学　位　論　文
Doctor's Thesis

Akt/PKB activity contributes to neuroprotective effects of sodium orthovanadate on delayed neuronal death following transient forebrain ischemia in gerbil hippocampus

（一過性前脳虚血後の砂ネズミ海馬遅発性神経細胞死に対する *o*-バナジウム酸ナトリウムによる神経保護効果および Akt/PKB 活性の関与）

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1. Summary

In transient forebrain ischemia, sodium orthovanadate as well as insulin-like growth factor-1 (IGF-1) rescued cells from delayed neuronal death in the hippocampal CA1 region. Adult Mongolian gerbils were subjected to 5-min forebrain ischemia. Immunoblotting analysis with anti-phospho-Akt/PKB (Akt) antibody showed that phosphorylation of Akt at serine-473 (Akt-Ser-473) in the CA1 region decreased immediately after ischemia and in turn transiently increased 6 hr after reperfusion. The decreased phosphorylation of Akt-Ser-473 was not observed in the CA3 region. Next, effects of intraventricular injection of orthovanadate and IGF-1, which are known to activate Akt were tested. Treatment with orthovanadate or IGF-1 30 min before ischemia blocked delayed neuronal death in the CA1 region. The neuroprotective effects of orthovanadate and IGF-1 were associated with preventing decreased Akt-Ser-473 phosphorylation in the CA1 region observed immediately after ischemia. Immunohistochemical studies with the anti-phospho-Akt-Ser-473 antibody also demonstrated that Akt was activated in the cell bodies and dendrites of pyramidal neurons and translocated from the cytoplasm to the nucleus following orthovanadate treatment. The orthovanadate treatment also prevented the decrease in phosphorylation of mitogen-activated protein kinase (MAPK). Pretreatment with combined blockade of phosphatidylinositol 3-kinase and MAPK pathways totally abolished the orthovanadate-induced neuroprotective effect. Furthermore NG108-15 cells were treated with NaCN to produce chemical hypoxia-induced apoptosis. Orthovanadte treatment inhibited apoptosis. NG108-15 cells transfected with dominant negative form of Akt were enhanced the chemical hypoxia-induced apoptosis and pretreatment with orthovanadate failed to prevent apoptosis. These results suggest that the decrease in both Akt and MAPK activities underlies neuronal vulnerability in the CA1 region following transient forebrain ischemia and that activation of Akt and MAPK by orthovanadate treatment rescues cells from delayed neuronal death in the hippocampus.

2. List of published papers

1. Neuroprotective Effect of Sodium Orthovanadate on Delayed Neuronal Death After Transient Forebrain Ischemia in Gerbil Hippocampus.

   Takayuki Kawano, Kohji Fukunaga, Yusuke Takeuchi, Motohiro Morioka, Shigetoshi Yano, Jun-ichiro Hamada, Yukitaka Ushio, Eishichi Miyamoto

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4. Abbreviations and Acronyms

CREB = cAMP-responsive element binding protein

DTT = dithiothreitol

FOXO = Forkhead box transcription factor, class O

GSK-3α/β = glycogen synthase kinase-3α/β

IGF-1 = insulin-like growth factor-1

IKK = IκB kinase

KRH = Krebs-Ringer Hepes solution

MAPK = mitogen-activated protein kinase

MCAO = middle cerebral artery occlusion

PCD = programmed cell death

PDK-1 = PIP_3-dependent protein kinase-1

PIP_3 = phosphatidylinositol-3,4,5-trisphosphate

PI3K = phosphatidylinositol 3-kinase

PKB = protein kinase B

PTP = protein tyrosine phosphatase

SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TNFα = tumor necrosis factor α

TUNEL = terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling

VEGF = vascular endothelial growth factor

5. Background and purpose

5.1. Brain ischemia and delayed neuronal death

5.1.1. Global ischemia and focal ischemia

Global ischemia occurs in the case of cardiac arrest in clinical scene. Even when resuscitation is performed and the patients are rescued from serious neurological deficits, some neurons would be lost in the specific regions of the brain, including the hippocampus, striatum, neocortical neurons of layers 3, 5 and 6, and so on (Brierley, 1976; Petit et al., 1987; Pulsinelli et al., 1982). Many researchers have made a forebrain ischemia model by clamping of the bilateral carotid arteries in Mongolian gerbils and rats. In the model, decrease in the blood flow is uniform throughout the brain regions, since collateral blood flow is negligible. In the global ischemic model, a unique phenomenon called delayed neuronal death occurs. After brief global ischemia (5 minutes in Mongolian gerbils and 20 minutes in rats), the pyramidal neurons in the CA1 region are lost 2-3 days after reperfusion without remarkable morphological changes at 1-2 days after ischemia (Kirino, 1982). In addition to the global ischemia, a focal cerebral ischemic model such as middle cerebral artery occlusion (MCAO) models was more preferentially used as a stroke model in humans. The brain injuries induced by the focal ischemia are divided into two types; 1) core of the injury (the focus of focal ischemia) which receives the relatively dense ischemia showing the strongest ischemic injury. 2) perifocal tissue (penumbra) which receives less dense ischemia because of collateral blood supplied from leptomeningeal branches of other major arteries. The ischemic injury in the penumbra is weaker than that in the core. The neuronal death in the penumbra of MCAO models showed an apoptotic cell death like delayed cell death in the global ischemia.

5.1.2. Mechanism underlying delayed neuronal death

Mechanism underlying delayed neuronal death is a major focus of current biomedical research to produce new specific therapeutic agents that protect nervous injures following ischemia (Fig. 1). Brain tissue requires a relatively high consumption of oxygen and glucose, and depends exclusively on oxidative phosphorylation for energy production. Focal and global impairments of cerebral blood flow stop the delivery of oxygen and glucose, and impair the energetic required to maintain ionic gradients through the plasma membranes (Martin et al., 1994). By the energy depletion, membrane potential is lost and thereby neurons and glial cells depolarize (Katsura et al., 1994). Consequently, somatodendritic and presynaptic voltage-dependent Ca^{2+} channels are activated and glutamate and aspartate, excitatory amino acids are released to extracellular space. The excitatory amino acids in turn activate the NMDA and non-NMDA receptors that account for Ca^{2+} overload into cells. The increased intracellular Ca^{2+} sequentially initiates cytoplasmic and nuclear events including activation of nitric oxide synthase (NOS), proteases, protein kinases, protein phosphatases, phospholipase A_2 and Ca^{2+}/Mg^{2+}-activated endonuclease. Furthermore, Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is inactivated following transient forebrain ischemia (Morioka et al., 1992).

After transient ischemia in the focal and global ischemia model, blood flow is restored by reperfusion. In combination with calcium overload, reoxygenation by reperfusion elicits cytotoxicity through downstream generation of reactive nitrogen and oxygen species that disrupt energy homeostasis through several modes of cellular damage. Excessive accumulation of calcium by mitochondria impairs oxidative phosphorylation and promotes production of reactive oxygen species such as superoxide (O_2^-) and hygrogen peroxide (H_2O_2) via electron transport chain. High mitochondrial calcium accumulation also alters permeability of the mitochondrial membrane, which inhibits mitochondrial ATP production and promotes necrosis. In addition, selective

permeability of the outer membrane release cytochrome c which activates caspases. Caspases, in turn cleave specific cytoplasmic and nuclear protein substrates to coordinate apoptosis. Hydrogen peroxide is formed from superoxide and itself converts to the highly reactive hydroxyl radical via iron catalyzed reactions. Ca^{2+} also activates NOS, a calmodulin-dependent enzyme to produce large amount of nitric oxide (NO). Superoxide and nitric oxide combine to form the much more reactive peroxynitrite anion ($ONOO^-$). Peroxynitrite damages the cell membrane and leads to oxidation and nitration of proteins containing aromatic amino acids such as tyrosine. DNA damage produced by either Ca^{2+}/Mg^{2+}-acitvated endonuclease, $ONOO^-$, or by hydroxyl radicals results in robust activation of poly (ADP-ribose) polymerase (PARP) activation with subsequent depletion of NAD levels. Since NAD is required for ATP production and since ATP is required for NAD synthesis, excessive PARP activation depletes the cellular energy pool and results in neuronal death.

Finally, the elevation of intracellular Ca^{2+}, production of oxygen free radicals by reperfusion and hypoxia itself trigger expression of a number of proinflammatory genes through activation of transcription factors, including NF-κB (O'Neill and Kaltschmidt, 1997), hypoxia inducible factor 1 (Ruscher et al., 1998), interferon regulatory factor 1 (Iadecola et al., 1999) and STAT3 (Planas et al., 1996). Thus, mediators of inflammation, such as platelet-activating factor, tumor necrosis factor α and interleukin 1β, are produced by injured cell (Rothwell and Hopkins, 1995). In rodent models of cerebral ischemia, as in patients with stroke, infiltrating neurophils produce inducible NOS (iNOS), an enzyme that produces toxic amount of NO (Forster et al., 1999). The pathogenic importance of NO production by iNOS is underscored by observations that pharmacological inhibition of iNOS reduces ischemic brain injury and that iNOS null mice have a reduction in ischemic damage (Iadecola, 1997; Iadecola et al., 1997). In addition, ischemic neurons express cyclooxygenase 2 (COX2) that produces superoxide and toxic prostanoids (Nogawa et al., 1997). These products through the inducible

genes associated with inflammation cause long-lasting pathological events accounting for cell death.

5.2. Survival signaling in neurons

5.2.1. Akt and downstream targets.

The AKR strain of mice exhibit a high incidence of leukemia and lymphomas from spontaneous thymoma (Staal et al., 1977). A retrovirus termed AKT8 was isolated from one of these lines derived from a spontaneous thymoma. The virus was demonstrated to transform only mink lung cells (CCL64) in culture, while the virus inoculated into newborn mice was shown to be tumorgenic (Staal and Hartley, 1988). The non-viral DNA component transduced from the mouse genome was subsequently identified, and two human homologues, Akt1 and Akt2, cloned (Staal, 1987). After the identification of the AKT8 retrovirus the cellular homologue of v-Akt was cloned independently by several groups and found to be a 57 kDa protein serine/threonine kinase (Coffer and Woodgett 1991; Jones et al., 1991; Bellacosa et al., 1991). The kinase shows most similarity to protein kinase A (PKA) and protein kinase C (PKC) and thus has been termed protein kinase B (PKB) (Coffer and Woodgett 1991), Related to A- and C-kinase (RAC-PK; (Jones et al., 1991)) or c-Akt (Bellacosa et al., 1991). Mammalian genomes contain three genes encoding PKBs (termed α/Akt1, β/Akt2 and γ). PKBβ and PKBγ are approximately 82 % identical with the α-isoform, although PKBγ lacks 23 amino acids at the C-terminus compared with the others (Konishi et al., 1995). Homologues have also been identified in the nematode worm *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster*, demonstrating wide evolutionary conservation (Waterston et al., 1992; Franke et al., 1994). Aktα and Aktβ are widely expressed, with highest levels in brain, thymus, heart and lung (Coffer and Woodgett

1991; Jones et al., 1991; Bellacosa et al., 1993). Expression of the γ-isoform is more restricted (high in brain and testes, lower in heart, spleen, lung and skeletal muscle), but all tissues contain at least one form of Akt. After Akt was cloned, several groups concurrently identified Akt as a downstream target of PI3K (Burgering and Coffer, 1995; Franke et al., 1995; Kohn et al., 1995). Stimulation of tyrosine kinase growth factor receptors (Hemmings, 1997) or GTP-binding (G) protein-coupled receptors activate PI3K (Murga et al., 1998; Krugmann et al., 1999), leading to production of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and phosphatidylinositol-3,4-bisphosphate, which bind to the Akt pleckstrin homology domain and promote dimerization and translocation of Akt to membranes (Datta et al., 1996). Such membrane translocation is an essential step in Akt activation, since addition of an N-terminal myristoylation sequence makes Akt constitutively active (Kohn et al., 1996; Andjelkovic et al., 1997). Akt activation is correlated with phosphorylation of Thr-308 in its catalytic domain and Ser-473 at the C terminus (Alessi et al., 1996). Mutagenesis of each residue to alanine revealed that both are required for full activation (Alessi et al., 1996). Although it is known that PIP_3-dependent protein kinase-1 (PDK-1) phosphorylates Akt at Thr-308 (Alessi et al., 1997; Stephens et al., 1998), the serine/threonine kinases including an integrin-linked kinase (Delcommenne et al., 1998), responsible for Ser-473 phosphorylation have not been established. Ca^{2+}/calmodulin-dependent protein kinase kinase can also phosphorylate Akt at Thr-308 both *in vitro* and *in vivo* in a PI3-K-independent manner (Yano et al., 1998).

Akt has been known to regulate glucose metabolism and protein synthesis. For example, activation of Akt is associated with stimulation of GLUT4 translocation (Kohn et al., 1996; Tanti et al., 1997; Hajduch et al., 1998). Hemmings and his co-workers have demonstrated that GSK-3 is a substrate of Akt, at least *in vitro* (Cross et al., 1995). Treatment of L6 myoblasts with insulin results in a 40-50% inhibition of GSK-3, which is eliminated by PI3K inhibitors. 6-Phosphofructose 2-kinase (PFK2) is responsible

for generating fructose 2,6-bisphosphate, a key allosteric activator of 6-phosphofructose-1-kinase, the rate-limiting enzyme in mammalian glycolysis. For example phosphorylation of PFK2 by stimulation with insulin results in enhanced its activity. It has been shown that $p70^{S6K}$, MAPKAP kinase-1 and Akt can all phosphorylate and activate PFK2 *in vitro* (Deprez et al., 1997). Thus phosphorylation of PFK2 by Akt may explain how glycolysis is stimulated by insulin. The stimulation of protein synthesis is another important response to insulin observed in a variety of cell types, especially in skeletal muscle and adipose tissue (Kimball et al., 1994). A critical event is the dissociation of 4E-binding protein (4E-BP1) from eukaryotic initiation factor 4E, which accounts for mRNA translation. Its phosphorylation by PI3K and $p70^{S6K}$ occur at multiple residues, since it is wortmannin- and rapamycin-sensitive (Azpiazu et al., 1996; Beretta et al., 1996; Fadden et al., 1997). Since Akt activation is also wortmannin-sensitive and contributes to the $p70^{S6K}$ activation (Burgering and Coffer, 1995), it may play a role in the regulation of insulin-mediated protein synthesis.

5.2.2 Akt and cell survival

Figure 2 shows the cascade of cell survival mediated by Akt induced by receptor tyrosine kinase activation.

In addition to a pivotal role of Akt in glucose metabolism and protein synthesis, much more attention have directed to roles of PI3K and its downstream target, Akt, in cell survival signaling (Ahmed et al., 1997; Datta et al., 1997; Dudek et al., 1997; Kauffmann et al., 1997; Kennedy et al., 1997; Kulik et al., 1997). Although accumulating evidences suggest diversity of the apoptotic machinery, there are a number of points at which Akt might act to promote survival and inhibit death. Most attractive targets for Akt include Bad, caspase 9, the Forkhead family of transcription factors, CREB and NF-κB.

