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Activation of brain protein phosphatase-1_I following cardiac arrest and resuscitation involving an interaction with 14-3-3γ

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Abstract

The intracellular signaling mechanisms that couple transient cerebral ischemia to cell death and neuroprotective mechanisms provide potential therapeutic targets for cardiac arrest. Protein phosphatase (PP)-1 is a major serine/threonine phosphatase that interacts with and dephosphorylates critical regulators of energy metabolism, ionic balance, and apoptosis. We report here that PP-1_I, a major regulated form of PP-1, is activated in brain by approximately twofold *in vivo* following cardiac arrest and resuscitation in a clinically relevant pig model of transient global cerebral ischemia and reperfusion. PP-1_I purified to near homogeneity from either control or ischemic pig brain consisted of the PP-1 catalytic subunit, the inhibitor-2 regulatory subunit, as well as the novel constituents 14-3-3γ, Rab GDP dissociation protein β, PFTAIRE kinase, and C-TAK1 kinase. PP-1_I purified from ischemic brain contained significantly less 14-3-3γ than PP-1_I purified from control brain, and purified 14-3-3γ directly inhibited the catalytic subunit of PP-1 and reconstituted PP-1_I. These findings suggest that activation of brain PP-1_I following global cerebral ischemia *in vivo* involves dissociation of 14-3-3γ, a novel inhibitory modulator of PP-1_I. This identifies modulation of PP-1_I by 14-3-3 in global cerebral ischemia as a potential signaling mechanism-based approach to neuroprotection.

Keywords

apoptosis; inhibitor-2; protein phosphorylation

Energy failure in global cerebral ischemia following cardiac arrest initiates massive ischemic cell injury and cell death (Siesjo 1988; Hou and MacManus 2002). Global cerebral ischemia induces irreversible cell death that results in severe brain damage and extremely poor neurological outcome following cardiac arrest that can be mitigated by prompt restoration of cerebral blood flow (Siesjo 1988; Siesjo *et al.* 1995; Martin *et al.* 1998; Lipton 1999; Liu *et al.* 2002; Bhardwaj *et al.* 2003). Ischemic cell damage continues into the post-ischemic reperfusion period following successful resuscitation as a result of reperfusion injury (Cao *et al.* 1988; Oliver *et al.* 1990; White *et al.* 2000). The cell signaling mechanisms that link cerebral ischemia to excitotoxicity, ionic dysregulation, cell death, and ischemic tolerance, an endogenous neuroprotective mechanism, are complex and not fully characterized. Energy depletion and membrane depolarization cause release of excitotoxic glutamate, activation of ionotropic NMDA- and α-amino-3-hydroxy-5-methylisoxazole-4-propionate-type glutamate receptors, and pathological Ca^{2+} influx that activates multiple

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cell signaling pathways involved in ischemic cell death (Siesjo 1988; Siesjo *et al.* 1995; Kristian and Siesjo 1998; Lipton 1999; Hou and MacManus 2002; Sugawara *et al.* 2004). The generation of reactive oxygen and nitrogen species upon reperfusion produces oxidative stress that is fundamental to reperfusion injury (Kontos 1985; Cao *et al.* 1988; Siesjo *et al.* 1989; Siesjo *et al.* 1995; Chan 2001; Oliver *et al.* 1990). The molecular mechanisms linking elevated Ca^{2+}, reactive oxygen and nitrogen species and other signaling pathways to cell death and survival remain to be delineated.

