*Current Perspective***Protein Tyrosine Phosphorylation in the Ischemic Brain**Norio Takagi^{1,*}¹*Department of Applied Biochemistry, Tokyo University of Pharmacy & Life Sciences,
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Abstract. Cerebral ischemia, a pathological condition in which brain tissue experiences a shortage of cerebral blood flow, is associated with cerebrovascular disease, brain trauma, epilepsy, and cardiac arrest. A reduction in blood flow leaves the brain tissue unsupplied with oxygen and glucose, thus leading to cell death in the ischemic core as well as subsequent peripheral injury in the penumbra. Neurons in the penumbra, where reperfusion occurs, are functionally inactive but still viable. Many biochemical changes, which may lead to neuronal cell death, thereby induce dysfunction of the central nervous system. However, the mechanisms responsible for ischemic stroke-induced cell damage remain to be determined. Protein phosphorylation has been implicated in the regulation of diverse cellular responses in the brain. Initially, tyrosine phosphorylation was considered to be involved in the regulation of cell growth and development. In addition, a variety of synaptic and cellular functions mediated by tyrosine phosphorylation in the brain were found to be associated with relatively high levels of protein tyrosine kinase activity. However, the involvement of this protein tyrosine kinase activity in ischemic cell death is still not fully understood. This review summarizes recent advances dealing with the possible implications of protein tyrosine phosphorylation in the ischemic brain.

Keywords: cerebral ischemia, stroke, tyrosine phosphorylation, tyrosine kinase, protein tyrosine phosphatase

1. Introduction

Stroke is a major cause of mortality, and stroke survivors suffer severe acquired disability, including motor dysfunction, post-stroke dementia, and depression. Although improved management of risk factors for ischemic stroke has contributed to a decline in the frequency of both primary and recurrent stroke, the only currently approved treatment for acute ischemic stroke is intravenous fibrinolysis achieved with recombinant tissue plasminogen activator (tPA). However, tPA must be administered within a short time window after the onset of symptoms in patients with acute ischemic stroke to optimize its therapeutic efficacy. Therefore, many acute stroke patients do not receive this treatment because of

the short therapeutic time window and/or the risk of hemorrhage. More study for an effective treatment of patients with stroke is required to determine the diverse pathophysiological responses after the onset of stroke (Table 1).

1.1. Cerebral ischemia

The pathophysiologic alterations are not the same for all cells in the ischemic brain. The progress of ischemic damage is determined by several factors including the duration of the ischemic episode and the properties of the affected cells (1, 2). An interruption in cerebral blood flow, which limits oxygen and glucose, causes energy depletion, leads to a disturbance in ionic gradients, and thereby a collapse of the membrane potential of neuronal cells. The subsequent membrane depolarization induces a marked increase in the release of neurotransmitters such as excitatory amino acids. Furthermore, energy failure impairs re-uptake of the excitatory neurotransmitters by the high-affinity transporters on the surrounding

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glial and neuronal cells. The excessive accumulation of extracellular glutamate over-stimulates their postsynaptic and/or extrasynaptic receptor ion channels in neurons, thereby inducing an increase in the level of intracellular Ca^{2+} , a principal event leading to cell death.

1.2. Protein phosphorylation

Cellular functions in the central nervous system are regulated by diverse intra- and extra-cellular responses. In particular, protein phosphorylation and dephosphorylation play crucial roles in various cellular functions. As protein kinase activity is abundant in the synaptic region, protein phosphorylation and dephosphorylation play a central role in the regulation of synaptic function under physiologic and pathophysiologic conditions (3, 4). Although tyrosine phosphorylation was once considered to be primarily involved in the regulation of cell growth and development, a number of tissues continue to reveal tyrosine phosphorylation in the adult animal, indicating an important role for tyrosine phosphorylation in the function of mature cells. In particular, post-mitotic neurons were found to express high levels of tyrosine kinase activity (5). The involvement of protein phosphorylation in a variety of synaptic functions under physiologic conditions has also been documented. Tyrosine phosphorylation of proteins is promoted in several stages after the start of reperfusion following cerebral ischemia. Increased tyrosine phosphorylation may contribute to the development of pathophysiologic alterations. In contrast, the amounts of several growth factors, which might be involved in protection and/or

repair of ischemic brain injury via their tyrosine kinase receptors, are increased in the late phase after cerebral ischemia. Thus, the pathophysiologic roles of protein tyrosine phosphorylation at an acute phase may be distinct from those at a late phase. The remainder of this review summarizes recent advances pointing to possible implications of protein tyrosine phosphorylation under pathophysiologic conditions, particularly cerebral ischemia.