Bad was identified on the basis of its ability to bind to Bcl-2, and analysis of its

primary structure reveals that Bad is similar to Bcl-2 exclusively within its BH3 domain (Yang et al.,1995). Bad directly interacts via its BH3 domain with prosurvival Bcl-2 family members such as Bcl-x_L, and its overexpression blocks Bcl-x_L–dependent cell survival (Yang et al., 1995; Ottilie et al., 1997; Zha et al., 1997). Bad is phosphorylated at Ser-136 by Akt and it leads to inactivation of Bad (Datta et al. 1997; Del Peso et al. 1997; Blume-Jensen et al. 1998). In the absence of survival factors and Akt activity, Bad is dephosphorylated. Dephosphorylated Bad interacts with Bcl-x_L, with concomitant inhibition of its function and thereby causes cell death. Akt can block cell death even after mitochondrial cytochrome c release.

In situ studies using ^{32}P-labeled cells revealed that Akt phosphorylates caspase 9 at Ser-196 (Cardone et al., 1998). The phosphorylation block cytochrome c-mediated caspase 9 activation *in vitro* (Cardone et al., 1998). In 293 cells, suppression of the caspase 9-induced cell death by Akt activity is partially dependent on the phosphorylation of Ser-196. These results suggest that Akt promotes survival through the inactivation of caspase 9 downstream of cytochrome c release.

The forkhead box transcription factor, class O (FOXO) is mammalian homologue of DAF-16, which are known to regulate life span of *Caenorhabditis elegans* (Lin et al., 1997) and includes subfamilies of forkhead transcription regulators such as AFX, FKHRL1 and FKHR. By stimulation with survival factors, FOXOs are phosphorylated by Akt and stay in the cytoplasm. Whereas once survival factors are depleted, FOXOs are dephosphorylated and translocate into the nucleus (Brunet et al., 1999; Takaishi et al., 1999; Nakae et al., 2000; Biggs III et al., 1999; Rena et al., 1999). In the nucleus, FOXOs activate target gene as transcription factor and induce apoptosis (Brunet at al., 1999; Dijkers et al., 2000; Nakamura et al., 2000; Tang et al., 1999). FOXOs -induced cell death is thought to be caused by expression of Fas ligand and Bim, a Bcl-2 family member (Brunet et al., 1999; Dijkers et al., 2000). The Akt-induced phosphorylation of FOXOs suppress their transcriptional activities induce apoptosis

(Brunet et al., 1999; Nakae et al., 2000; Tang et al., 1999).

The transcriptional activator CREB was shown to be phosphorylated by Akt both *in vitro* and within cells at Ser-133 (Du and Montminy, 1998). The phosphorylation of Ser-133 increases the binding of CREB to CBP and enhances CREB-mediated transcription (Du and Montminy, 1998). In PC12 cells stimulated by IGF-1, blockade of PI3K activity leads to a decrease in CREB phosphorylation at Ser-133 and partially inhibits CREB transcriptional activation (Pugazhenthi et al. 1999). The relevance of the phosphorylation of CREB by Akt in survival signaling is also still unclear, although there is evidence that CREB regulates expression of genes critical for survival such as the gene encoding the cytokine and BDNF (Shieh et al., 1998; Tao et al., 1998).

Akt also promotes survival by activating anti-apoptotic NF-κB signaling through IκB kinase (IKK) activation (Romashkova and Makarov, 1999). The ability of Akt to regulate NF-κB activity is associated with the phosphorylation of IKK at a critical regulatory site, Thr-23 (Ozes et al. 1999). The genes induced by NF-κB to promote survival are also identified, which include pro-survival Bcl-2 family member, Bfl-1/A1 and the caspase inhibitors, c-IAP1 and c-IAP2 (Chu et al., 1997; Zong et al., 1999). Thus Akt may promote survival through direct phosphorylation of the Bcl-2 family and the caspases, and/or through their gene expressions.

5.2.3. MAPK and cell survival

The mitogen-activated protein kinase or extracellular signal-regulated kinases (MAPK or ERK) regulated a diverse array of functions, such as cell growth, proliferation, differentiation, and cell survival. Such actions have been extensively examined in the context of growth factor signaling via receptor tyrosine kinases. Tyrosine kinase growth factor receptors activate MAPK by a complex mechanism involving the SH2/3/protein Grb2, the exchange protein Sos, and Ras as shown in Figure 2. The GTP-bound Ras protein binds to the Raf kinase and initiates a protein

kinase cascade that leads to MAPK activation. MAPK kinase kinase has been designated c-Raf or MEKK that phosphorylates and activates MEK, the MAPK kinase. Activated MEK phosphorylates and activates MAPK. Subsequently, the activated MAPK translocates into the nucleus where many of the physiological targets for MAPK are located. These targets include transcription factors that are regulated by MAPK-dependent phosphorylation (e.g., Elk-1, c-Myc, c-Jun, c-Fos, and C/EBP beta). Thus the MAPK pathway represents a significant mechanism of signal transduction by growth factor receptors from the cell surface to the nucleus that results in the regulation of gene expression.

Neuronal survival is regulated by both neuronal growth factors and neuronal activity in a synergic manner. In mammalian systems, an anti-apoptotic role for MAPK in neurons has been proposed, and recent studies show that MAPK can protect certain populations of neurons against specific insults (Meyer-Franke et al., 1998; Anderson and Tolkovsky, 1999). However, the most interesting data linking MAPK with neuronal survival have come from the examination of photoreceptor cell apoptosis in *Drosophila*. Genetic analysis has demonstrated that activation of a Ras-dependent pathway leads to the inactivation of the pro-apoptotic molecule, Hid. The inactivation occurs by direct MAPK-mediated phosphorylation of Hid and MAPK-dependent downregulation of Hid expression (Bergmann et al., 1998; Kurada and White, 1998). Whether such a pathway is conserved in mammalian neuronal system remains to be determined. In contrast to the anti-apoptotic actions of MAPK, recent reports suggest that sustained overstimulation of MAPK signaling cascades may actually promote neuronal necrotic cell death (Runden et al., 1998; Murray et al., 1998). In brain ischemic insults, transient activation of MAP kinase is evident in the hippocampus (Campos and Kindy, 1992) and vulnerable CA1 region (Shamloo et al., 1999; Gu et al., 2001). However, relevance of the activation of MAP kinase remains to be unclear. Figure 3 shows the cascade of cell survival mediated by Akt and ERK induced by

receptor tyrosin kinase activation.

5.2.4. Insulin-like growth factor-1 and cell survival

Receptor tyrosine kinase ligands such as insulin-like growth factor-1 (IGF-1) promote cell survival through Akt activation and concomitant PI3K activation (Datta et al., 1997; D'Mello et al., 1997; Dudek et al., 1997; Galli et al., 1995; Miller et al., 1997; Párrizas. et al., 1997; Kulik et al., 1997). Indeed, IGF-1 prevents neuronal death after transient forebrain ischemia (Gluckman et al., 1992; Zhu and Auer, 1994; Tagami et al., 1997) and after hypoxic-ischemic injury (Guan et al., 1993; Guan et al., 1996). Topical application of IGF-1 also reduces the infarction volume in rat cortex following transient middle cerebral artery occlusion (Wang et al., 2000). Although the mechanisms underlying neuroprotection by IGF-1 are not fully understood, PI3K and Akt signaling may be required for IGF-1-induced neuroprotection.

5.2.5. Vanadium compound and cell survival

Vanadium, which is a transition metal, atomic number 23, atmic weight 50.9415, was discovered by Sefström. Usually, vanadium ions bind with the oxygen atom to form oxo compounds such as VO_3^- as vanadate and VO^{2+} as vanadyl forms. The vanadic form of V^{3+} is very unstable under air and oxidizes to vanadyl or vanadate form which is stable (Chasteen, 1983; Kustin and Macara, 1982; Rehder, 1995). Orthovanadate is a phosphate analog and generally thought to bind as a transition state analog to the phosphoryl transfer enzymes and inhibit ATPases such as Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase and phosphatases including acid or alkaline phosphatase, and phosphoprotein tyrosine phosphatases (Simons 1979; Swarup et al. 1982). Other enzymes that are inhibited by vanadate include RNase, dynein ATPase, phosphoglucomutase and glucose-6-phosphatase. Furthermore, vanadate but not vanadyl indirectly activated non-receptor protein tyrosine kinase in cell-free

experiments (Elberg et al., 1994). Thus, vanadate activates tyrosine kinases and affects intracellular tyrosine phosphorylation levels via inhibition of non-selective protein tyrosine phosphatases. Vanadate, its derivative pervanadate and vanadyl compounds mimic insulin in stimulating glucose transport, lipogenesis and protein synthesis and in inhibiting lipolysis (Heffetz et al., 1990; Shechter, 1990; Shisheva and Shechter, 1993; Posner et al., 1994; Fantus et al., 1994). The most prominent effects of vanadium are observed in rats made insulin-deficient and diabetic by injection of streptozotocin (model of type I diabetes). Oral administration of vanadate or vanadyl causes a fall in blood glucose levels within 2-5 days. The antidiabetic effects of vanadium have also been investigated in genetically obese, hyperinsulinaemic and insulin-resistant rats and mice (models of type II diabetes). Vanadate improved glucose homeostasis in ob/ob and db/db mice, in which a marked insulin resistance leads to overt diabetes despite very high plasma insulin levels (Brichard et al., 1990; Meyerovitch et al., 1991).

The studies on the insulin-like properties of vanadium *in vitro* and in animal studies have allowed clinical studies to begin. Two groups have administrated small doses of vanadyl sulphate or sodium metavanadate to diabetic patients for a period of two to three weeks. These doses are about 100-fold lower than those used in most animal studies. Beneficial effects were, however, observed. Vanadium treatment improved insulin sensitivity of patients with non-insulin-dependent diabetes mellitus (Goldfine et al., 1995; Cohen et al., 1995; Brichard and Henquin, 1995). In recent report, type II diabetic patients were treated with vanadyl sulfate and percutaneous biopsies of the quadriceps muscle were performed. The activity of PI3K was measured in muscle homogenates and was found to increase 4.2-fold after vanadium treatment (Goldfine et al., 2000). Since vanadate and pervanadate can activate Akt and mitogen-activated protein (MAP) kinase signaling (Zhao et al., 1996; Meier et al., 1998; Wijkander et al., 1997; D'Onofrio et al. 1994; Zhao et al. 1996; Hadari et al. 1992), these protein kinases are potentially important for vanadium compound-induced insulin-like actions.

Orthovanadate is known to protect cells from apoptosis but in some situation induces growth inhibition and apoptosis. One hundred μM orthovanadate evoked neuronal cell death of cultured dentate gyrus cells at a dose previously shown to be protective against apoptosis in other cell types (Figiel and Kaczmarek 1997). At concentration of 5-10μM, orthovanadte displays cytotoxicity towards proliferation primary cultures and tumor cell lines (Cruz et al. 1995). While vanadate inhibits apoptosis induced by TNFα in endothelial cells (Yang et al., 1996) and the mouse connective tissue cell line L-929 (Totpal et al., 1992), by extracellular ATP in the murine mastocytoma cell line and a murine leukaemia cell line (Bronte et al., 1996), and by K252a in malignant glioma cells (Chin et al., 1999). Thus the effects of the orthovanadate on the cell survival is controversial between non-neuronal a neuronal cells.

5.3. Therapeutic strategies and proposal.

Strokes kill about 5 million people each year, making cerebrovascular disease the second leading cause of death worldwide (WHO). At least 15 million others have non-fatal strokes annually, and about a third are disabled as a consequence (Bonita, 1992; Hankey and Warlow, 1999). Many of disabled patients are due to neurological deficits because of ischemia. Extensive studies using brain ischemic models have been documented the molecular basis of necrosis and apoptosis following brain injury. The understanding for the signaling cascades of the neuronal death clarified molecular target for therapeutic agents to rescue neurons from the ischemic injury. For example, glutamate receptor antagonists and Ca^{2+} channel blockers to inhibit Ca^{2+} overload. Inhibitors for Ca^{2+}-dependent enzymes such as NOS and proteases including caspases are also potential therapeutic agents. Furthermore, radical scavengers are currently introduced to clinical use. The previous strategies of neuroprotection progressed

toward to inhibition the death signaling. Hypothesis that potentiation of survival signaling during brain damage also potential therapeutic tool is proposed. In this context, stimulation of Akt pathway to protect neurons from ischemic injury were performed and, thereby IGF-1 and vanadate was selected as stimulator of Akt pathway. Especially, the neuroprotective effects of vanadate were focused, because vanadium naturally exists in the human body and some clinical studies using vanadium compounds were successful as anti-diabetic agent.

The author hypothesized that orthovanadate as well as IGF-1 would lead to Akt phosphorylation *in vivo* and thereby inhibit the delayed neuronal death after transient forebrain ischemia in gerbil hippocampal CA1 region. Thus, the possibility of new therapeutic agent for cerebral ischemic insults was documented.

6. Methods

6.1. Cell culture

Hippocampal organotypic cultures were prepared from 7-9-day-old neonates of Wistar rats as previously described (Stoppini et al., 1991). Slice cultures were maintained for 10 days before stimulation with orthovanadate (Sigma, St. Louis, MO) or IGF-1 (Sigma). Neonatal rat hippocampal cell cultures were prepared according to the methods described before (Fukunaga et al., 1992). Cultures were maintained for 16 days before stimulation with IGF-1.

6.2. Induction of ischemia

Adult male Mongolian gerbils weighing 60-80 g were housed under constant environmental conditions (temperature, 22 ± 2 °C; humidity, 55 ± 5% and a 12:12 h light/dark cycle) in the Animal Research Center, Kumamoto University. The gerbils were anesthetized and maintained with 2 % halothane in a mixture of 30 % O_2 and 70 % N_2O. The rectal temperature, monitored with a digital thermometer inserted 2 cm into the anus, was maintained at 37-38 °C throughout the operation by placing gerbils under a heat lamp and warming them with a blanket. The bilateral common carotid arteries were surgically exposed and quickly occluded with aneurysm clips. After 5-min occlusion, the clips were removed to restore blood flow. After awakening from anesthesia, gerbils were returned to home cages and allowed to take food and water before experiments. Control animals were sham-operated and received the same experimental procedures except for occlusion of arteries. Ischemic and sham-operated gerbils were killed by decapitation at the indicated times and hippocampal regions were dissected from their brains. All animal experiments were approved by the Committee

of Animal Experiments at Kumamoto University School of Medicine.