Cell death in cerebral ischemia occurs by both necrotic and apoptotic mechanisms that are highly regulated by protein phosphorylation mechanisms (Wieloch *et al.* 1996). Protein phosphorylation is also involved in endogenous neuroprotective mechanisms such as ischemic pre-conditioning (Dirnagl *et al.* 2003). While considerable evidence links protein kinases to the control of ischemic cell death (Aronowski *et al.* 1992; Hu and Wieloch 1995; Blanck *et al.* 2000; Bright *et al.* 2004), relatively little is known about the role of protein phosphatases (PPs). PP-1, PP-2A, and PP-2B have all been implicated in the biochemical regulation of apoptosis (Klumpp and Krieglstein 2002; Garcia *et al.* 2003; Van Hoof and Goris 2003). The catalytic subunit of PP-1 (PP-1_{cat}) binds and/or dephosphorylates multiple proteins involved in apoptosis, including Bad, Bcl-2, and Rb (Ayllon *et al.* 2001; Wang *et al.* 2001), and in excitotoxicity, including α-amino-3-hydroxy-5-methylisoxazole-4-propionate- and NMDA-type glutamate receptors (Greengard 2001). Based on these key connections, we hypothesized that activation of PP-1 might be a component of the cell signaling pathways that link cerebral ischemia to cell death. PP-1_I, which consists of PP-1_{cat}, inhibitor-2 (I-2), and a regulatory protein kinase, is a major form of PP-1 in brain that requires phosphorylation of T72 of the regulatory subunit I-2 for activation of catalytic activity (Yang and Fung 1985; Tung and Reed 1989; Agarwal-Mawal and Paudel 2001). Here, we show that brain PP-1_I is activated following cardiac arrest in a clinically relevant pig model of cardiac arrest and resuscitation *in vivo*. The mechanism by which PP-1_I is activated appears to involve reduced association with a novel inhibitory PP-1 regulatory protein identified as 14-3-3γ.

Experimental procedures

Materials

ATP, benzamidine, DEAE Sepharose, poly-L-lysine agarose, phenylmethylsulfonyl fluoride (PMSF), protamine, phosphorylase *b*, phosphorylase kinase, catalytic subunit of cAMP-dependent protein kinase (PKA), bovine serum albumin and pentobarbital were from Sigma-Aldrich (St Louis, MO, USA). Tiletamine and zolazepam were from Reading (Carros, France). Xylazine was from Bayer AG (Leverkuser-Bayerwerk, Germany). GE Healthcare (Piscataway, NJ, USA) provided [γ-^{32}P]ATP. Anti-PP-1_{cat}, anti-I-2, and anti-14-3-3γ were from Chemicon (Temecula, CA, USA).

Protein preparation

Full-length human dopamine- and cyclic AMP-regulated neuronal phosphoprotein Mr 32 kDa (DARPP-32), PP-1 catalytic subunit α (PP-$1\alpha_{cat}$), I-2, Bad and phosphorylase kinase γ were expressed in bacteria using the pTrcHis-Topo vector and purified by chromatography on Ni-agarose 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). DARPP-32 was phosphorylated by PKA as described (Hemmings *et al.* 1984b). ^{32}P-labeled Bad was prepared by phosphorylation with PKA as described (Lizcano *et al.* 2000). PP-1_I devoid of activating kinase was reconstituted by incubating purified recombinant PP-$1\alpha_{cat}$ (300 μg) and I-2 (200 μg) in 50 mM imidazole-Cl pH 7.2, 0.2 mM EGTA and 0.1% (v/v) 2-mercaptothanol at

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30°C for 30 min. Reconstituted PP-1_I was then purified by gel filtration on Superdex 200 as described (Tung and Cohen 1984).

Cardiac arrest model

Experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals as approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College. Female Yorkshire pigs (~25 kg) were sedated with tiletamine (2.2 mg/kg i.m.), zolazepam (2.2 mg/kg i.m.), and xylazine (2.2 mg/kg i.m.), and transported to the laboratory. An intravenous catheter was placed, anesthesia was induced with pentobarbital (5 mg/kg i.v.), the trachea intubated, and mechanical ventilation initiated with room air. Anesthesia was maintained throughout the procedure by intravenous infusion of pentobarbital (8 mg/kg/h) in 0.9% NaCl at 5 mL/kg/h. Temperature was maintained at 37 ± 0.5°C by a warming blanket. Following insertion of femoral venous and arterial catheters for rapid drug administration and blood pressure monitoring, respectively, a median sternotomy was performed to expose the heart. Ventricular fibrillation was induced by rapid pacing (rate of 800, output of 16 mA) and confirmed by visual inspection, electrocardiography, and loss of pulsatile arterial pressure. Ventilation was discontinued at the onset of fibrillation. After 10 min of cardiac arrest, ventilation with 100% O_2 was initiated and spontaneous cardiac function was restored by intravenous administration of lidocaine (1.5 mg/kg), epinephrine (0.5 mg), and sodium bicarbonate (10 mEq) with open chest cardiac massage at 60/min for 2 min, followed by internal defibrillation (20–50 J). After defibrillation and resumption of effective circulation, systolic blood pressure was maintained > 60 mmHg by intermittent injection of 50–100 μg epinephrine as required. Following 2 h of reperfusion and ventilation with 100% O_2, animals were killed by intracardiac injection of 10 mL saturated KCl, and the brain was quickly removed (< 3 min) through a ventral approach through the palate and chilled on ice. Control animals were treated identically except that a fatal dose of KCl was injected without ventricular fibrillation and resuscitation.

