Protein phosphatase-2A is activated in pig brain following cardiac arrest and resuscitation

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Abstract Protein phosphatase-2A (PP-2A) interacts with several regulators of cell death pathways and is therefore a potential component of signaling pathways linking global cerebral ischemia to cell death. Using a novel procedure to quantify PP-2A activity, we find that cardiac arrest with resuscitation and reperfusion leads to activation of PP-2A by 1.6-fold in pig brain extract and by 3.4-fold after partial purification of PP-2A. This is the first demonstration of PP-2A activation in a clinically relevant model of transient global cerebral ischemia. These results suggest that inhibition of PP-2A activity may be neuroprotective in global cerebral ischemia.

Keywords Apoptosis · Necrotic cell death · Inhibitor-1 · Inhibitor-2 · Okadaic acid · Tautomycin

Introduction

Global cerebral ischemia triggers a series of cellular reactions that culminate in reversible or permanent cell injury (Siesjo 1988). Irreversible cell injury in the form of apoptotic and necrotic cell death contributes to brain damage associated with the extremely poor neurological outcome following cardiac arrest (Farber *et al.* 1981;

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Siesjo 1988; Choi 1992; Martin *et al.* 1998; Lipton 1999; Giffard 2000; Love 2003). Cell injury occurs both during the ischemic period and during the post-ischemic reperfusion period after successful resuscitation and return of spontaneous circulation as a result of reperfusion injury (Grinyo 1997). The cell signaling pathways that link global cerebral ischemia to the induction of cell death are not fully characterized. Depletion of ATP, loss of Na^+ pump function and ischemic depolarization lead to release of vesicular and cytoplasmic glutamate which activates ionotropic NMDA and AMPA glutamate receptors (excitotoxicity). The resulting increase in intracellular Ca^{2+} is a triggering event that activates multiple cell signaling pathways involved in ischemia-induced cell death (Olney 1969; Rothman and Olney 1986; Siesjo 1988; Choi 1992; Goldberg and Choi 1993; Lipton and Rosenberg 1994; Siesjo *et al.* 1995; Lipton 1999), but the mechanisms linking high Ca^{2+} and other intracellular signals (e.g. reactive oxygen species) to cell death are unknown.

Cell death due to global cerebral ischemia is highly regulated by protein phosphorylation and dephosphorylation (Small and Bucharan 1996; Wieloch *et al.* 1996). While the role of protein kinases in the control of cell death and/or poor neurological outcome in brain ischemia is well documented (Aronowski *et al.* 1992; Churn *et al.* 1992; Hu and Wieloch 1995; Shamloo and Wieloch 1999; Blanck *et al.* 2000; Shamloo *et al.* 2000; Bright *et al.* 2004), comparatively little is known about the protein phosphatases involved. Protein phosphatases previously implicated in the control of apoptotic cell death include protein phosphatase-1, protein phosphatase-2A and protein phosphatase-2B (Klumpp and Krieglstein 2002; Garcia *et al.* 2003; Van Hoof and Goris 2003). Protein phosphatase-2A (PP-2A) is of particular interest because of its role in the induction of both apoptotic and necrotic (oncolytic) cell death (Kleinberger 2000; Chiang *et al.* 2002; Cicchillitti *et al.* 2003; Tamura *et al.* 2004; Janoo *et al.* 2005). Protein phosphatase-2A is a highly regulated hetero-oligomer that exists as multiple isoforms with varying distributions and substrate specificities (Cohen 1989; Jannssens and Goris 2001). We hypothesized that a form of PP-2A may be activated in global cerebral ischemia, and may link global cerebral ischemia to the control of apoptosis and necrotic cell death. We now present evidence that PP-2A is activated in pig brain following cardiac arrest with resuscitation and reperfusion.

Materials and methods

Materials

ATP, benzamidine, DEAE sepharose, poly-L-lysine agarose, phenylmethylsulfonyl fluoride (PMSF), protamine, phosphorylase b, PKA and bovine serum albumin were from Sigma (Saint Louis, MO). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was from GE Healthcare (Piscataway, NJ, USA).

Protein preparation

Bacterially expressed human inhibitor-1, human inhibitor-2 and human phosphorylase kinase γ were purified by chromatography on a Ni-agarose column as

recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). ^{32}P -labeled phosphorylase a was prepared by phosphorylation of phosphorylase b by phosphorylase kinase as described (Cohen *et al.* 1988). Inhibitor-1 was phosphorylated by PKA as described (Hemmings *et al.* 1984). Protein concentration was determined by the method of Bradford (Bradford 1976) using bovine serum albumin as standard.