2. Tyrosine phosphorylation of glutamate receptors in the ischemic brain

Neuronal cell death induced by ischemic stroke has been traditionally thought to result from glutamate receptor-mediated excitotoxicity (6). Glutamate receptors can be divided into functionally distinct metabotropic and ionotropic ones. The ionotropic glutamate receptors can be subdivided into three pharmacologically distinct subfamilies based on their affinity for *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate.

The NMDA receptor is a heteromer that consists of complexes of a ubiquitously expressed GluN1 subunit and four distinct types of GluN2 subunits (GluN2A-2D). Whereas GluN1 is the principal subunit for NMDA-receptor-channel activity, GluN2 subunits contribute to the variety of receptor functions. Intracellular calcium is an important messenger in diverse cellular responses in the brain, but excessive Ca^{2+} loading via the NMDA receptor can promote inappropriate processes, including

Table 1. Tyrosine phosphorylation-regulated events after cerebral ischemia

Targets	Phosphorylation or dephosphorylation	Observed effects (acute or late phase of post-stroke)	Reference No.
GluN2A GluN2B	Tyrosine phosphorylation	NMDA receptor-mediated Ca^{2+} influx (acute)	9
GluN2B	Tyrosine phosphorylation	Binding to the SH2 domain of PI3-kinase (acute)	15, 16
Various proteins	Inhibition of dephosphorylation by STEP KO	Exacerbation of stroke-induced tissue damage (acute)	21
PSD-95	Tyrosine phosphorylation	Interaction of PSD-95 with Src/Fyn (up-regulation of NMDA receptor function, acute)	22
Eph-A2 receptor	Decrease in tyrosine phosphorylation of EphA receptor by deletion	Promotion of tight junction formation (acute – subacute)	24
		Reduction in the level of pro-apoptotic proteins (acute – subacute)	24
Eph-A receptors	Tyrosine phosphorylation	Inhibition of axonal sprouting (subacute – late)	25
Occludin	Tyrosine phosphorylation	Increase in vascular permeability (acute)	29
Src	Inhibition of phosphorylation by src family inhibitor PPI	Increase in the expression of tight junction protein (acute – subacute)	30
Tie2	Activation of tyrosine kinase receptor Tie2 by Ang-1	Reduction in stroke-induced BBB leakage (acute)	32
Receptor for growth and neurotrophic factors	Tyrosine phosphorylation of tyrosine kinase receptors	Protection from ischemic injuries (late phase of post-stroke)	35, 38, 39, 40
		Increase in vascular permeability (in acute phase of post-stroke, VEGF)	37

activation of calcium-sensitive proteases and nitric oxide synthase. In addition, free-radical generation occurs in the mitochondria secondary to calcium influx through the NMDA receptor. Therefore, excessive Ca^{2+} influx through the NMDA receptor has been implicated to play a crucial role in the progression of ischemic damage.

Both GluN2A and GluN2B subunits of the NMDA receptor have been shown to be phosphorylated on their tyrosine residues (7, 8). Furthermore, it was earlier demonstrated that NMDA receptor-mediated currents are potentiated by tyrosine phosphorylation (9). Also, NMDA receptor-mediated Ca^{2+} influx is enhanced when tyrosine residues of GluN2A and GluN2B are phosphorylated (7, 10). Thus, the finding that cerebral ischemia and reperfusion facilitate tyrosine phosphorylation of GluN2A and GluN2B subunits suggests that these tyrosine-phosphorylated subunits play a key role in neuronal damage mediated by excessive Ca^{2+} influx after ischemic stroke (11–13).