6.3. Administration of wortmannin, sodium orthovanadate, IGF-1 or U0126

For intraventricular injection, animals were anesthetized and placed on a stereotaxic operation frame. After making one burr hole at the right parietal skull 2.0 mm posterior from bregma and 3.5 mm lateral, a Hamilton syringe was inserted into the ventricle at a depth of 2.5 mm from the cortical surface. When drug infusion was carried out twice, the first injection was made on the left side and the second on the right side. For example, 2 μl of 100 μM wortmannin (Wako Pure Chemical Industries, Osaka, Japan) was injected into the ventricle via a Hamilton syringe at a flow rate of 0.4 μl/min on the left side, and 15 min later 2 μl of 5 mM orthovanadate was injected on the right side. Thirty minutes later, animals were subjected to 5-min forebrain ischemia as described above. Sodium orthovanadate (5 mM) and IGF-1 (25 μg/ml) were dissolved in phosphate-buffered saline (PBS) and wortmannin (100 μM) was in PBS with 2 % dimethylsulfoxide (DMSO). U0126 (500μM) (Calbiochem-Novabiochem, San Diego, CA) was dissolved in PBS with 5 % DMSO.

6.4. Tissue preparation for double immunofluorescence staining

At 0, 2, 6 h, and 7 days after 5-min ischemia, gerbils were anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused through the left ventricle of the heart with ice-cold PBS followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (PB). For immunohistochemical studies using anti-phospho-Ser-473 antibody, animals were perfused with PBS containing 30 mM sodium pyrophosphate and 50 mM NaF, followed by 4 % paraformaldehyde in 0.1 M PB containing 30 mM sodium pyrophosphate and 50 mM NaF to prevent dephosphorylation. The brains were removed and post-fixed

overnight at 4 °C in the same fixative solution. Coronal brain sections (30 μm in thickness) at the level of the hippocampus were prepared using a vibratome. The sections were incubated by floating in the following media: 30 min in 0.1M PB containing 0.01 % Triton-X100; 1h in PBS with 3 % bovine serum albumin (BSA)(blocking solution); overnight with polyclonal anti-phospho-Ser-473 antibody (diluted 1: 50) (Upstate biotechnology, Lake Placid, NY) and monoclonal anti-Akt1 antibody (diluted 1: 500) (Transduction Lab., Lexington, KY) in blocking solution. The sections were then labeled for 2 h with both Alexa 594-labeled anti-sheep IgG (Molecular Probes, Eugene, OR) and fluorescein-labeled anti-mouse IgG (Leinco Technologies, Ballwin, MO) in PBS, which are diluted 1: 200. After several washes with PBS, the sections were mounted on glass slides with coverslips and analyzed using a confocal laser microscope (Fluoview, Olympus, Tokyo, Japan). To normalize immunoreactivity with anti-phospho-Ser-473 antibody, hippocampal slices without changing the confocal laser intensity and laser aperture were analyzed. Furthermore, sections were double-stained with anti-phospho-Ser-473 and anti-Akt1 antibodies. The intensity of immunoreactivity with anti-Akt1 antibody between control and ischemic hippocampal slices were confirmed to be the same.

6.5. Gel electrophoresis and immunoblotting

Gerbils were killed by decapitation immediately after occlusion or at the indicated times after reperfusion. After brains were removed and rinsed once with cold PBS, CA1 and CA3 regions of the hippocampus were dissected out in cold PBS under a microscope. Each tissue sample was stored in a test tube and kept at -80 °C before use. Frozen tissues were homogenized with a hand homogenizer and sonicated with a Sonifier-250 (Branson, Danbury, CT) at 0 °C in 0.2 ml of the homogenization buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 % Triton X-100, 4 mM EGTA, 10 mM EDTA,

0.5 M NaCl, 1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 50 mM NaF, 50 μg/ml of leupeptin, 25 μg/ml of pepstatin A, 50 μg/ml of trypsin inhibitor and 1 mM dithiothreitol (DTT). Insoluble materials were removed by a 10-min centrifugation at 15000*g*. Following determination of protein content in each supernatant fraction using Bradford's solution, each sample containing equivalent amounts of protein was applied to 10 % acrylamide denaturing gel (SDS-PAGE) (Laemmli, 1970). After electrophoresis, proteins were transferred for 2 h at 70 V to an Immobilon PVDF membrane (Millipore) (Towbin et al., 1979). Blotting membranes were incubated for 1 h in PBS containing 3 % BSA (blocking solution) at room temperature and incubated overnight at 4 °C with a 1:200 dilution of anti-phospho-Ser-473 antibody (New England Bio Labs, Beverly, MA) or a 1:1000 dilution of anti-Akt1 antibody in blocking solution. After several washes with PBS containing 0.1% Tween-20 (PBS-T), the membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech, Arlington Heights, IL) or anti-sheep IgG antibody (Leinco Technologies) diluted 1:10000. The membranes were then processed with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech). The images were scanned and analyzed semiquantitatively using the NIH image (public domain software developed at the United States National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image). Similar methods were used for immunoblotting with anti-phospho-MAP kinase (Promega, Madison,WI) and anti-MAP kinase (pan ERK) antibodies (Transduction Lab).

6.6. Histopathological analysis

After 7 days, gerbils were anesthetized and perfusion-fixed with 4 % paraformaldehyde as described. Hippocampal serial sections (30 μm) were stained

with 5 μM propidium iodide (Molecular Probes) diluted in PBS and were inspected under a confocal laser microscope. Viable and non-viable neurons in the pyramidal cell layer of hippocampal CA1 regions were counted from sections 1.4-1.8 mm posterior to bregma. Viable neurons were defined as cells with normal morphological properties exhibiting round nuclei stained with propidium iodide. Four sections were obtained from 5-8 animals in each condition. The total number of the viable cells from the left and right hippocampi for each animal was calculated as an average of two randomly chosen 1350 x 1000 μm area from the four different slices counted by two independent observers without knowledge of treatment conditions. Sections were also stained with cresyl violet to count viable neurons, and the numbers of viable cells were the same as the numbers stained with propidium iodide.

6.7. Akt kinase assay

Akt activity was measured with an Akt assay kit (New England Bio Labs) according to the manufacturer's protocol with minor modifications. Dissected hippocampal tissues were homogenized with a hand homogenizer in lysis buffer. Extracts containing equivalent amounts of protein were incubated with a slurry of immobilized Akt antibody for 2 h at 4 °C. Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$. Immunoprecipitates were then subjected to an *in vitro* kinase assay in kinase buffer with 400 μM ATP and 1 μg of GSK-3α/β crosstide (New England Bio Labs) for 30 min at 30 °C. The reaction was stopped by adding SDS-sample buffer. Phosphorylation of Ser-21 of GSK-3α/β crosstide was analyzed by immunoblotting with anti-phospho-GSK-3α/β antibody.

6.8. Transfection of dominant negative Akt in NG108-15 cells

The cDNA of Myc-His tagged dominant negative Akt1 in pUSEamp (+) expression vector was purchased from Upstate biotechnology (termed pUSE-d.n.Akt). NG108-15 cells were grown in Dulbecco's modified Eagle's medium containing additives, as described (Higashida et al., 1986). NG108-15 cells (3 x 10^5 cells/dish) were plated in a 35-mm dish (Becton Dickinson Labware, Franklin Lakes, NJ) and cultured in the standard medium for 24h. The cells were transfected with the cDNA of pUSE-d.n.Akt (6 μg of DNA), using 10 μl of FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) in 1 ml of the serum-free medium for 6h. The culture medium was changed to the standard medium, and the cells were cultured for 48h. Following serum deprivation for 30 min, cultures were treated with or without orthovanadate (50 μM in serum-free medium) for 30 min. To induce a chemical hypoxia, cultures were exposed to 1 mM NaCN (Sigma) in Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, NY) with 10 mM glucose for 24h.

6.9. Detection of NaCN induced apoptosis

To detect NaCN-induced apoptosis in NG108-15 cells, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labeling (TUNEL) method using apoptosis detection system, fluorescein (Promega, Madison, WI) was performed according to the manufacturer's protocol with minor modifications. Cells transfected with pUSE-d.n.Akt were identified by immunofluorescence staining with anti-Myc Tag antibody (Upstate biotechnology). Cells were incubated 10 min with PBS containing 0.2 % Triton X-100; 30 min with PBS with 3 % BSA (blocking solution); overnight with monoclonal anti-Myc Tag antibody (diluted 1: 200) in blocking solution. The cells were then labeled for 30 min with Alexa 594-labeled anti-mouse IgG

(Molecular Probes) in PBS, which are diluted 1: 200. After several washes with PBS, the cells were analyzed using a confocal laser microscope.

6.10. Statistical analysis

All values reported here were expressed as means ± SE. Overall statistical significance for differences among groups was tested by one-way analysis of variance (ANOVA), followed by multiple comparisons between each group or between control and other groups using Dunnett's multiple comparison test. $P < 0.05$ was considered significant.

7. Results

7.1. Akt activation with orthovanadate and IGF-1 in rat hippocampal slice cultures

Vanadate is a general protein tyrosine phosphatase inhibitor (Swarup et al., 1982). Vanadate and IGF-1 can activate Akt in non-neuronal cells (Alessi et al., 1996; Meier et al., 1998). Whether orthovanadate and IGF-1 stimulate Akt activity in the hippocampal neurons of slice culture was tested. Fifty μM orthovanadate maximally stimulated Akt activity (Fig. 4A). Akt activation was maximally observed 15 min after treatment and was sustained up to 120 min (Fig. 4B). Likewise, IGF-1 markedly increased Akt-Ser-473 phosphorylation. The maximal activation of Akt was obtained with 50 ng/ml of IGF-1 30 min after stimulation (Figs. 4C and D). Increased Akt-Ser-473 phosphorylation was apparent 120 min after stimulation. Levels of total Akt protein were unchanged during stimulation in these experiments.

7.2. IGF-1 activate Akt in rat hippocampal neuron

Hippocampal neuron with or without stimulated with 50 ng/ml IGF-1 were stained with anti-phospho-Akt-Ser-473 antibody. In control, the immunoreactivity for phospho-Akt was observed mainly in cell bodies neurons. In these cells, immunostaining was observed in a punctate pattern and localized to the submembrane area (Fig. 5A). Following IGF-1 treatment, immunoreactivity seen markedly increased in dendrites and nucleus as well as in the cell bodies of the neurons (Fig. 5C). Pre-treatment with 200 nM wortmannin inhibited the Akt activation effects of IGF-1 (Fig. 5D). IGF-1 activates Akt through PI3K in rat hippocampal neurons.

7.3. Changes in activity of Akt after transient forebrain ischemia

Changes in activity of Akt were examined after transient forebrain ischemia. To evaluate Akt activities following 5-min transient forebrain ischemia, phosphorylation of Akt at Ser-473 was measured, which is required for Akt activation. Lethal ischemia, which caused death of about 90 % of the pyramidal cells in the CA1 region resulted in dephosphorylation of Akt-Ser-473 in the CA1 region immediately (0 h) after ischemia without changes in Akt protein levels (Fig. 6A). Akt phosphorylation then transiently increased 6 h after reperfusion and returned to the basal level observed in sham-operated animals within 1 day of reperfusion. Akt activity was determined by an immunocomplex kinase assay using GSK-3α/β crosstide as substrate. Akt kinase activity following 5-min ischemia decreased to 12 % of that of sham-operated gerbils and increased to 240 % 6 h after reperfusion (Fig. 6B). Changes in Akt activity were correlated with changes in Akt-Ser-473 phosphorylation. In contrast with the CA1 region, decreased Akt-Ser-473 phosphorylation was not evident in the CA3 region immediately after induced ischemia (Fig. 6C). Total Akt protein levels were unchanged in the CA3 region in samples from both sham operated and ischemic animals (data not shown).

7.4. In vivo phosphorylation of Akt with orthovanadate and IGF-1

Next, whether treatment with orthovanadate or IGF-1 *in vivo* increases in both Akt phosphorylation and its activity were examined. Fifteen min after intraventricular administration of orthovanadate and IGF-1, Akt-Ser-473 phosphorylation significantly increased to 194 and 154 %, respectively, of levels seen in sham-operated animals to which the vehicle was administered. Increased Akt phosphorylation was transient and returned to a basal level within 30 min of administration (Fig. 7A). Consistent with Akt phosphorylation, Akt kinase activity as assessed by GSK3α/β phosphorylation

significantly increased (187 % of that of sham-operated animals) 15 min after administration of orthovanadate (Fig. 7B). Intraventricular administration of selective PI3-K inhibitor, wortmannin, 15 min before orthovanadate treatment prevented the orthovanadate-induced Akt phosphorylation (Fig. 7C). Thus orthovanadate activates Akt through PI3-K pathway.

7.5. Immunohistochemical localization of activated Akt after administration of orthovanadate

Next, whether intraventricular administration of orthovanadate induces Akt activation in pyramidal neurons was examined. Hippocampal slices obtained from sham-operated and orthovanadate-treated gerbils were double-stained with conventional anti-Akt antibody and anti-phospho-Akt-Ser-473 antibody. In sham-operated animals, the immunoreactivity for conventional Akt was observed mainly in cell bodies and dendrites of pyramidal neurons and was weak in surrounding non-neuronal cells (Fig. 8A). By contrast, active Akt was predominantly localized in cell bodies of pyramidal neurons in the hippocampus (Fig. 8C). In these cells, immunostaining was observed in a punctate pattern and localized to the submembrane area (Fig. 8C). Following orthovanadate treatment, immunoreactivity seen with anti-phospho-Akt antibody markedly increased in dendrites as well as in the cell bodies of pyramidal neurons (Fig. 8D) without changes in immunoreactivity against conventional Akt antibody (Fig. 8B). In the cell bodies, active Akt was evident in the nucleus as well as in the cytoplasm (Figs. 8D and H).

7.6. Prevention of Akt inactivation with orthovanadate and IGF-1 in CA1 region

Levels of Akt-Ser-473 phosphorylation following ischemia after treatment with

orthovanadate or IGF-1 were next examined. Intraventricular administration of orthovanadate 30 min before ischemia prevented the dephosphorylation of Akt-Ser-473 observed immediately after 5-min ischemia in the CA1 region (Fig. 9A, C). Levels of total Akt protein were unaffected (Fig. 9A). In addition, increased Akt-Ser-473 phosphorylation following orthovanadate treatment was observed 2 and 6 h after ischemia whereas the significant increase was observed 6 h in ischemic animals without orthovanadate. Similarly, administration of IGF-1 eliminated the ischemia-induced dephosphorylation of Akt (Fig. 9B, C). Using the immunocomplex kinase assay, pretreatment with orthovanadate rescued the ischemia-induced decrease in Akt activity observed after 5-min ischemia was also confirmed (Fig. 9D).

