the risk of developing HNSCC in the oral cavity (1, 2). In spite of considerable advances in our understanding of the molecular alterations that occur in this malignancy, the 5-year survival rate has stubbornly remained at approximately 50%, due to resistance to therapy; cancer recurrence following surgi-cal resection even when followed by chemo- or radiotherapy; and the development of second, unrelated malignancies (1–3).

Key genetic alterations known to exist in HNSCC include (a) overexpression of the growth factor receptor EGFR; (b) muta-tions in the tumor suppressor gene *p53*; (c) mutation or overexpression of the onco-genes *K-ras* or *H-ras*; (d) increased levels of the cell-cycle regulator and proto-onco-gene cyclin D1, the cytokine IL-6, the tran-scription factor runt-related transcription factor 2 (RUNX3), and the inflammatory mediator COX2; (e) excessive activation of PI3K/Akt, STAT3, and NF-κB pathways fundamental to cell proliferation and sur-vival; (f) germline mutations in the Fan-coni anemia/breast cancer susceptibility gene (Fanc/Brca) pathway, which coordi-