The tyrosine-phosphorylated GluN2 subunit can also be associated with several proteins that contain the Src homology 2 (SH2) domain (14). Among tyrosine residues on GluN2 subunits, Tyr1336, located in the intracellular C-terminal domain of GluN2B, is phosphorylated and may bind to the SH2 domain of the p85 subunit of PI3-kinase. Tyrosine phosphorylation of GluN2B is enhanced, and the binding of tyrosine phosphorylated GluN2B subunits to the SH2 domain of PI3-kinase is increased, after cerebral ischemia (15, 16). Interestingly, the SH2 domain of PI3-kinase does not bind to the GluN2A subunit after cerebral ischemia, although a larger increase in the tyrosine phosphorylation of GluN2A than in that of GluN2B is observed. It has been shown that binding of proteins through their phospho-tyrosine residues to the SH2 domains of p85 enhances PI3-kinase activity (17). Therefore, cerebral ischemia-induced tyrosine phosphorylation of GluN2 subunits of the NMDA receptor can alter intracellular signaling pathways via the interaction of the NMDA receptor with SH2 domain-containing proteins, an event that may contribute to an important pathophysiological alteration after ischemic stroke (15, 16).

Src family kinases, which are highly expressed in the central nervous system, act as a key modulator to regulate the localization of GluN2 subunits of the NMDA receptor in intracellular and synaptic pools (18). For instance, tyrosine phosphorylation of the GluN2B subunit at its Y1472 residue plays a pivotal role in the regulation of NMDA-receptor expression at synaptic membrane surfaces and in PSD-95 binding to the GluN2B C-terminus for NMDA-receptor stability on the plasma membrane (19). Therefore, enhanced tyrosine phosphorylation of GluN2 subunits affects the localization

of the NMDA receptor after ischemic stroke.

Both protein kinases and phosphatases are involved in regulating the phosphorylation state of proteins. In this sense, striatal-enriched phosphatase (STEP), a protein tyrosine phosphatase, is widely expressed in the CNS, especially in the striatum, and dephosphorylates a number of proteins, including the GluN2 subunit of the NMDA receptor. STEP has been implicated in synaptic function under physiologic and pathophysiologic conditions. As STEP dephosphorylates tyrosine residues on the GluN2B subunit of the NMDA receptor, an action that induces the removal of these subunits from the synaptic membrane and a reduction in NMDA-receptor function, STEP has been implicated in excitotoxicity through the NMDA receptor after ischemic stroke. Indeed, the amount and the activity of STEP are reduced after transient cerebral ischemia (20). Furthermore, ischemic stroke-induced tissue damage, as well as neurological deficits, is exacerbated in STEP KO mice, suggesting a neuroprotective role for STEP-mediated dephosphorylation of proteins after ischemic stroke (21).

3. Tyrosine phosphorylation of PSD-95 in the ischemic brain

Postsynaptic density protein 95 (PSD-95), a major scaffold protein at the postsynaptic density in excitatory synapses, is one of the members of the family of membrane-associated guanylate kinases (MAGUKs), which also includes PSD-93, SAP102, and SAP97. PSD-95 has several protein-binding domains, including three N-terminal PDZ (PSD-95, Discs large, Zonula occludens-1) domains, a Src homology 3 (SH3) domain, and a C-terminal guanylate kinase-like domain. PSD-95 binds to the intracellular C-terminal end of GluN2 subunits of the NMDA receptor through its PDZ1 and/or PDZ2 domains and interacts with various intracellular signaling molecules as well as with other scaffold proteins. For example, neuronal nitric oxide synthase (nNOS), a Ca^{2+} /calmodulin-activated enzyme, interacts with PSD-95 via its PDZ domain and is efficiently activated by Ca^{2+} influx through the NMDA receptor.

Src family protein tyrosine kinases interact with the PDZ3 domain of PSD-95 via its SH2 domain. It was demonstrated that brain ischemia and reperfusion enhance the tyrosine phosphorylation of PSD-95 and the interaction of it with Src/Fyn kinases (22). PSD-95 has been implicated in the up-regulation of NMDA-receptor function. Interestingly, a mutation of Tyr523 in PSD-95 decreases Src/Fyn-dependent tyrosine phosphorylation of PSD-95 and also abolishes the facilitating effect of PSD-95 on NMDA receptor function (22). Therefore, the tyrosine phosphorylation of PSD-95 after

an ischemic stroke contributes to pathologic activation of the NMDA receptor at the postsynaptic density, which may be ultimately associated with ischemic brain injury.