7.7. Immunohistochemical localization of activated Akt after ischemia

To confirm that changes in Akt activity and Ser-473 phosphorylation occurred in neurons of the CA1 region, active Akt was visualized using the anti-phospho-Akt-Ser-473 antibody. Consistent with decreased Akt activity after ischemia, the intensity of immunofluorescence against anti-Akt-Ser-473 markedly decreased in the pyramidal neurons of ischemia-induced animals, but weak and punctate staining remained in the cell bodies (Fig. 10B) compared to the negative control staining (Fig. 10E). Increased Akt-Ser-473 phosphorylation observed 6 h after ischemia was evident in the cell bodies of pyramidal neurons (Fig. 10C). In addition, increased immunostaining was also observed in stratum lacunosum, stratum radiatum and stratum oriens in the CA1 region (Fig. 10C), suggesting that Akt may be activated in the surrounding non-neuronal cells. Furthermore, treatment with orthovanadate prevented the decrease in immunoreactivity induced by ischemia (Fig. 10D).

7.8. Orthovanadate and IGF-1 protection against delayed neuronal death in the CA1

hippocampus after transient forebrain ischemia

Since orthovanadate and IGF-1 prevented the dephosphorylation of Akt activity in CA1 pyramidal cells, their neuroprotective effects on ischemia-induced delayed neuronal death were examined. Seven days after transient forebrain ischemia severe neuronal losses of greater than 90 % compared to sham-operated animals occurred in the CA1 region (Fig. 11C, D). By contrast, treatment with orthovanadate (Fig. 11E, F) or IGF-1 (Fig. 11G, H) protected neurons from delayed neuronal death, resulting in survival of 80 and 90 % of neurons relative to controls. Furthermore, pre-treatment with wortmannin, a selective PI3-K inhibitor, significantly reduced the neuroprotective effect of orthovanadate (Fig. 11I, J, K). Intraventricular administration of wortmannin (2 μl of 100 μM in PBS) significantly inhibited both basal and orthovanadate-induced *in vivo* phosphorylation of Akt-Ser-473 was confirmed (Yano et al., 2001 and in Fig. 7C).

7.9. Neuroprotective effects of post-treatment with orthovanadate

Time courses of neuroprotective effects of post-treatment with orthovanadate after ischemia were examined. As shown in Fig. 11K, pre-treatment with orthovanadate largely rescued cells from delayed neuronal death. Post-ischemic intraventricular administration of orthovanadate also significantly rescued cells by post-treatment at 30 min and 1 h, but not at 2 h after reperfusion (Fig. 12). Akt phosphorylation at 1 h after reperfusion when orthovanadate treatment was clearly neuroprotective, returned to control level of the sham-operated animals (130 % of the control, without statistically significance).

To eliminate the possibility that the neuroprotective effect of orthovanadate could be due to hypothermia, body temperature was monitored after 5-min ischemia with or without orthovanadate treatment. No difference in body temperatures was observed at

any time point recorded up to 24 h before or after ischemia between the vehicle-treated and orthovanadate-treated groups (data not shown).

7.10. Involvement of the MAP kinase pathway in neuroprotection with orthovanadate

As shown in Fig. 11K, orthovanadate-induced neuroprotection was partially inhibited by wortmannin treatment, suggesting that Akt activation is partly responsible for such neuroprotection. On the other hand, wortmannin treatment completely inhibited orthovanadate-induced Akt activation (Fig. 7C), suggesting that other signaling mediating survival such as MAP kinase pathway is activated by orthovanadate treatment. Treatment with 50-100 μM orthovanadate stimulated MAP kinase up to 200 % of controls in cultured hippocampal slices. The maximal activation of MAP kinase was obtained up to 300 % of control with 200 μM of orthovanadate 30 min after stimulation. In addition, phosphorylation of MAP kinase required for its activation decreased in the CA1 region immediately after ischemia without changes in MAP kinase protein levels (Fig. 13A). Intraventricular administration of orthovanadate prevented the decrease in phosphorylation of MAP kinase (Fig. 13B). In order to verify involvement of MAP kinase in the orthovanadate-induced neuroprotection, the effects of U0126 were analyzed, a specific MAP kinase kinase inhibitor, on neuroprotective effects of orthovanadate. Injection of 2 μl of 500 μM U0126 or a combination of U0126 plus wortmannin had no apparent effect on cell viability without ischemia (Fig. 13C). Pretreatment with U0126 significantly reduced the neuroprotective effects of orthovanadate and a combination of both wortmannin and U0126 totally suppressed orthovanadate-induced neuroprotection (Fig. 13C). These results suggest that, in addition to the Akt pathway, the MAP kinase pathway is involved in orthovanadate-induced neuroprotective effects.

7.11. Orthovanadate prevents NaCN-induced apoptosis of NG108-15 through Akt activation

To confirm involvement of Akt activation in the orthovanadate-induced neuroprotection, the neuroprotective effects of orthovanadate in NG108-15 cells transfected with dominant negative form of Akt were tested. To produce chemical hypoxia-induced apoptosis, NG108-15 was treated with 1 mM NaCN for 24h. In control experiments, cells were double-stained with TUNEL and propidium iodide. Twenty-five percentage of total cells were TUNEL positive (Fig. 14A and C) following NaCN treatments and pre-incubation with 50 μM orthovanadate significantly decreased the number of TUNEL positive cells to 5.2% (Fig. 14B and D). In the experiment with transfected cells, cells were double-stained with TUNEL and anti-Myc Tag antibody to evaluate apoptosis only in cells expressed dominant negative form of Akt (Fig. 14E, F, G and H). Although expression of the dominant negative Akt itself slightly increased the number of TUNEL positive cells, the TUNEL positive cells largely increased to 82 % of cells expressed dominant negative Akt following NaCN treatments (Fig. 14I). Consistent with our hypothesis, the expression of the dominant negative Akt enhanced the chemical hypoxia-induced apoptosis and pretreatment with orthovanadate failed to prevent apoptosis in cells expressed with dominant negative Akt. Small but significant neuroprotective effect still remained after treatment with orthovanadate even in the transfected cells, probably due to partial contribution of MAP kinase signaling in the orthovanadate effect. These data demonstrate orthovanadate-induced Akt activation underlies its neuroprotective effect.

8. Discussion

Here using a global cerebral ischemic model in the gerbil, intraventricular administration of orthovanadate or IGF-1 blocks delayed neuronal death following ischemia was demonstrated. Specifically, treatment with orthovanadate or IGF-1 rescued decreases in Akt activity following ischemia, transiently stimulated Akt activity in hippocampal neurons *in vivo* and, was completely ineffective when animals were pretreated with both wortmannin and U0126, suggesting that orthovanadate may exert neuroprotective action via both Akt and MAP kinase activation. Since decreased Akt activity observed immediately after ischemia was seen only in the CA1 but not the CA3 region, decreased Akt and MAP kinase activities underlie the vulnerability of CA1 pyramidal neurons following ischemia were suggested. Since orthovanadate inhibits protein tyrosine phosphatase, its neuroprotective effects may be due to stimulation of protein tyrosine phosphorylation. Thus, treatment with orthovanadate activates pathways downstream of both Akt and MAP kinase, which in turn promote protection from neuronal death. Furthermore, brief exposure to ischemic insults caused activation of Akt and thereby induced ischemic tolerance (Yano et al. 2001). Here, the Akt pathway was primarily analyzed.

Akt was inactivated *in vivo* by dephosphorylation immediately following ischemia, probably due to the activity of protein phosphatase 2A. Protein phosphatase 2A but not protein phosphatase 1, inactivates Akt *in vitro* (Andjelkovic et al., 1996). Since Ca^{2+} mobilization through NMDA receptors is stimulated during ischemic conditions (Choi et al., 1995), it is possible that activation of the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, might result in dephosphorylation of Akt. However, using recombinant Akt protein this hypothesis was tested by assaying dephosphorylation of Akt-Ser-473 by calcineurin *in vitro* and calcineurin did not dephosphorylate Akt-Ser-473 was showed (data not shown). In addition, we recently reported decreased

calcineurin activity immediately after forebrain ischemia in a four-vessel occlusion model of rat, in which protein phosphatase 2A activity did not change (Morioka et al., 1999). Thus, protein phosphatase 2A but not protein phosphatase 1 and calcineurin may be major protein phosphatase that accounts for the dephosphorylation of Akt.

In contrast to the decrease in Akt activity observed following 5-min ischemia, phospho-Akt increased above baseline levels 6 h after reperfusion following ischemia. Previous studies suggest that protein tyrosine phosphorylation is upregulated following forebrain ischemia (Ohtsuki et al., 1996; Hu and Wieloch, 1994; Shamloo and Wieloch, 1999). In the ischemic gerbil hippocampus, protein tyrosine phosphorylation significantly increased up to 200 % of that of sham-operated animals 3-12 hr after ischemia (Ohtsuki et al., 1996). Kinases downstream of tyrosine kinase signaling such as mitogen-activated protein (MAP) kinase were also activated following ischemia (Hu and Wieloch, 1994), although more persistently than was Akt in the present study (Shamloo et al., 1999). After ischemia and reperfusion, changes in levels of several cytokines and growth factors have also been reported. Tumor necrosis factor α (TNFα) is induced 1.5 h after reperfusion in mouse hippocampus after 30-min transient global ischemia (Uno et al., 1997). Vascular endothelial growth factor (VEGF) mRNA also increases 12 h after reperfusion with a peak at 24 h in hippocampus after transient forebrain ischemia in rats (Lee et al., 1999). After transient middle cerebral artery occlusion in rats, both the mRNA and protein levels of VEGF increase 1 hour after reperfusion, with a peak at 3 h (Hayashi et al., 1997). IGF-1 also accumulates in blood vessels of the damaged hemisphere within 5 h after hypoxic-ischemia injury (Beilharz et al., 1998). Furthermore, TNFα and VEGF activate PI3-K (Pastorino et al., 1999) and Akt (Gerber et al., 1998; Fujio and Walsh, 1999), respectively. Taken together, increased Akt phosphorylation observed 6 h after reperfusion may be due to multiple factors, such as growth factors and cytokines that activate protein tyrosine kinases.

The characteristic distribution of active Akt suggests several downstream targets.

L-type, voltage dependent Ca^{2+} channels in the plasma membranes of the cell body and dendrites of pyramidal cells could represent a potential target, since Akt-dependent Ca^{2+} channel activation is required for IGF-1-induced neuroprotection in cerebellar granule cells (Blair et al., 1999). Bad and caspase 9 may also function in Akt-induced neuroprotection. Bad, a proapoptotic protein of the Bcl-2 family, is phosphorylated by Akt with concomitant dissociation from Bcl-x_L, which in turn prevents mitochondrial damage, such as release of cytochrome c (Datta et al., 1997; Kennedy et al., 1999; Zha et al., 1996). Similarly, phosphorylation and subsequent inactivation of caspase 9 inhibits apoptotic signaling (Cardone et al., 1998). Release of cytochrome c from mitochondria and activation of a caspase-like protease with a concomitant decrease in Akt activity was documented in a two-vessel occlusion model in rat (Ouyang et al., 1999). Furthermore, activation of Akt in nuclei of pyramidal neurons may play a pivotal role in expression of anti-apoptotic proteins through activation of transcription factors, such as the cAMP-responsive element binding protein (CREB) and Forkhead family transcription factors. For example, activation of Akt signaling pathways leads to CREB phosphorylation (Du and Montminy, 1998), and IGF-1-induces CREB phosphorylation through the Akt pathway (Pugazhenthi et al., 1999). Activation of CREB also stimulates IGF-1 induced Bcl-2 expression through the PI3-K pathway (Pugazhenthi et al., 2000). A Forkhead family transcription factor is also directly phosphorylated by Akt and inhibits expression of proapoptotic proteins such as the Fas ligand (Brunet et al., 1999). Furthermore, IGF-1 could phosphorylate Forkhead family transcription factor, FKHRL1, through PI3-K/Akt pathway in rat pheochromocytoma (PC12) cells (Zheng et al., 2000).

Although IGF-1 attenuates apoptosis of hippocampal neurons induced by cerebral ischemia (Gluckman et al., 1992; Guan et al., 1993; Zhu and Auer, 1994; Guan et al., 1996; Tagami et al., 1997; Wang et al., 2000), the penetration of peptides such as growth factors into the hippocampus is generally slow when administered into the ventricle and

very inefficient when administrated to peripheral tissues. Vanadate, vanadium complexes and vanadyl compounds can produce insulinomimetic effects in the liver, adipocytes and muscle when administered in peripheral tissues (McNeill et al., 1995; Bevan et al., 1995). The biological activity of these vanadate and vanadium complexes is not, however, restricted to non-neuronal cells. Differentiation and neurite outgrowth of PC12 cells and neurite outgrowth of human neuroblastoma SH-SY5Y cells are stimulated by treatment with orthovanadate (Rogers et al., 1994). Although vanadate and vanadium complexes stimulate proliferation in mouse fibroblasts (Krady et al., 1997), they inhibit proliferation of NB41 neuroblastoma cells and C6 glioma cells (Faure et al., 1995). Inhibition of protein tyrosine phosphatases appears to underlie the biological actions of vanadate species (Meier et al., 1998).

The effects of vanadium compounds are of particular interest since these are known to therapeutic agents for insulin-deficient and diabetic rats (Heyliger et al., 1985; Brichard et al., 1988; Dai et al., 1994; Blondel et al., 1990) and for genetically obese, hyperinsulinaemic and insulin-resistant rats and mice (Brichard et al., 1990; Meyerovitch et al., 1991; Brichard et al., 1989). Furthermore, clinical studies of vanadyl sulfate or sodium metavanadate report improved insulin sensitivity of patients with non-insulin-dependent diabetes mellitus (Goldfine et al., 1995; Cohen et al., 1995; Brichard and Henquin, 1995).

Vanadate species activate the MAP kinase pathway (Zhao et al., 1996). Among the vanadate species, pervanadate, which is prepared from a combination of vanadate and H_2O_2, is the most potent stimulant of both Akt and MAP kinase in situ (Zhao et al., 1996; Meier et al., 1998). However, the half-life of pervanadate is 1-2 h in aqueous solution and its stability in biological systems is questionable (Morinville et al., 1998). Moreover, H_2O_2 itself is toxic for cells. Although prolonged incubation for 48 h with 100 μM orthovanadate has been reported to induce apoptosis of cultured neurons from rat dentate gyrus (Figiel et al., 1997), intraventricular administration of orthovanadate

did not produce neurotoxic effects on hippocampal pyramidal neurons in our study. Our observations are in fact consistent with vanadate-induced inhibition of apoptosis induced by TNFα in endothelial cells (Yang et al., 1996) and the mouse connective tissue cell line L-929 (Totpal et al., 1992), by extracellular ATP in the murine mastocytoma cell line and a murine leukaemia cell line (Bronte et al., 1996), and by K252a in malignant glioma cells (Chin et al., 1999). Despite the weak activation by orthovanadate of MAP kinase in situ (Zhao et al., 1996) and Akt in the present study, orthovanadate exhibited a neuroprotective effect equivalent to that of IGF-1 when administrated into the ventricle. Interestingly, post-administration of orthovanadate also resulted in a neuroprotective effect 30 min and 1 h after reperfusion as shown in Figure 12.