Preparation of brain extracts

The rostral brain (~100 g; including cerebral cortex, hippocampus, basal ganglia, thalamus, and hypothalamus) from a control animal and an experimental animal that underwent cardiac arrest/resuscitation on the same day were treated identically for each experiment. Each brain was homogenized in five volumes of ice-cold 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 at 4°C. The supernatant (soluble extract) was collected, analyzed for PP-1_I activity, and used for purification of PP-1_I (see below).

Assay of PP-1_I

Brain PP-1_I was assayed for its ability to dephosphorylate added ^{32}P-labeled phosphorylase *a* following pre-incubation with ATP and Mg^{2+} to allow activation by endogenous protein kinase activity. PP-1_I is the only known Mg^{2+}/ATP-dependent phosphorylase a phosphatase (Cohen *et al.* 1988). The assay consisted of 0.01 mL of brain extract or partially purified PP-1_I in Dilution Buffer (50 mM imidazole–Cl pH 7.3, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, and 1 mg/mL bovine serum albumin); 0.01 mL of Assay Buffer (50 mM imidazole–Cl pH 7.2, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mg/mL bovine serum albumin, ±0.375 mM ATP/3.75 mM $MgCl_2$, ±300 nM phospho-T34-DARPP-32 (a specific inhibitor of PP-1; Hemmings *et al.* 1984a,b); and 0.01 mL of 30 μM ^{32}P-labeled phosphorylase a in Dilution Buffer plus 75 mM caffeine. PP-1_I was pre-incubated with Mg^{2+}/ATP in Assay Buffer for 5 min at 30°C prior to initiation of the reaction with ^{32}P-

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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 *g* for 5 min in a microcentrifuge, and 0.2 mL of the supernatant containing released [^{32}P]phosphate was analyzed for Cerenkov radiation in a liquid scintillation spectrometer. One unit of PP-1_I catalyzes the dephosphorylation of 1 nmol of phosphate per min at 30°C in aMg^{2+}/ATP-dependent manner. Basal PP-1 activity was determined as above with the omission of Mg^{2+}/ATP.

Purification of PP-1_I from control and ischemic brain following cardiac arrest and resuscitation

Purification procedures were performed at 4°C. Soluble brain extract from each forebrain (100 g) 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 eluted with Buffer A plus 300 mM NaCl (flow rate 60 mL/h; 7 mL fractions). Active fractions of PP-1_I were collected, diluted 10-fold 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 Buffer A plus 50 mM NaCl, 100 mM NaCl, 250 mM NaCl, and 500 mM NaCl (flow rate 30 mL/h; 4.5 mL fractions). PP-1_I from the 250 mM NaCl fraction was collected and PP-1_I activity was determined. Total PP-1_I activity of the partially purified enzyme from the poly-L-lysine agarose chromatography step was quantified as the area under the peak of activity (units). The pooled fractions were concentrated by vacuum dialysis, and separated on a Superdex 200 column (1.5 × 60 cm) equilibrated in Buffer A plus 200 mM NaCl. Active fractions from the Superdex 200 column were collected and purified on a Mono Q column (0.5 × 10 cm) using a gradient of NaCl in Buffer A as recommended by the manufacturer (GE Healthcare). Active fractions of PP-1_I from the Mono Q column were collected, concentrated by vacuum dialysis, and stored at –20°C in Buffer A with 50% (v/v) glycerol.

Identification of proteins by mass spectrometry

The PP-1_I complex was separated on sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (Laemmli 1970), and protein bands identified by Sypro Ruby staining (Molecular Probes, Portland, OR, USA) were excised and washed three times in 50 mM NH_4HCO_3 (pH 8.8) in 50% (v/v) acetonitrile. Gel slices were incubated at 30°C overnight with 1 μg of trypsin in 0.5 mL 50 mM NH_4HCO_3 and 0.05% (v/v) Zwittergent-3–16 (Sigma-Aldrich). Released tryptic peptides were dried in a rotary evaporator and reconstituted in 60% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid. Matrix-assisted laser desorption time of flight mass spectrometry spectra were obtained on a Voyager RP instrument (Framington, MA, USA) at The Rockefeller University Protein Core Facility (New York, NY, USA) averaged over 100–300 laser shots. The identities of peptides were determined by searching a human protein sequence database (Henzel *et al.* 1993; Shevchenko *et al.* 1996).