Assay of protein phosphatase-2A

PP-2A was assayed for its ability to dephosphorylate ^{32}P -labeled phosphorylase a following activation by protamine. The assay consisted of 0.01 ml of PP-2A in 50 mM imidazole–Cl pH 7.2, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mg/ml bovine serum albumin (enzyme diluent buffer); 0.01 ml 50 mM imidazole–Cl pH 7.2, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mg/ml bovine serum albumin, 100 nM phospho- T^{35} -inhibitor-1 or 1 μM inhibitor-2 and 30 $\mu\text{g}/\text{ml}$ protamine sulfate (enzyme assay buffer); and 0.01 ml of ^{32}P -labeled phosphorylase a in enzyme diluent buffer plus 75 mM caffeine. PP-2A was preincubated with protamine in enzyme assay buffer for 5 min at 30°C prior to initiation of protein phosphatase assay with ^{32}P -labeled phosphorylase a. Reactions proceeded for 10 min at 30°C and were terminated with 0.2 ml of 25% (v/v) trichloroacetic acid. The resulting suspension was centrifuged at 10,000 $\times g$ for 5 min, and 0.2 ml of the supernatant which contained the released [^{32}P]-phosphate was analyzed in a liquid scintillation spectrophotometer by Cerenkov counting. One unit of enzyme activity catalyzes the dephosphorylation of 1 nmol of phosphate per minute at 30°C.

Ischemia and reperfusion

Animal experiments were approved by the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University. The pig model of transient global cerebral ischemia was performed as described in (Gedeborg *et al.* 2001; Liu *et al.* 2002) with some modifications. Female Yorkshire pigs (~25 kg) were sedated with tiletamine/zolazepam (4.4 mg/kg IM) and xylazine (2.2 mg/kg IM). Anesthesia was induced with pentobarbital (5 mg/kg bolus IV), the trachea intubated, and mechanical ventilation initiated with 21% oxygen. Anesthesia was maintained by intravenous infusion of pentobarbital (8 mg/kg per h) with 0.9% NaCl at 5 ml/kg per h. Temperature was maintained at 37 $\pm$ 0.5°C by a warming blanket. A femoral venous catheter and a femoral arterial catheter were placed for rapid drug infusion and arterial pressure monitoring. Median sternotomy was performed to expose the heart. Ventricular fibrillation was induced by rapid pacing (rate of 800, output of 16 mA) and was confirmed by electrocardiography and loss of systolic blood pressure (<20 mmHg). Ventilation was discontinued at the onset of fibrillation. After 10 min of cardiac arrest, animals were resuscitated by IV administration of lidocaine (1.5 mg/kg), epinephrine (500 μg), and sodium bicarbonate (6 mEq) with open chest cardiac massage at 60/min for 2 min, followed by internal defibrillation (20J–50J) and ventilation with 100% O₂. After defibrillation, systolic blood pressure was maintained at >60 mmHg with intermittent bolus of epinephrine. Following 2 h of reperfusion and ventilation with 100% O₂, animals were sacrificed with saturated

KCl (10 ml) IV, and the brain was removed quickly (<3 min) through a ventral approach by removing the mandible and extraction through the palate. Control animals were treated identically with a sham operation, except that a fatal dose of KCl was injected without ventricular fibrillation and resuscitation.

Partial purification of PP-2A

Brains (~80 g) from a control animal and an animal that underwent cardiac arrest/resuscitation on the same day were treated identically for each experiment. Each brain was cut into small pieces and homogenized in five volumes of 50 mM imidazole–Cl pH 7.3, 2 mM EDTA, 2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF and 5% (v/v) glycerol using 6×30 s pulses at low speed in a blender. The homogenate was centrifuged at 10,000×g for 30 min and the supernatant (the soluble extract) was collected, diluted twofold in 25 mM imidazole–Cl pH 7.3, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF and 10% (v/v) glycerol (buffer A), and loaded onto a DEAE sepharose column (2.5×20 cm) equilibrated in buffer A. The column was washed with 300 ml of buffer A plus 50 mM NaCl and then eluted with buffer A plus 300 mM NaCl. Active fractions of PP-2A were collected, diluted tenfold with buffer A, and loaded onto a poly-L-lysine column (1.5×10 cm) equilibrated in buffer A. The column was washed with 150 ml of buffer A and eluted successively with 150 ml of buffer A plus 50, 100, 250 or 500 mM NaCl. The active fractions from each elution were collected, concentrated by vacuum dialysis and stored at –20°C in buffer A plus 50% (v/v) for up to 3 months.