9. Conclusions

Akt and MAP kinase activation contributes to neuroprotection after transient forebrain ischemia induced by treatment with orthovanadate has been shown. Figure 15 shows the speculated pathways involved in the mechanism of neuroprotection by orthovanadate. Activated Akt was predominantly located in pyramidal neurons, particularly in the nuclei and dendrites. The characteristic localization of the active Akt provides new insight regarding target molecules acting to protect neurons from apoptosis. Furthermore, expression of dominant negative form of Akt enhanced hypoxia-induced apoptosis and largely prevented the orthovanadate-induced neuroprotective effect. These downstream targets of orthovanadate-induced Akt activation are now under investigation. In the present study, the author suggests that sodium orthovanadate has potential pharmacological effects and is useful therapeutic agents for delayed neuronal death after transient forebrain ischemia. Further investigation is required for the future application of vanadium against ischemic patients in clinical scene.

10. References

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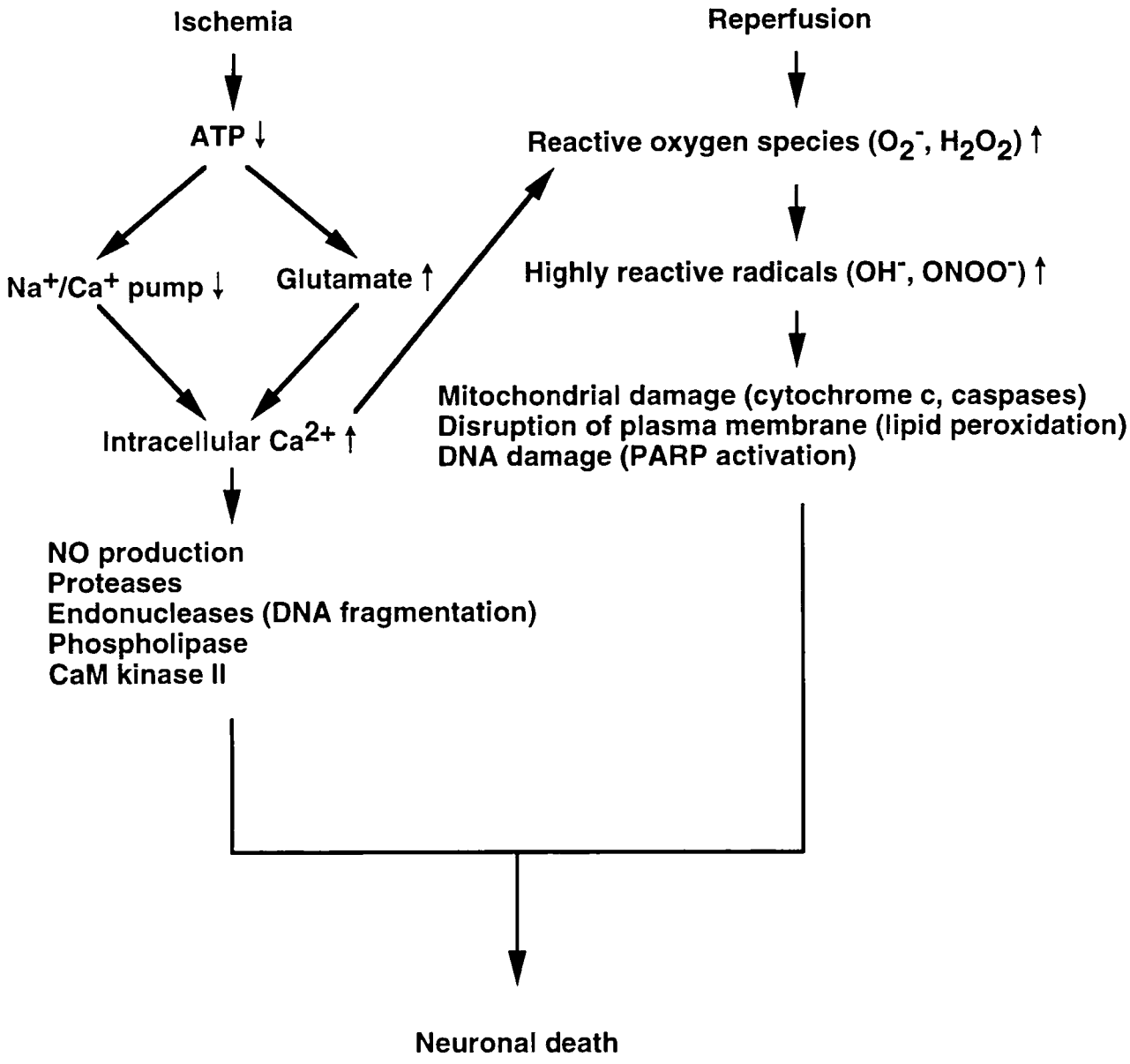

Figure 1. Intracellular cascades of neuronal death by ischemia

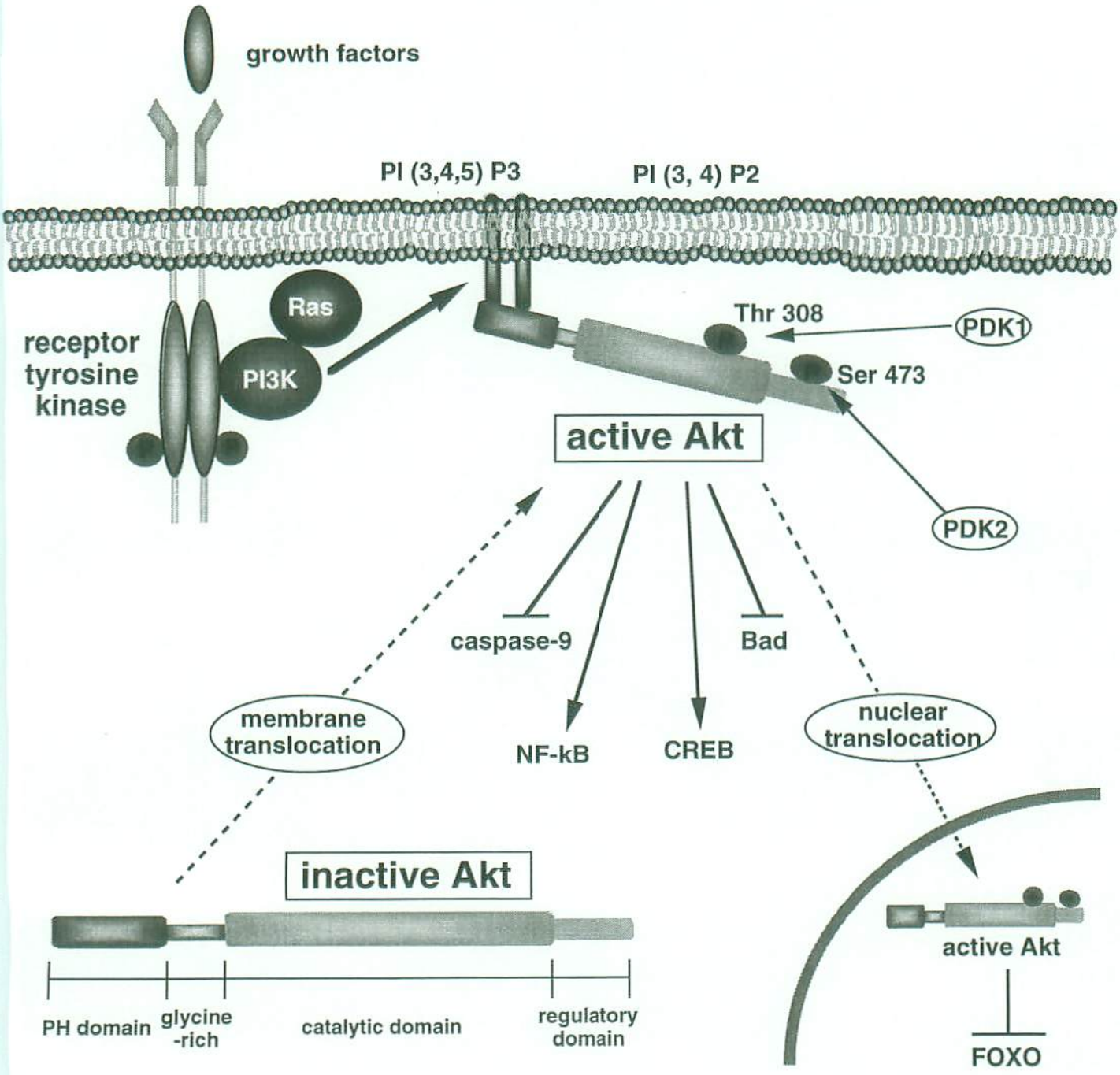

Figure 2. **Akt is activated by growth factors and necessary for cell survival.** Stimulation of tyrosine kinase growth factor receptors or GTP-binding protein-coupled receptors activates PI3K, leading to production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) and phosphatidylinositol-3,4 -bisphosphate, which bind to the Akt pleckstrin homology domain and promote translocation of Akt to cell membranes. Such membrane translocation is an essential step in Akt activation. Akt activation is correlated with phosphorylation of Thr-308 in its catalytic domain and Ser-473 at the C terminus. Activated Akt phosphorylate several substrates, such as Bad, FOXOs, caspase 9, CREB and NF-κB, and mediate cell survival.

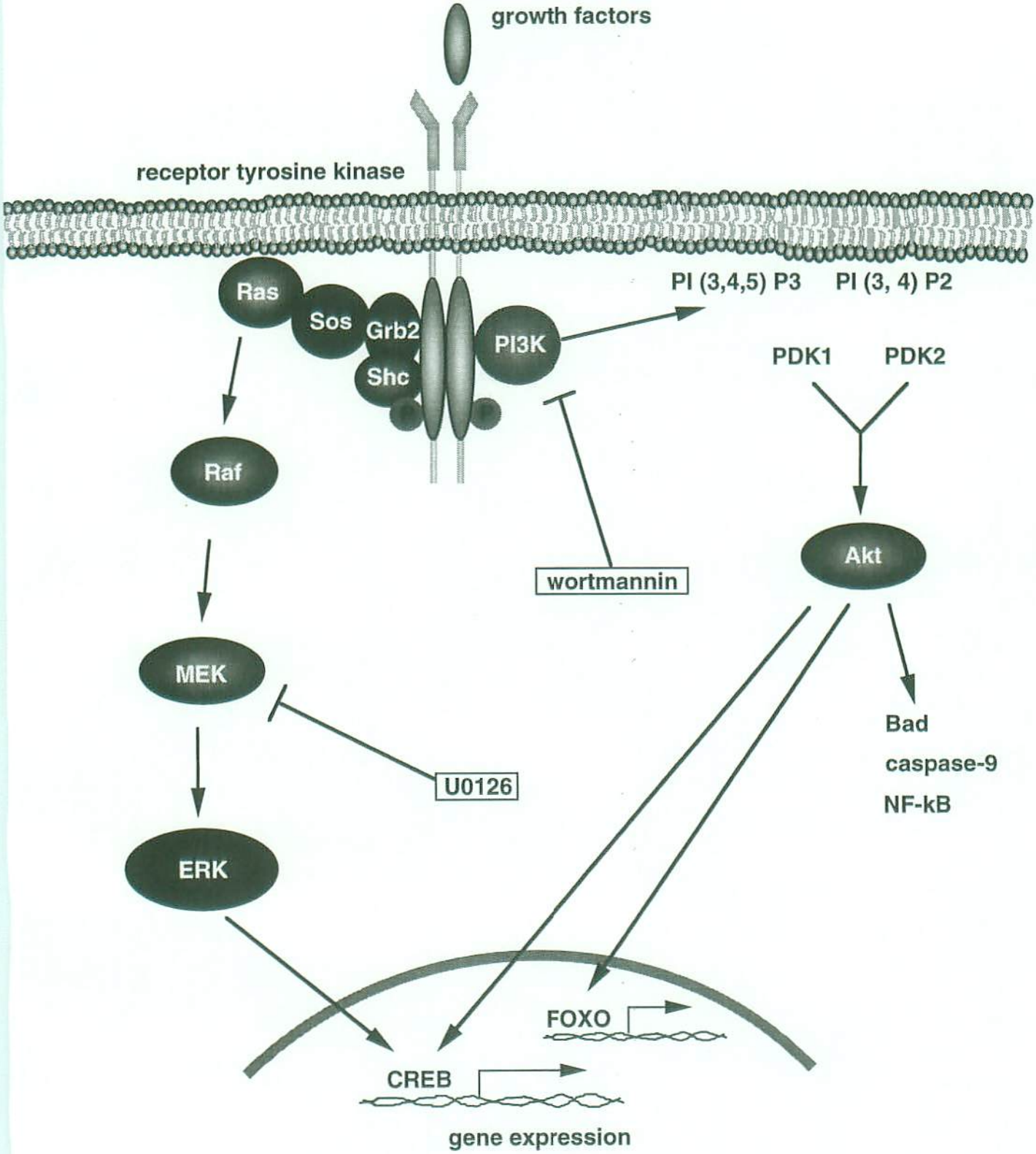

Figure 3. **Growth factors activate both PI3K-Akt and MAPK pathways through receptor tyrosine kinase activation.** Growth factors mediate the survival of neurons by binding to activate receptor tyrosine kinase. PI3K-Akt pathway and MEK/MAPK pathway are the major effectors of growth factor induced cell survival. Activated Akt phosphrylates several substrates to cell survive such as Bad, caspase 9, NF-κB, FOXO and CREB. Activated ERK may induce anti-apoptotic gene expressions mediated by CREB. Wortmannin and U0126 are pharmacological inhibitors that inhibit activities of PI3K and MEK, respectively.