Miscellaneous methods

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard. Immunoblotting was performed as described (Burnette 1981) except that immunoreactivity was detected with alkaline phosphatase conjugated anti-mouse or anti-rabbit secondary antibody using 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium for colorimetric detection according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Gels and immunoblots were scanned on a flat bed gel scanner

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(PhosphorImager; Molecular Dynamics, Sunnyvale, CA, USA) and analyzed using NIH Image (http://rsb.info.nih.gov/nih-image/).

Statistical analysis

Differences between groups were determined by the Student *t*-test, with $p < 0.05$ accepted as statistically significant.

Results

PP-1_I activity in pig brain

Soluble extracts of pig brain contain large amounts of spontaneously active PP activity because of PP-1 and PP-2A using phosphorylase *a* as a representative phosphoserine substrate (Tung and Reed 1989; Tung *et al.* 1997). PP-1_I, also known as Mg^{2+}/ATP-dependent PP-1, is a complex of the PP-1_{cat} with the I-2 regulatory subunit (Hemmings *et al.* 1982; Tung and Reed 1989). It is activated via phosphorylation of T72 of I-2 by multiple protein kinases *in vitro*, although the physiologically relevant kinase(s) *in vivo* is unknown (Holmes *et al.* 1987; Wang *et al.* 1995; Agarwal-Mawal and Paudel 2001; Leach *et al.* 2003). The contribution of PP-1_I to total brain phosphorylase phosphatase activity was determined as the Mg^{2+}/ATP-dependent PP-1 activity using a maximally effective concentration of phospho-T34-DARPP-32, a specific inhibitor of PP-1 to define PP-1 activity (Hemmings *et al.* 1984a). PP-1_I accounted for 22% of total soluble brain phosphorylase phosphatase activity (PP-1 + PP-2A) and 31% of the total PP-1 activity in control pig brain extract (Fig. 1).

PP-1_I activation by global cerebral ischemia

The activity of PP-1_I was determined in control pig brain and brain from pigs subjected to 10 min of cardiac arrest followed by resuscitation and 2 h of reperfusion. Brain PP-1_I was activated 1.6-fold following ischemia/reperfusion compared with control in crude extracts (Fig. 2). There was no activation of spontaneously active PP-1 (data not shown). Indirect determination of PP-1_I activity in crude extracts can be inaccurate as Mg^{2+}/ATP can inhibit free PP-1 and PP-2A (Ingebritsen and Cohen 1983). Measurement of PP-1_I activity in the absence of free PP-1 and PP-2A was achieved by partial purification using DEAE Sepharose and poly-L-lysine agarose chromatography to separate interfering phosphatases and inhibitors (Yang and Fung 1985; Tung and Reed 1989; Tung *et al.* 1997). Total PP-1_I activity, quantified as the area under the peak of Mg^{2+}/ATP-dependent PP-1 activity eluted from poly-L-lysine agarose, was 2.2-fold greater in brain subjected to cardiac arrest and resuscitation compared with control brain (Fig. 3).

Purification and properties of PP-1_I from control and ischemic brain

Protein phosphatase-1_I was purified to near homogeneity from control and ischemic brain by successive chromatography of soluble extracts on DEAE Sepharose, poly-L-lysine agarose, Superdex 200 and Mono Q. Analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis showed that PP-1_I from control and ischemic brain contained six major proteins with apparent molecular masses 81, 53, 50, 37, 32, and 31 kDa (Fig. 4). These proteins were identified by matrix-assisted laser desorption time of flight mass spectrometry as C-TAK1 kinase (81 kDa), PFTAIRE kinase (53 kDa), Rab GDP dissociation inhibitor protein β (50 kDa), PP-$1\alpha_{cat}$ (37 kDa), 14-3-3γ (32 kDa), and PP-1 I-2 (31 kDa) (Fig. 4b). All three isoforms of PP-1_{cat} (α, β, and γ) were identified in purified PP-1_I by immunoblotting with isoform-specific antibodies (data not shown). Purified PP-1_I from control and ischemic brain eluted with an apparent molecular mass of 140 and 145 kDa, respectively, by gel filtration (Fig. 5). Taken together with the mass spectrometry data, the