4. Ephrins and their receptors (Ephs) in the ischemic brain

The Ephs are a family of tyrosine kinase transmembrane receptors, and ephrin ligands are anchored to the cell membrane. The Ephs consist of nine Class A Eph receptors and five Class B Eph receptors, which interact with five glycosyl phosphatidylinositol (GPI)-linked cell surface-bound type-A ephrin ligands and three transmembrane type-B ephrin ligands, respectively, in humans. Ephrin signaling via their receptors plays a key role during embryogenesis and also in the regulation of axon guidance and synaptic plasticity as well as the promotion of long-term potentiation in the central nervous system (23). Accumulating evidence has indicated the roles of ephrins and their Ephs in pathologic conditions of the central nervous system. For example, the Eph-A2 receptor, which is predominantly localized in neurons and endothelial cells in the penumbra, has been shown to mediate inflammation and angiogenesis during ischemic injury. In this sense, the deletion of Eph-A2 receptors promotes tight junction formation of endothelial cells in the brain and reduces the level of pro-apoptotic proteins. These effects could be associated with protection against ischemic stroke-induced blood-brain barrier damage and neuronal cell death (24). In addition, it was demonstrated that ephrin-A5 mRNA expression is markedly increased in reactive astrocytes after ischemic stroke and that up-regulation of ephrin-A5 inhibits axonal sprouting through tyrosine phosphorylation of EphA receptors (25). In contrast, blocking ephrin-A signaling by knockdown of ephrin-A5 with siRNA or EphA decoys induces axonal sprouting (25). Therefore, ephrin-A signaling through EphA tyrosine kinase receptors may play a crucial role in neuroplasticity, activity-dependent axonal sprouting, and motor recovery after an ischemic stroke. These findings suggest that Eph and ephrin would be promising therapeutic targets for the treatment of ischemic stroke.

5. Tyrosine phosphorylation of occludin in the ischemic brain

Increased vascular permeability and disruption of the blood-brain barrier (BBB) after an ischemic stroke can be initiating factors for the development of cerebral infarctions. The BBB is formed by the interaction of membrane-associated accessory proteins such as occludin and claudin of brain capillary endothelial cells together

with pericytes, the basal lamina, and astroglial cells. Zonula occludens (ZO)-1 is a cytoplasmic tight junctional accessory protein that connects tight junctions to the actin cytoskeleton.

It was earlier shown that the assembly of tight junctions is regulated by protein tyrosine kinases in epithelial monolayers (26, 27), suggesting the involvement of protein tyrosine phosphorylation at tight junctions in the regulation of BBB function. In this sense, it was demonstrated *in vitro* that occludin appears to be phosphorylated on its tyrosine residues by src family tyrosine kinase, and this tyrosine phosphorylation of occludin plays a pivotal role in the interaction of occludin with ZO-1 molecules (28).

Although pathophysiological changes in tight junctional proteins under *in vivo* ischemic conditions are not fully understood, the tyrosine phosphorylation of occludin is increased, which may have been caused by an increased activity of src, after an ischemic stroke. Furthermore, it was demonstrated that the amounts of occludin and ZO-1 proteins in isolated brain capillaries are decreased after ischemic stroke. Therefore, an increase in the tyrosine phosphorylation of occludin and a decrease in the level of tight junctional proteins have been implicated in the impairment of BBB function after ischemic stroke. In this sense, it was demonstrated that treatment with the src-family tyrosine kinase inhibitor PP2 reduces ischemia-induced tyrosine phosphorylation of occludin, which is accompanied by attenuation of the vascular permeability and the development of a cerebral infarction after transient focal cerebral ischemia (29). Interestingly, it was recently demonstrated that another src-family tyrosine kinase inhibitor, PP1, also reduces cerebral ischemia-induced vascular permeability and infarct size, as well as also increases the expression of tight junction protein ZO-1 and modulates the levels of angiogenic factor (30). Therefore, the blocking of tyrosine phosphorylation of occludin in the tight junction of brain capillaries could be an appropriate strategy to prevent dysfunction of the BBB, whose dysfunction may lead to secondary brain damage after an ischemic stroke.

6. Role of angiotensin in the ischemic brain

The angiotensin superfamily consists of four distinct types of proteins [Angiotensin (Ang)-1 to Ang-4]. Angiotensins bind to the receptor tyrosine kinase Tie2, which is expressed specifically on endothelial cells and act synergistically with VEGF during pathophysiological angiogenesis (31). Ang-1 is known as an agonist for Tie2, whereas Ang-2 has been mainly described as an antagonist for it.