Statistics

Data were analyzed by the Student's *t* test; $p < 0.05$ was considered statistically significant (Motulsky 2003). Concentration–effect data were fitted to a sigmoidal curve to determine IC_{50} values using Graphpad Prism v. 4.02 (Graphpad, San Diego, CA, USA).

Results

Soluble pig brain extracts contain high levels of phosphorylase phosphatase activity due to PP-1 and PP-2A (Yang and Fung 1985; Tung and Reed 1989; Tung 1997). PP-2A activity can be determined in the presence of a specific inhibitor of PP-1 (phospho-Thr³⁵-inhibitor-1 or inhibitor-2) and protamine sulfate which activates PP-2A but inhibits PP-1 (Fig. 1) (Ingebritsen and Cohen 1983; Ingebritsen *et al.* 1983; Tung *et al.* 1985). To investigate the role of PP-2A in transient global cerebral ischemia, the pig model of brain ischemia following cardiac arrest and resuscitation was used (Gedeborg *et al.* 2001; Liu *et al.* 2002). Whole brain PP-2A was activated by 1.6-fold following cardiac arrest with resuscitation and reperfusion compared to control (Fig. 2). More accurate measurement of PP-2A in brain extracts was obtained by partial purification of PP-2A using DEAE Sepharose and poly-L-lysine agarose chromatography (Fig. 3; Tung *et al.* 1985; Tung *et al.* 1997). The major peak of PP-

Fig. 1 Measurement of PP-2A activity in pig brain. Soluble brain extract was prepared as described in [Materials and methods](#). Phosphorylase phosphatase activity was determined in the absence or presence of 100 nM phospho-Thr³⁵-inhibitor-1 or 1 μ M inhibitor-2 plus 10 μ g/ml protamine sulfate. Both specific PP-1 inhibitors produced similar inhibition. Phosphorylase phosphatase activity measured in the presence of 100 nM phospho-Thr³⁵-inhibitor-1 or 1 μ M inhibitor-2 and protamine is defined as PP-2A activity. Mean $\pm$ SEM ($n=3$)

2A activity eluted at 500 mM NaCl after chromatography on poly-L-lysine agarose (Fig. 3b). Total PP-2A activity, quantified as the area under the peak, increased by 3.4-fold in ischemic following cardiac arrest and resuscitation with reperfusion compared to control (Fig. 3c).

Partially purified PP-2A from control and ischemic pig brain was not inhibited by phospho-inhibitor-1 or inhibitor-2, but was completely inhibited by okadaic acid ($IC_{50}=2.1$ and 5.4 nM, respectively) or tautomycin (0.33 and 0.86 nM, respectively),

Fig. 2 Activation of brain PP-2A by cardiac arrest and resuscitation. The activity of PP-2A in soluble brain extract was determined in control pigs and in pigs subjected to 10 min of cardiac arrest followed by resuscitation and reperfusion for 2 h. PP-2A activity was quantified as described in Fig. 1. Mean $\pm$ SEM ($n=3$). * $p<0.05$ vs control (Student's t test)

Fig. 3 Quantification of PP-2A activity from control and ischemic brain following partial purification. Soluble brain extracts from a control (non-ischemic) pig and a pig subjected to 10 min of cardiac arrest followed by resuscitation and reperfusion for 2 h were prepared as described in [Materials and methods](#). **a** Extracts were loaded onto a DEAE sepharose column (2.5×20 cm) which was washed and eluted at a flow rate of 60 ml/h with collection of 7 ml fractions. PP-2A activity from control (*square*) and ischemic (*circle*) brain was determined as described in experimental procedures and Fig. 1. Data are shown for a single representative experiment ($n=3$). **b** Active fractions from the DEAE column were pooled as indicated and loaded onto a poly-L-lysine agarose column which was washed and eluted at a flow rate of 30 ml/h with collection of 4.5 ml fractions. PP-2A activity from control (*square*) and ischemic (*circle*) brain was determined. Data are shown for a single representative experiment ($n=3$). **c** Total PP-2A activity from the poly-L-lysine agarose chromatography step was quantified as the area under the peak of activity. Mean $\pm$ SEM ($n=3$). * $p<0.05$ vs control (Student's t test)

confirming them as type 2A protein phosphatases (Fig. 4; Ingebritsen and Cohen 1983; Ingebritsen *et al.* 1983; Cohen *et al.* 1990; Hori *et al.* 1991).