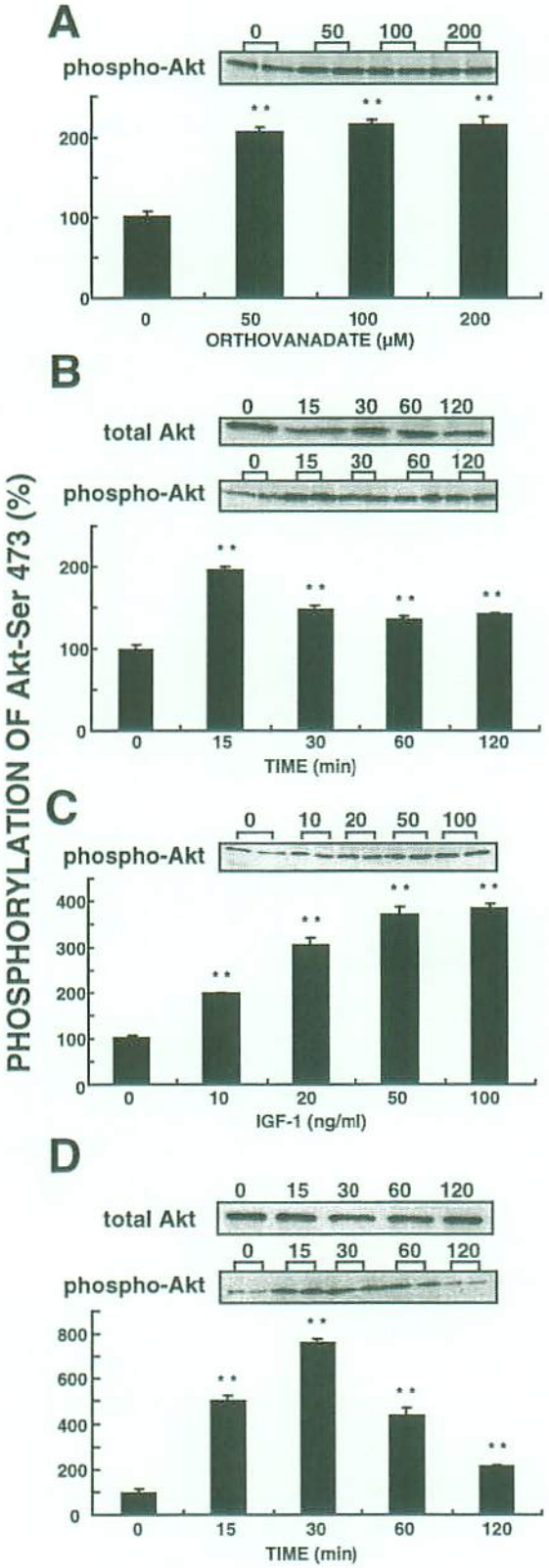

Figure 4. **Activation of Akt by stimulation with orthovanadate or IGF-1 in cultured hippocampal slices.** Hippocampal slices were cultured for 7-9 days and were incubated for 1 h with Krebs-Ringer Hepes solution (KRH). Hippocampal slices were then stimulated for 30 min with orthovanadate or IGF-1 at the indicated concentrations (in **A** and **C**) or treated for the indicated times with orthovanadate (100 μM) or IGF-1 (50 ng/ml) (in **B** and **D**). Using cell extracts obtained from two slices, immunoblotting analysis was performed with anti-phospho-Ser-473 and anti-Akt antibodies. Quantitative analysis of relative phospho-Ser-473 levels was performed by densitometric analysis of the immunoblot. Data are means ± SD (n=4) and represented as percentage of the control value without stimulants. Asterisks denote a significant difference between control and stimulated slices (**$p < 0.01$).

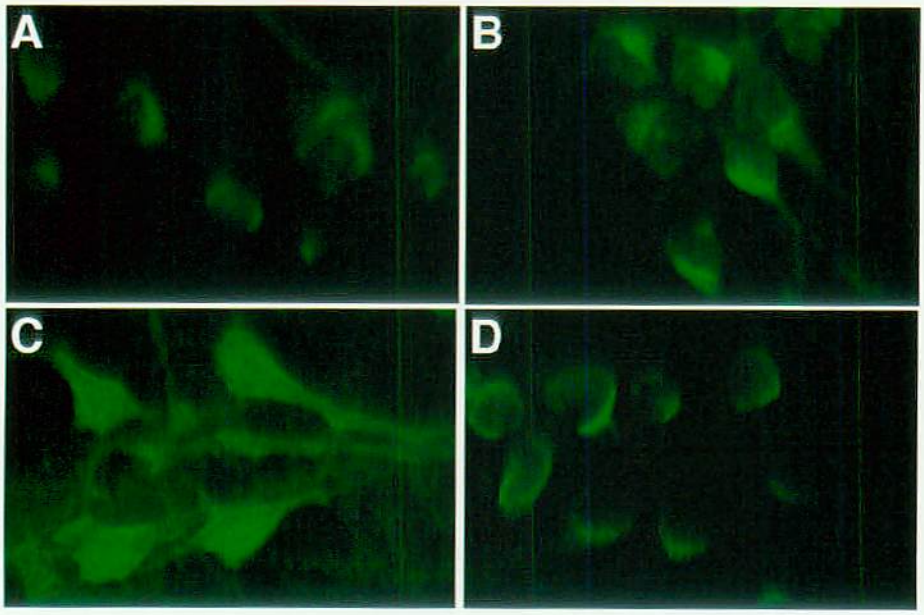

Figure 5. **Immunohistochemical localization of active Akt in dispersed hippocampal neuron.** Dispersed hippocampal neuron were cultured 16 days and immunofluorescence staining were performed with anti-phospho Akt antibody followed by with Alexia 488 conjugated anti-sheep IgG. Dispersed hippocampal neurons were incubated 1 hour with KRH and we then stimulated for 15 or 30 min with or without IGF-1 at 50 ng/ml (A, B and C). Pre-incubation with wortmannin with 200 nM prevented the activation of Akt stimulated with 50 ng/ml IGF-1 for 30 min (D).

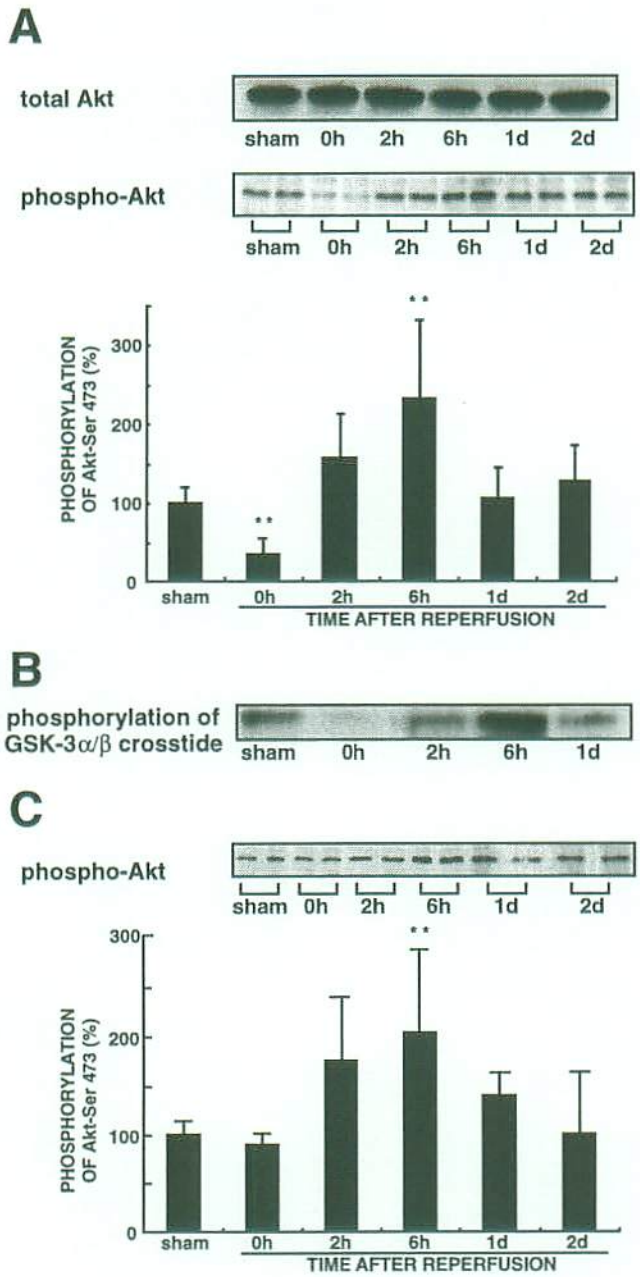

Figure 6. **Changes in Akt-Ser-473 phosphorylation and Akt activity after transient forebrain ischemia.** Extracts were obtained from the hippocampal CA1 or CA3 region from sham-operated animals or from 5-min ischemic animals at the indicated times. **A,** Representative image of immunoblot using either anti-Akt antibody (total Akt) or anti-phospho-Ser-473 antibody (phospho-Akt) using cell extracts from the CA1 region. Quantitative analysis of relative phospho-Ser-473 levels was performed by densitometric analysis of the immunoblots. Data are expressed as percentage of values of sham-operated animals (means ± SD, n = 5). **B,** Akt activity in the CA1 region following 5-min ischemia was measured using GSK-3α/β crosstide as substrate as described in Materials and Methods. **C,** Representative image of immunoblot using anti-phospho-Ser-473 antibody (phospho-Akt) using cell extracts from the CA3 region. Data are expressed as percentage of values of sham-operated animals (means ± SD, n = 5). Asterisks denote a significant difference between sham-operated and ischemic groups (**$p < 0.01$).

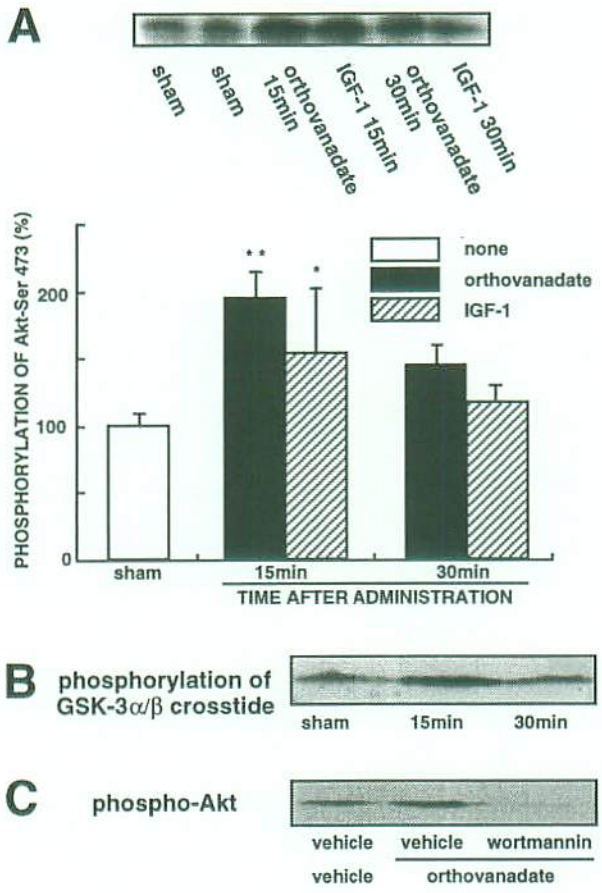

Figure 7. **Effects of IGF-1 and orthovanadate on phosphorylation and activity of Akt in the hippocampal CA1 region without ischemia. A,** Quantitative analysis of relative phospho-Akt Ser-473 levels following intraventricular administration of orthovanadate or IGF-1. Gerbils were administrated 2 μl of 5 mM orthovanadate or 25 μg/ml of IGF-1 and decapitated 15 min or 30 min after administration. Extracts from the CA1 regions were subjected to immunoblotting analysis with anti-phospho-Akt-Ser-473 antibody. Results are means ± SD (n = 5) and expressed as percentage of sham-operated animals treated with the vehicle. Statistically significant differences are indicated by *asterisks* (*$p < 0.05$; **$p < 0.01$). **B**, Akt activity following intraventricular administration of orthovanadate. Akt activity in extracts from the CA1 region was measured by immunocomplex kinase assay using GSK3 crosstide 15 and 30 min after administration of orthovanadate. The changes in Akt activity were highly correlated with Ser-473 phosphorylation shown in panel A. **C,** Effect of wortmannin on orthovanadate-induced Akt activation. Gerbils were intraventricular administration 2 μl of vehicle or 100 μM wortmannin 15 min before administration of vehicle or orthovanadate as described above. Representative image of immunoblot using anti-phospho-Akt-Ser-473 antibody using cell extracts from CA1 regions.

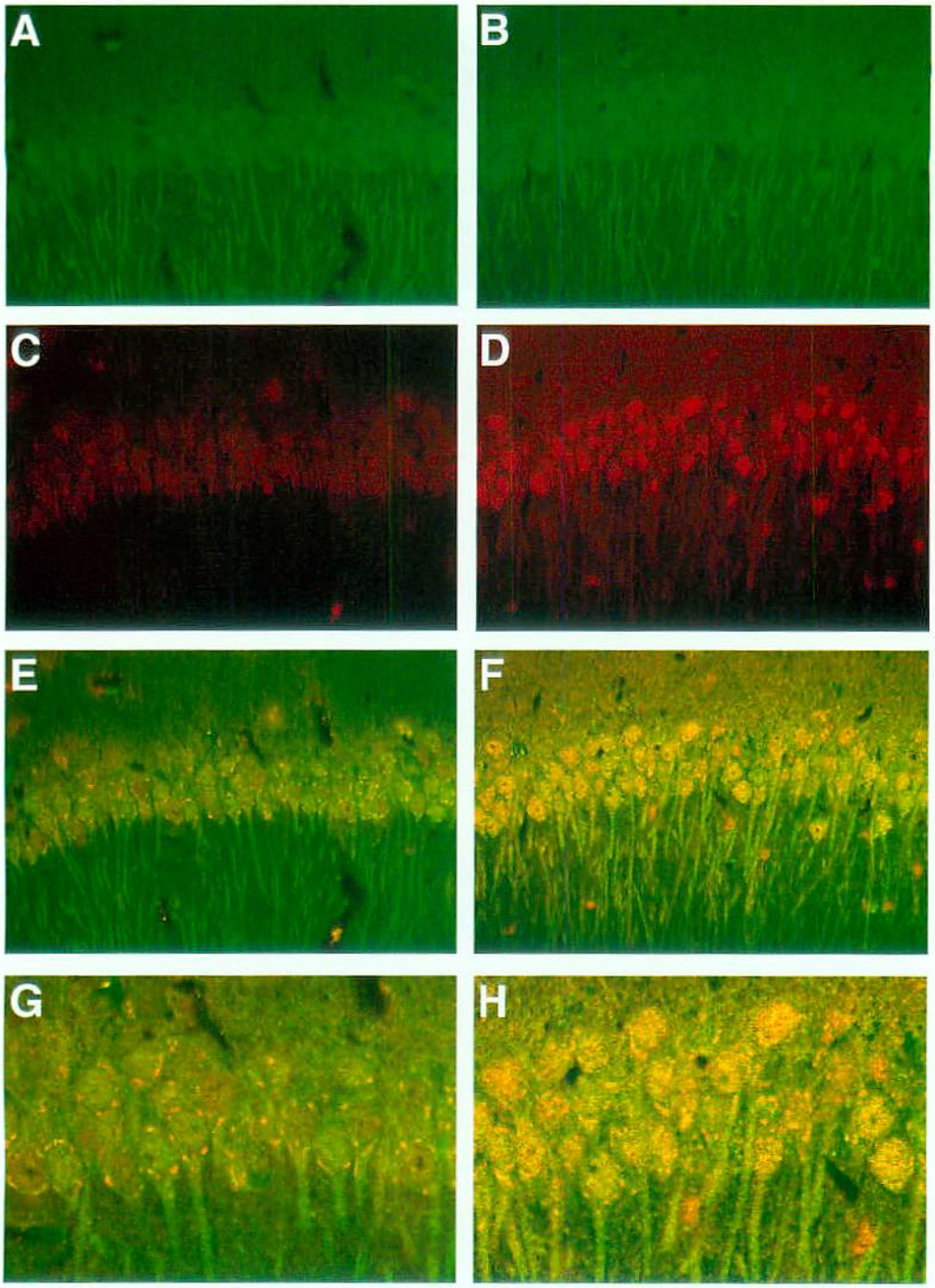

Figure 8. **Immunohistochemical localization of active Akt in hippocampal CA1 region.** Gerbils were perfused and fixed with 4 % paraformaldehyde after treatment with or without orthovanadate. In cases of sham-operated (**A, C, E** and **G**) and orthovanadate treated animals (**B, D, F** and **H**), gerbils were perfused and fixed 15 min after intraventricular administration of the vehicle or orthovanadate. Hippocampal slices were double-immunostained with anti-Akt antibody (**A** and **B**), followed by fluorescein-conjugated anti-mouse IgG and anti-phospho-Akt-Ser-473 antibody (**C** and **D**), followed by with Alexa 594-conjugated anti-sheep IgG. Confocal images of anti-Akt antibody (green) and anti-phospho-Akt antibody (red) were merged in **E** and **F**. **G** and **H** are high magnification images of **E** and **F**, respectively. Similar results were obtained from four independent experiments.