It has been shown that administration of recombinant

adenoviruses expressing Ang-1 reduces ischemic stroke-induced BBB leakage and decreases the lesion volume through Tie2 (32). Ischemic stroke-induced neuronal cell death is accompanied by an increase in vascular permeability. BBB leakage could be a consequence of the increase in VEGF expression, an increase concomitant with that of Ang-2. Direct effects of Ang-2 on the Tie2 receptor on endothelial cells have been reported, and Ang-2 is now considered to act as a context-dependent agonist for Tie2 (33). Furthermore, exogenous Ang-2 is a protective factor during the acute phase of ischemic stroke. This protective effect of Ang-2 on neurons is not associated with neuroprotection; rather, the beneficial effect of Ang-2 under ischemic conditions in the brain seems to be restricted to its vasoprotective capacity. For example, VEGF decreases the expression of tight-junction protein claudin5 and adherens junction protein VE-cadherin in the ischemic brain; and these effects are eliminated in the presence of Ang-2 (34). Although the role of Ang-2 is still controversial, its receptor tyrosine kinase could be, at least, involved in the pathogenesis of ischemic stroke.

7. Growth factors and neurotrophic factors in the ischemic brain

In addition to the possible role of enhanced tyrosine phosphorylation of proteins in cerebral ischemia-induced pathogenesis, it has been considered that growth factors or neurotrophic factors, including IGF, FGF, BDNF, and GDNF, attenuate cell damage after cerebral ischemia by inhibiting the activation of apoptotic pathways through their tyrosine-kinase receptors. For example, stimulation of IGF-1 receptor with intraventricular injection of IGF-1 exerts protective effects through the activation of PI-3 kinase/Akt pathways in hippocampal CA1 neurons after transient global brain ischemia (35). Although vascular endothelial growth factor (VEGF), known as a vascular permeability factor, enhances angiogenesis, newly formed blood vessels at the acute phase after cerebral or retinal ischemia–reperfusion tend to be immature and permeable (36). Therefore, these newly formed and immature vessels can exacerbate cerebral edema, thus leading to deterioration of an ischemic lesion (37). In contrast, a delayed administration of VEGF reduces infarct size and improves neurological functions, indicating a potent protective effect against ischemic brain injury through multiple mechanisms (38).

Hepatocyte growth factor (HGF) activates the c-Met/HGF receptor to evoke diverse cellular responses, such as mitogenic, angiogenic, and anti-apoptotic activities in various types of cells. The administration of human recombinant HGF or human HGF gene attenuates

ischemic brain injury by stimulating angiogenesis without cerebral edema and by protecting neurons and cerebral endothelial cells from injury (39, 40). These findings suggest that the stimulation of tyrosine kinase receptors for growth factors in the late phase after an ischemic insult plays a key role in brain remodeling.

8. Conclusions

Ischemic stroke-induced cell death and/or neurological dysfunction is not mediated by a single molecular cascade. In addition, a number of proteins, including enzymes, ion-channel proteins, growth factor receptors, etc. can be tyrosine phosphorylated in different ways. For example, t-PA, which is usually used for thrombolysis after a stroke, binds to the receptor LRP-1, resulting in rapid tyrosine phosphorylation of it (41). Although hyperosmolar mannitol is used to prevent deterioration from cerebral swelling, mannitol-induced BBB opening is caused by protein tyrosine phosphorylation (42). These findings suggest that it is important to identify the specific tyrosine kinases and tyrosine phosphatases involved in the ischemia-induced tyrosine phosphorylation of individual proteins and the mechanisms by which the activities of these enzymes are regulated in response to ischemic stimuli and/or stroke therapies. Furthermore, tyrosine phosphorylation of proteins is promoted in several stages after the start of reperfusion following cerebral ischemia. The amounts of several growth factors are increased during the late phase after cerebral ischemia, which might be involved in protection and/or repair of ischemic brain injury via their tyrosine-kinase receptors. The pathophysiologic roles of protein tyrosine phosphorylation at the acute phase may be distinct from those at the late phase. Therefore, a greater understanding of tyrosine phosphorylation (when?, where?, how?) occurring in the ischemic brain is required to determine targets for the treatment of ischemic brain injuries.

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