Discussion

Cardiac arrest followed by resuscitation and reperfusion increased the activity of a form of brain PP-2A. This represents the first demonstration of PP-2A activation by transient global cerebral ischemia in a clinically relevant animal model of cardiac arrest and resuscitation with reperfusion. PP-2A interacts with and dephosphorylates several proteins that are dephosphorylated upon initiation of apoptosis, including Bcl2, Bad and Rb (Chiang *et al.* 2002; Cicchillitti *et al.* 2003; Tamura *et al.* 2004). PP-2A is also involved in the induction of necrotic (oncolytic) cell death in cell culture (Janoo *et al.* 2005). These findings suggest that a form of PP-2A may be an important component of the cell signaling pathway(s) that link ischemia to the control of cell death in transient global cerebral ischemia.

Protein phosphatase activation has been previously inferred in *in vitro* models of ischemia by the use of nonspecific inhibitors that target both PP-1 and PP-2A (Yung and Tolkovsky 2003). Using careful biochemical analysis, we were able to demonstrate directly the activation of PP-2A in transient global cerebral ischemia *in vivo*. This result is at variance with that published by Martin de la Vega *et al.* (2001, 2002) which used a rat model of transient global cerebral ischemia to demonstrate that PP-2A is inhibited in brain. The conflicting results may be due to

Fig. 4 Sensitivity of PP-2A from control and ischemic pig brain to various protein phosphatase inhibitors. PP-2A from control (a) and ischemic pig brain (b) obtained after partial purification by chromatography on DEAE Sepharose and poly-L-lysine agarose was assayed with various concentrations of phospho-Thr³⁵-inhibitor-1 (circle) inhibitor-2 (square), okadaic acid (star), and tautomycin (diamond). One hundred percent activity is equivalent to 0.005 U

the different methods used to achieve transient global cerebral ischemia and also to improper assay of PP-2A. There are several forms of PP-2A in brain. Activation of one form of PP-2A may imply depletion of another. The form of PP-2A that becomes activated in brain following cardiac arrest and resuscitation is currently being purified to near homogeneity. The mechanism of the pathophysiological activation of PP-2A in global cerebral ischemia and other diseases is unknown, as are the mechanisms that underlie its physiological activation. Viral proteins, including adenovirus E4orf4 protein and HIV-1 Vpr-derived C-terminal peptide, interact with and activate protein phosphatase-2A₀ and/or protein phosphatase-2A₁ (Shtrichman *et al.* 1999; Janoo *et al.* 2005). An intriguing hypothesis is that cellular homologues of these viral activators mediate PP-2A activation *in vivo*.

Cell death as a result of global cerebral ischemia is thought to be mediated in part by glutamate-induced elevation of intracellular Ca²⁺ which activates Ca²⁺-dependent protein kinases, protein phosphatases and proteases (Wieloch *et al.* 1996). At least two Ca²⁺-dependent protein kinases, Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C δ have been implicated as downstream components of Ca²⁺ cytotoxicity in global cerebral ischemia (Hu and Wieloch 1995; Bright *et al.* 2004) but their roles in activating cell death pathways and/or PP-2A are not known. This demonstration of a greater than threefold activation of PP-2A in global cerebral ischemia that is maintained through multiple purification steps suggests a covalent modification (e.g. phosphorylation) and/or stable molecular interaction (e.g. recruitment of an activating factor or subunit). Further characterization of the form of PP-2A activated in global cerebral ischemia should help clarify the mechanism(s) involved.