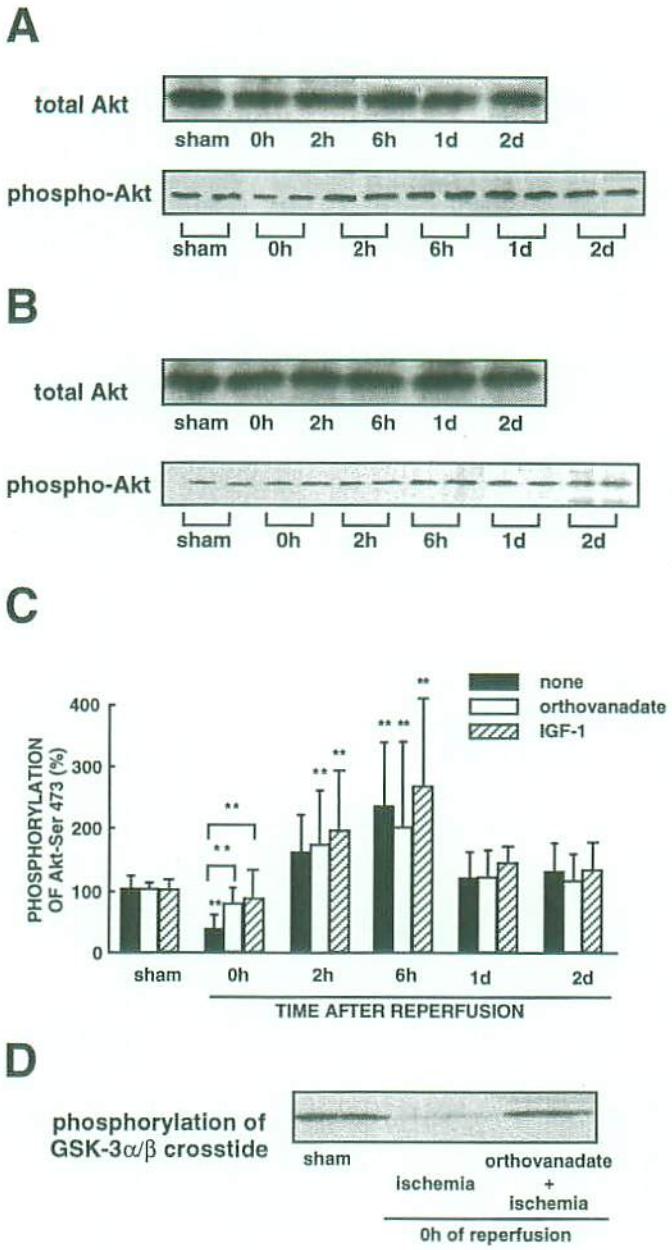

Figure 9. **Effects of orthovanadate and IGF-1 treatment on phosphorylation of Akt following transient forebrain ischemia.** Gerbils were intraventricularly administered 2 µl of 5 mM orthovanadate (**A**) or 2 µl of 25 µg/ml of IGF-1 (**B**) and, 30 min later, 5-min ischemia was performed as described in Materials and Methods. Representative image of immunoblot with either anti-Akt antibody (total Akt) or anti-phospho-Ser-473 antibody (phospho-Akt) after treatment with orthovanadate (**A**) or IGF-1 (**B**) and quantitative analysis of relative phospho-Ser-473 levels in ischemic gerbils with or without drug treatments (**C**). Extracts were prepared from the CA1 region of sham-operated animals and of 5-min ischemic animals at 0 h, 2 h, 6 h, 1 day (1 d) and 2 days (2d) after reperfusion. Results are means ± SD (n = 5) and are expressed as percentage of sham-operated animals. Statistically significant differences from sham-operated animals and between no treatment (none) and drug treatments at 0 h are indicated by *asterisks* (* $p < 0.05$; ** $p < 0.01$). **D**, Akt activity was also measured immediately after 5-min ischemia with or without orthovanadate treatment. The preservation by orthovanadate of Akt activity was correlated with recovery of Akt-Ser-473 phosphorylation by orthovanadate in 0 h seen in Fig. 9A.

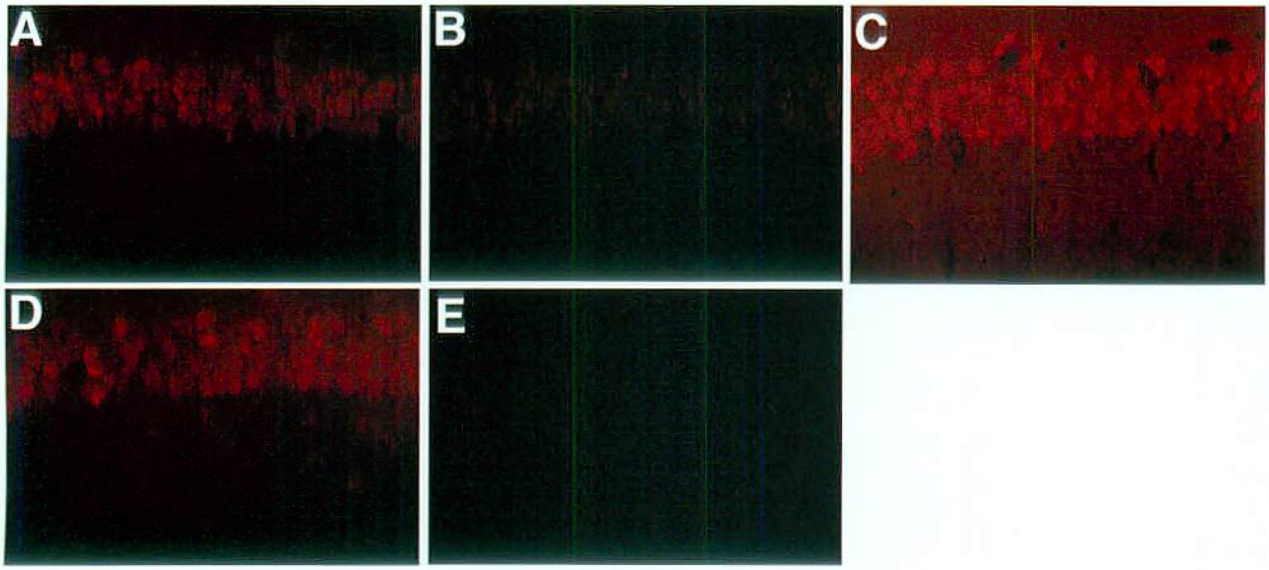

Figure 10. **Immunohistochemical localization of active Akt in the hippocampal CA1 region.** Gerbils were perfused and fixed with 4 % paraformaldehyde with or without ischemia. Confocal laser scanning light microscopic analysis of the hippocampal CA1 pyramidal cell layer was done after immunostaining with anti-phospho-Akt-Ser-473 antibody. Hippocampal slices were obtained from sham-operated gerbils (**A**) and gerbils 0 h (**B**) and 6 h (**C**) after ischemia and gerbils treated with orthovanadate 0 h (**D**) after ischemia. **E,** Negative control stained without primary antibody. Similar results were obtained from four independent experiments.

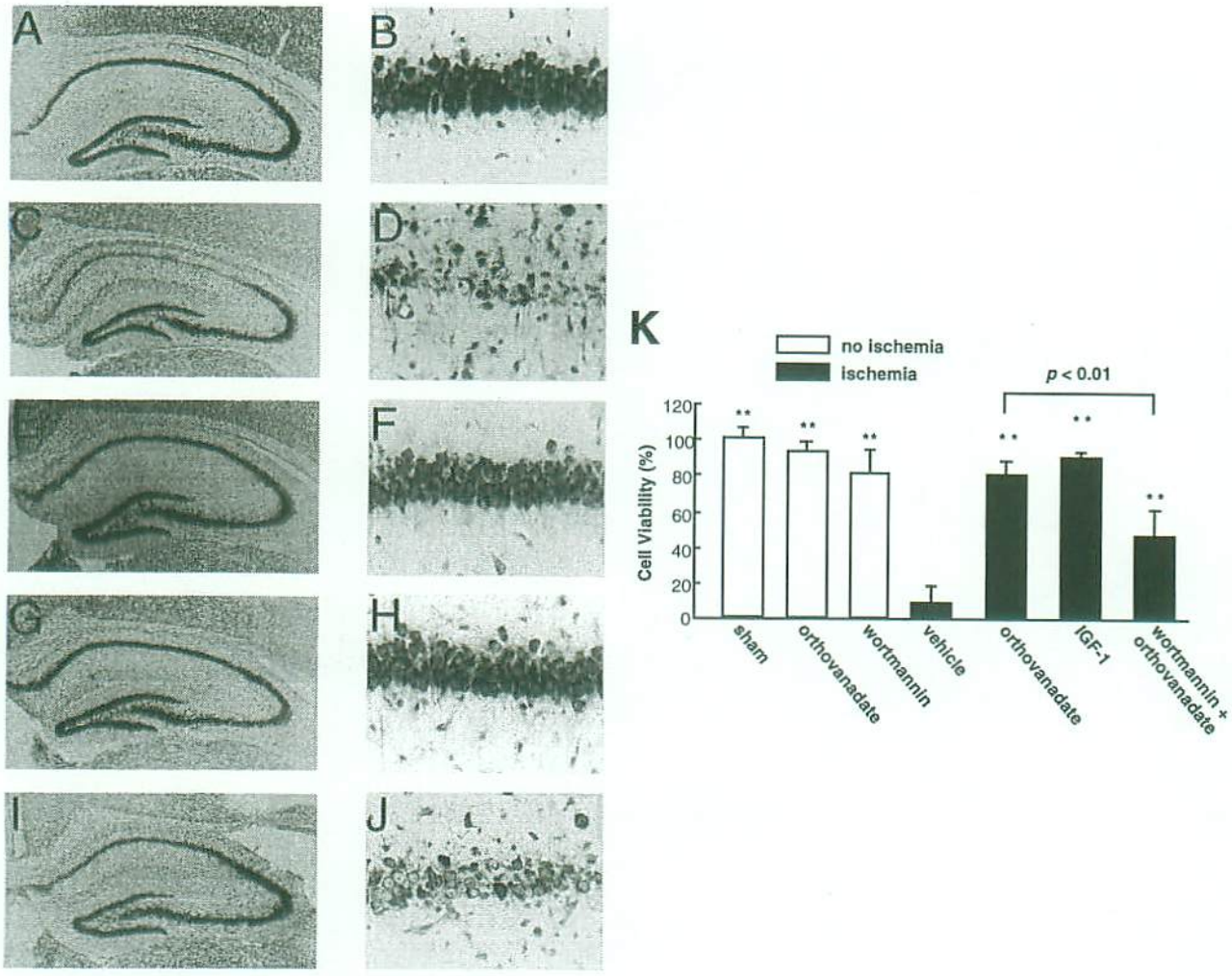

Figure 11. **Histological changes in the hippocampal CA1 pyramidal cell layer following transient forebrain ischemia.** The vehicle or drugs were injected into the right cerebral ventricle 30 min before ischemia. Histology was performed 7 days after sham operation or reperfusion in gerbils subjected to 5-min ischemia. Hippocampal slices were stained with propidium iodide and observed using a confocal laser microscope. The histological images were obtained from the right hippocampal CA1 region. Representative histological sections of hippocampus (**A, C, E, G** and **I**) and the hippocampal CA1 region (**B, D, F, H** and **J**) in sham operated animals (**A** and **B**), in 5-min ischemic animals treated vehicle (2μl) (**C** and **D**), in 5-min ischemic animals treated 5 mM orthovanadate (2μl) (**E** and **F**), in 5-min ischemic animals treated with 25μg/ml IGF-1 (2μl) (**G** and **H**), and in 5-min ischemic animals treated with 2 μl of 100 nM wortmannin and 5 mM orthovanadate (**I** and **J**). **K**, Quantitative analysis of survival of neurons in the CA1 region of the hippocampus with or without administration of drugs. The total number of surviving cells was counted on both sides of the hippocampal CA1 region. Surviving neurons marked by large, round nuclei in the CA1 region were counted in each image and expressed as a percentage of that observed in sham-operated gerbils (sham, n = 8; orthovanadate, n = 5; wortmannin, n = 5; vehicle, n = 5; orthovanadate treatment plus ischemia, n = 8; IGF-1 treatment plus ischemia, n = 8; wortmannin and orthovanadate treatment plus ischemia, n = 8). Results are means ± SD. Statistically significant differences versus vehicle-treated animals are indicated by *asterisks* (**$p < 0.01$).

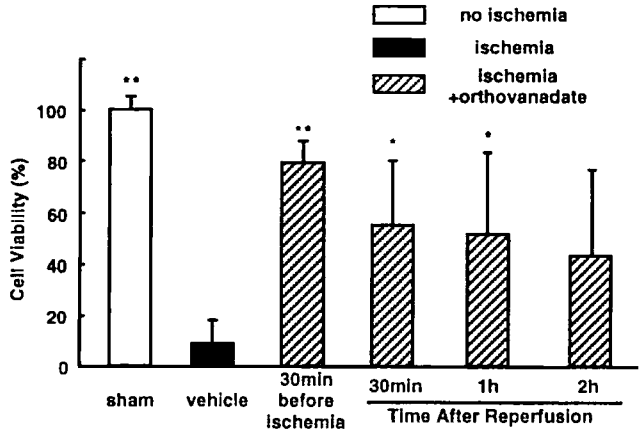

Figure 12. **Effects of post-treatment with orthovanadate on ischemia-induced delayed neuronal death in the hippocampal CA1 region.** Two μl of orthovanadate was injected into the right cerebral ventricle 30 min, 1 or 2 h after reperfusion in ischemic animals. Surviving neurons in the CA1 region were quantitatively analyzed as in Fig. 11. The data from sham-operated and ischemic animals treated with the vehicle and pretreated with orthovanadate were the same as those shown in Fig. 11. Results are means ± SD (n = 5) and are expressed as percentage of sham-operated animals. Statistically significant differences are indicated by *asterisks* (*$p < 0.05$, **$p < 0.01$).

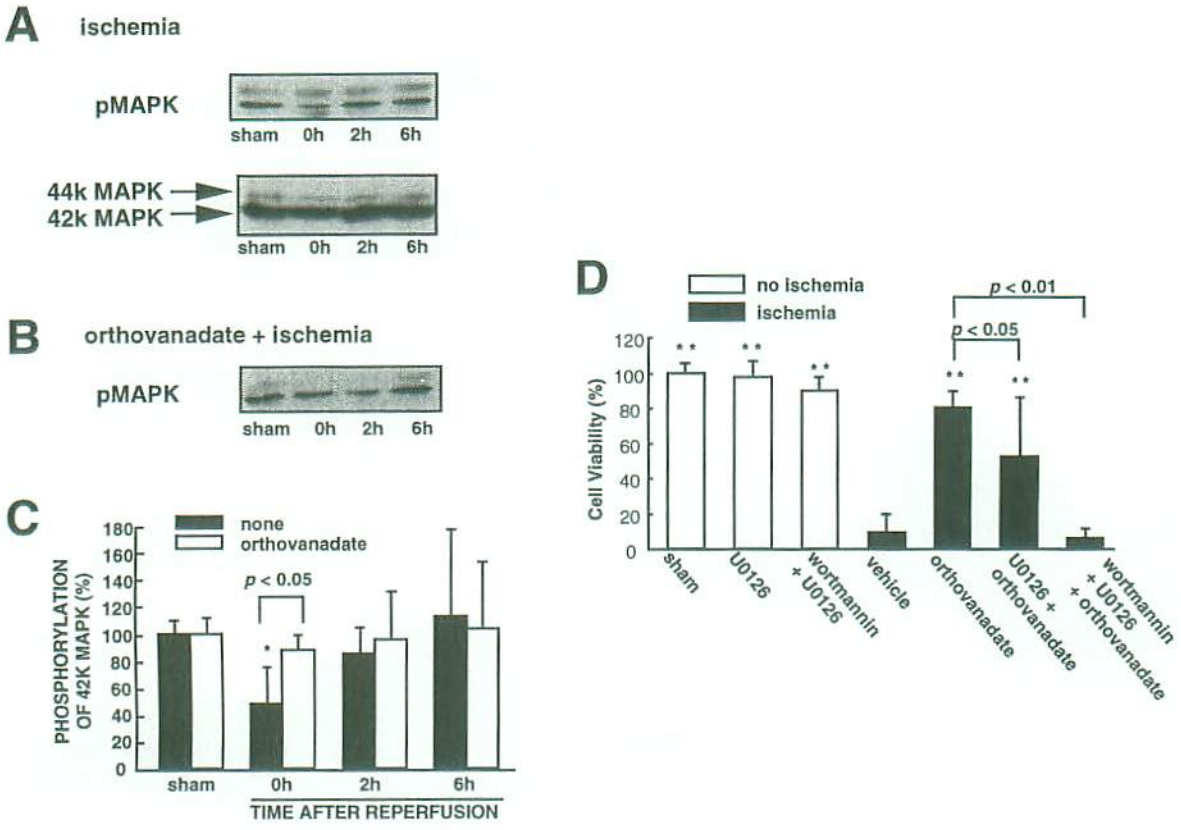

Figure 13. **Changes in MAP kinase phosphorylation and effects of U0126 and wortmannin on orthovanadate-induced neuroprotection following forebrain ischemia. A,** Representative image of immunoblot using either anti-phospho MAP kinase antibody, which recognizes dual phosphorylation of Thr-183 and Tyr-185 within the activation loop of MAP kinase, or conventional anti-MAP kinase antibody using cell extracts from the CA1 region. Extracts were obtained from the hippocampal CA1 region of sham-operated animals (sham) or 5-min ischemic animals at the indicated times. A significant decrease in 42 kDa MAP kinase phosphorylation was observed immediately (0 h) after ischemia without changes in protein levels of MAP kinase. **B,** Prevention of ischemic-induced dephosphorylation of MAP kinase by treatment with orthovanadate. Representative image of immunoblot with anti-phospho MAP kinase antibody using cell extracts from the CA1 region. **C**, Quantitative analysis of surviving neurons in the CA1 region of the hippocampus. Two μl of 500 μM U0126 and 100 μM wortmannin were administrated into the left ventricle and 15 min later orthovanadate was injected into the right ventricle. Thirty minutes later, animals were subjected to 5-min ischemia and 7 days later, cell viability was quantified as described in Materials and Methods. Data from sham-operated and ischemic animals with or without orthovanadate were originally the same as those in Fig. 11K. Results are means ± SD (n = 5) and are expressed as percentage of control (sham operated) animals. Statistically significant differences versus ischemic animals with the vehicle are indicated by *asterisks* (**$p < 0.01$). Treatments with U0126 and U0126 plus wortmannin significantly prevented orthovanadate-induced neuroprotection (*$p < 0.05$, **$p < 0.01$).

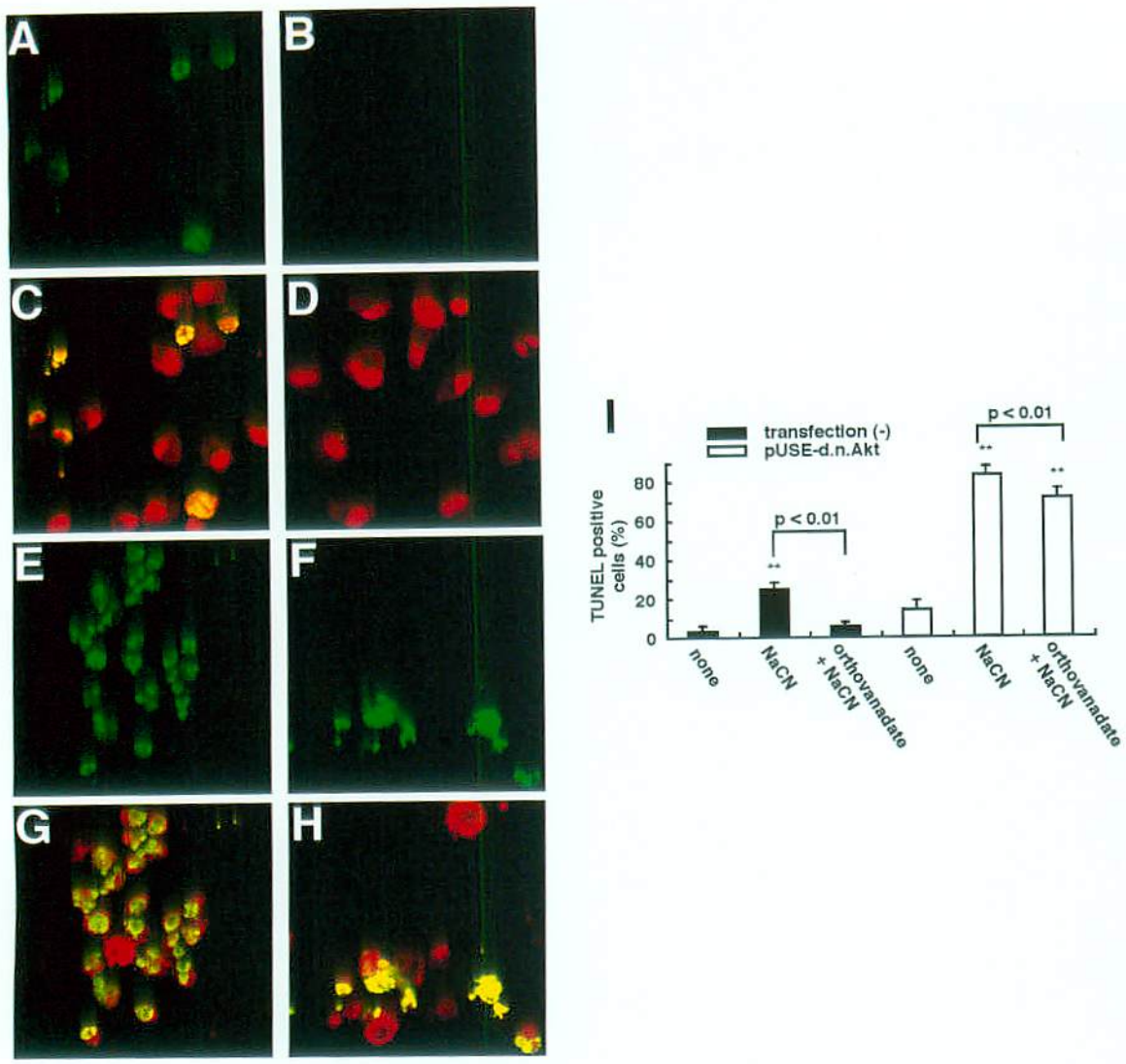

Figure 14. **Effects of expression of dominant negative Akt on orthovanadate-induced neuroprotection in NG108-15 cells. A, B, C and D;** NG108-15 cells were treated with 1 mM NaCN for 24h with (B and D) or without (A and C) pre-treatment of 50 μM orthovanadate for 30 min. After fixation with 4 % paraformaldehyde in PBS, all cells were stained with propidium iodide (*red* in C and D) and apoptotic cells were detected with TUNEL staining (*green* in A, B, C and D). **E, F, G and H;** NG108-15 cells were transfected with Myc-His tagged dominant negative Akt1 in pUSEamp(+) expression vector. Two days after transfection, cells were incubated with (F and H) or without (E and G) 50 μM orthovanadate for 30min and in turn treated with 1mM NaCN for 24h. Transfected cells were identified by immunofluorescence staining using anti-Myc Tag antibody (*red* in G and H). Apoptotic cells were identified with TUNEL staining (*green* in E, F, G and H). Quantitative analysis of TUNEL positive cells were performed. At least 600 cells were counted for each condition. Data are means ± SD (n = 4). Statistically significant differences versus none-treated cells are indicated by *asterisks* (**p< 0.01). Data were statistically significant ($p < 0.01$) between NaCN and orthovanadate + NaCN in both non-transfected cells and transfected cells.

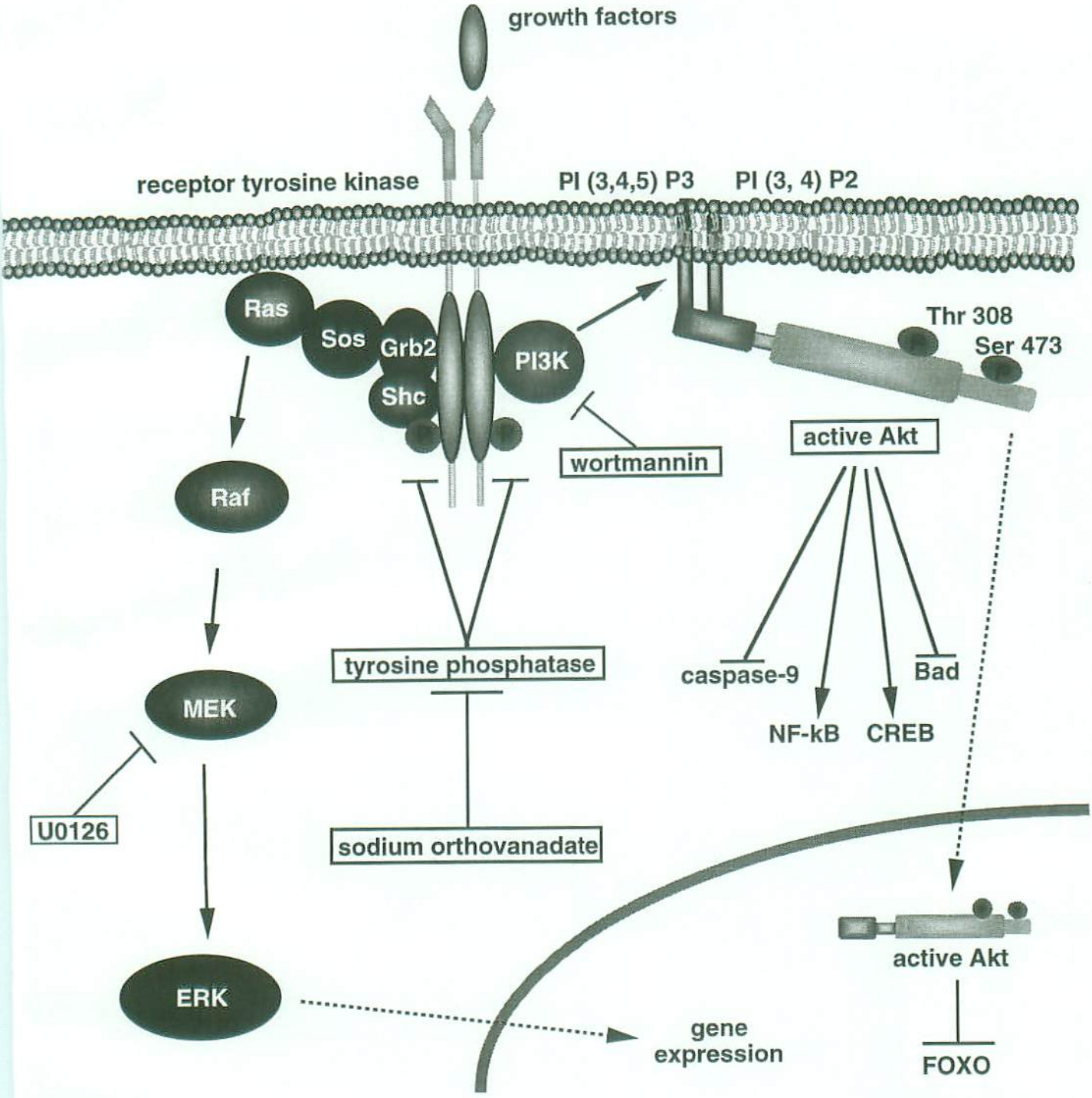

Figure 15. **Sodium orthovanadate rescue CA1 pyramidal neurons from delayed neuronal death after transient forebrain ischemia through PI3K-Akt and MAPK pathways**. Phosphorylation of receptor tyrosine kinase is important for activation of down stream substrates. Sodium orthovanadate is a potent tyrosine phosphatase inhibitor and induces the phosphorylation of receptor tyrosine kinase. PI3K-Akt pathway is activated and activated Akt phosphrylates several substrates to cell survive. Activated receptor tyrosine kinase also activates Ras-Raf-MEK-ERK cascade and may induce anti-apoptotic gene expressins.