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References

- Aronowski J, Grotto JC, Waxhan MN (1992) Ischemia-induced translocation of Ca²⁺/calmodulin-dependent protein kinase II: potential role in neuronal damage. *J Neurochem* 58:1743–1753
- Blanck TJ, Halle M, Xu F, Heerdt P, Beckmann J, Kang R, Adamo A, Hemmings Jr HC (2000) Isoflurane pretreatment ameliorates postischemic neurological dysfunction and preserves hippocampal Ca²⁺/calmodulin-dependent protein kinase II in a canine cardiac arrest model. *Anesthesiology* 93:1285–1283
- Bradford AA (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 76:248–254
- Bright R, Raval AP, Dembner JM, Perez-Pinzon MA, Steinberg GK, Yenari MA, Mochly-Rosen D (2004) Protein kinase C δ mediates cerebral reperfusion injury *in vivo*. *J Neurosci* 24:6880–6888
- Chiang CW, Harris G, Ellig C, Masters SC, Subramanian R, Shenolikar S, Wadzinski BE, Yang E (2002) Protein phosphatase-2A activates proapoptotic function of BAD in interleukin-3-dependent lymphoid cells by a mechanism requiring 14–3–3 dissociation. *Blood* 2001(97):1289–1297
- Choi DW (1992) Excitotoxic cell death. *J Neurobiol* 23:1261–1276
- Churn SB, Taft WC, Billingsley MS, Sankaran B, DeLorenzo RJ (1992) Global forebrain ischemia induces a posttranslational modification of multifunctional calcium- and calmodulin-dependent kinase II. *J Neurochem* 59:1221–1232
- Cicchillitti L, Fasanaro P, Biglioli P, Capogrossi MC, Martelli F (2003) Oxidative stress induces protein phosphatase-2A-dependent dephosphorylation of the pocket proteins pRb, p107, and p130. *J Biol Chem* 278:19509–19517

- Cohen P (1989) The structure and regulation of protein phosphatases. *Annu Rev Biochem* 58:453–508
- Cohen P, Alemany S, Hemmings BA, Resink TJ, Stralfors P, Tung HYL (1988) Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. *Methods Enzymol* 159:390–409
- Cohen P, Holmes CF, Tsukitani Y (1990) Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem Sci* 15:98–102
- Farber JL, Chien KR, Mittenacht Jr S (1981) The pathogenesis of irreversible cell injury in ischemia. *Am J Pathol* 102:271–281
- Garcia A, Cayla X, Guernon J, Dassaue F, Hospital V, Rebollo MP, Fleisher A, Rebollo A (2003) Serine/threonine protein phosphatases PP1 and PP2A are key players in apoptosis. *Biochimie* 85:721–726
- Gedeborg R, Silander HC, Rubertsson S, Wiklund L (2001) Cerebral ischaemia in experimental cardiopulmonary resuscitation -comparison of epinephrine and aortic occlusion. *Resuscitation* 50:319–329
- Giffard RG (2000) Cell apoptosis in cerebral pathophysiology. *Eur J Anaesthesiol* 17:9–10
- Goldberg MP, Choi DW (1993) Combined oxygen and glucose deprivation in cortical cell culture. *J Neurosci* 13:3510–3524
- Grinyo JM (1997) Reperfusion injury. *Transplant Proc* 29:622–632
- Hemmings Jr HC, Williams KR, Konigsberg WH, Greengard P (1984) DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated neuronal phosphoprotein. I. Amino acid sequence around the phosphorylated threonine. *J Biol Chem* 259:14486–14490
- Hori M, Magae J, Han Y-G, Hartshorne DJ, Haraki H (1991) A novel protein phosphatase inhibitor, tautomycin. *FEBS Lett* 285:145–148
- Hu B-R, Wieloch T (1995) Persistent translocation of Ca^{2+} /calmodulin dependent protein kinase II to synaptic junctions in the vulnerable hippocampal CA1 region following transient ischemia. *J Neurochem* 64:277–284
- Ingebritsen TS, Cohen P (1983) The protein phosphatases involved in cellular regulation. 1. Classification and substrate specificities. *Eur J Biochem* 132:255–261
- Ingebritsen TS, Stewart AA, Cohen P (1983) The protein phosphatases involved in cellular regulation. 6. Measurement of type-1 and type-2 protein phosphatases in extracts of mammalian tissues; an assessment of their physiological roles. *Eur J Biochem* 132:297–307
- Jannsens V, Goris J (2001) protein phosphatase-2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signaling. *Biochem J* 353:417–439
- Janoo A, Morrow PW, Tung HYL (2005) Activation of protein phosphatase-2A1 by HIV-1 Vpr cell death causing peptide in intact CD4+ T cells and in vitro. *J Cell Biochem* 94:816–825
- Kleinberger T (2000) Induction of apoptosis by adenovirus E4orf4 protein. *Apoptosis* 5:211–215
- Klump S, Krieglstein J (2002) Serine/threonine protein phosphatases in apoptosis. *Curr Opin Pharmacol* 2:458–462
- Lipton P (1999) Ischemic cell death in brain neurons. *Physiol Rev* 79:1431–1568
- Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med* 330:613–622
- Liu X, Nozari A, Basu S, Ronquist G, Rubertsson S, Wiklund L (2002) Neurological outcome after experimental cardiopulmonary resuscitation: a result of delayed and potentially treatable neuronal injury. *Acta Anaesthesiol Scand* 46:537–546
- Love S (2003) Apoptosis and brain ischemia. *Prog Neuro-Psychopharmacol Biol Psychiatry* 27:267–282
- Martin de la Vega C, Burda J, Salinas M (2001) Ischemia-induced inhibition of the initiation factor 2a phosphatase activity in the rat brain. *NeuroReport* 12:1021–1025
- Martin de la Vega C, Burda J, Lobo MVT, Salinas M (2002) Cerebral postischemic reperfusion-induced demethylation of the protein phosphatase-2A catalytic subunit. *J Neurosci Res* 69:540–549
- Martin LJ, Al-Abdulla NA, Brambrink AM, Kirsch JR, Sieber FE, Portera-Cailliau C (1998) Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: a perspective on contributions of apoptosis and necrosis. *Brain Res Bull* 46:281–309
- Motulsky HJ (2003) Prism 4 statistics guide—statistical analyses for laboratory and clinical researchers. Graphpad Software, Inc. San Diego, CA
- Olney JW (1969) Brain lesion, obesity and other disturbances in mice treated with monosodium glutamate. *Science* 164:719–721
- Rothman SM, Olney JW (1986) Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Ann Neurol* 19:105–111
- Shamloo M, Wieloch T (1999) Changes in protein tyrosine phosphorylation in the rat brain after cerebral ischemia in a model of ischemic tolerance. *J Cerebral Blood Flow Metabol* 19:173–183

- Shamloo M, Kamme F, Wieloch T (2000) Subcellular distribution and autophosphorylation of calcium/calmodulin-dependent protein kinase II- α in rat hippocampus in a model of ischemic tolerance. *Neuroscience* 96:665–674
- Shtrichman R, Sharf R, Barr H, Dobner T, Kleinberger T (1999) Induction of apoptosis by adenovirus E4orf4 protein is specific in transformed cells and requires an interaction with protein phosphatase-2A. *Proc Natl Acad Sci* 96:10080–10085
- Siesjö BK (1988) Mechanisms of ischemic brain damage. *Crit Care Med* 16:954–963
- Siesjö BK, Zhao Q, Pahlmark K, Siesjö P, Katsura K, Folbergrova J (1995) Glutamate, calcium and free radicals as mediators of ischemic brain damage. *Ann Thorac Surg* 59:1316–1320
- Small DL, Buchanan AM (1996) Mechanisms of cerebral ischemia: intracellular cascades and therapeutic interventions. *J Cardiothorac Vasc Anesth* 10:139–146
- Tamura Y, Smizu S, Osada H (2004) The phosphorylation status and anti-apoptotic activity of Bcl-2 are regulated by ERK and protein phosphatase-2A on the mitochondria. *FEBS Lett* 569:249–255
- Tung HYL, Reed LJ (1989) Purification and characterization of protein phosphatase-II activating kinase from bovine brain cytosolic and particulate fractions. *J Biol Chem* 264:2985–2990
- Tung HYL, Alemany S, Cohen P (1985) The protein phosphatases involved in cellular regulation. 2. Purification, subunit structure and properties of protein phosphatase-2A0, 2A1 and 2A2 from rabbit skeletal muscle. *Eur J Biochem* 148:253–263
- Tung HYL, De Rocquigny H, Zhao L-J, Cayla X, Roques BP, Ozon R (1997) Direct activation of protein phosphatase-2A0 by HIV-1 encoded protein complex NCp7:Vpr. *FEBS Lett* 401:197–201
- Van Hoof C, Goris J (2003) Phosphatases in apoptosis: to be or not to be, PP2A is in the heart of the question. *Biochim Biophys Acta* 1640:97–104
- Wieloch T, Hu BR, Boris-Moller A, Cardell M, Kamme F, Kurihara J, Sakata K (1996) Intracellular signal transduction in the postischemic brain. *Adv Neurol* 71:371–387
- Yang SD, Fung Y-L (1985) Identification and characterization of an ATP.Mg-dependent protein phosphatase from pig brain. *J Biol Chem* 260:13464–13470
- Yung HW, Tolkovsky AM (2003) Erasure of kinase phosphorylation in astrocytes during oxygen-glucose deprivation is controlled by ATP levels and activation of protein phosphatases. *J Neurochem* 86:1281–1288