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results suggest that the purified preparations of $PP\text{-}1_I$ contain several complexes. Complexes in the purified $PP\text{-}1_I$ preparation consistent with the observed molecular mass data include $PP\text{-}1_{cat}$: I-2 : 14-3-3γ : PFTAIRE kinase (~140 kDa); $PP\text{-}1_{cat}$: I-2 : C-TAK1 (~144 kDa); and $PP\text{-}1_{cat}$: I-2 : 14-3-3γ (~95 kDa), although other combinations are possible.

Confirmation of a $PP\text{-}1_I$: I-2 : 14-3-3γ complex by co-immunoprecipitation

The presence of $PP\text{-}1\alpha_{cat}$, I-2, and 14-3-3γ in $PP\text{-}1_I$ purified from control brain was confirmed by immunoblotting with specific antibodies (Fig. 6a). Immunoprecipitation of purified $PP\text{-}1_I$ with anti-$PP\text{-}1\alpha_{cat}$ followed by immunoblotting with anti-14-3-3γ and anti-I-2, or immunoprecipitation with anti-14-3-3γ followed by immunoblotting with anti-$PP\text{-}1\alpha_{cat}$ and anti-I-2 confirmed that 14-3-3γ interacts with these proteins within $PP\text{-}1_I$ (Fig. 6b and c). Various 14-3-3 isoforms can bind $PP\text{-}1_{cat}$ (Margolis *et al.* 2003; Huang *et al.* 2004; Pozuelo Rubio *et al.* 2004), and inhibit PP activity in cell extracts or purified $PP\text{-}1_{cat}$ *in vitro* (Chen and Wagner 1994; Muslin *et al.* 1996; Banik *et al.* 1997; Margolis *et al.* 2003). We therefore determined the effect of purified 14-3-3γ on the activities of $PP\text{-}1\alpha_{cat}$ and a minimal form of $PP\text{-}1_I$ reconstituted from purified $PP\text{-}1_{cat}$ and I-2. 14-3-3γ inhibited both $PP\text{-}1\alpha_{cat}$ and reconstituted $PP\text{-}1_I$ activity using phospho-Bad as a relevant substrate with IC_{50} values of ~350 nM (Fig. 7).

Mechanism of $PP\text{-}1_I$ activation in global cerebral ischemia

Having identified 14-3-3γ as a novel inhibitory modulator of native brain $PP\text{-}1_I$, we compared the amounts of 14-3-3γ present in $PP\text{-}1_I$ purified from control versus ischemic pig brain. Densitometric scans indicated that the relative amount of 14-3-3γ was significantly reduced in $PP\text{-}1_I$ purified from ischemic versus control brain, while the relative amounts of the other identified proteins were not significantly different (Fig. 8a–c). The finding of reduced 14-3-3γ in ischemic versus control brain was confirmed by quantitative immunoblotting for 14-3-3γ (Fig. 8d–f).

Discussion

Protein phosphatase-1_I is a highly regulated form of PP-1 that is involved in the regulation of multiple targets relevant to cerebral ischemia, in particular excitatory ion channels and cell death mediators (Cohen 1989; Greengard 2001; Garcia *et al.* 2003). We found that brain $PP\text{-}1_I$ was significantly activated by global cerebral ischemia using a clinically relevant model of ischemia/reperfusion. This is the first direct demonstration that a regulated form of PP-1 ($PP\text{-}1_I$) is activated by transient global cerebral ischemia *in vivo*. As PP-1 plays such a prominent role in the dephosphorylation of several regulators of cell death (e.g. Bad, Bcl-2, and Rb) (Ayllon *et al.* 2001, 2002; Wang *et al.* 2001), our results suggest that activation of $PP\text{-}1_I$ contributes to cell death in ischemia and reperfusion. $PP\text{-}1_I$ could also dephosphorylate other phospho-proteins that contribute to neurotoxicity in ischemia and reperfusion such that $PP\text{-}1_I$ might also have a neuroprotective role. Further analysis of this signaling pathway should resolve this question. Characterization of $PP\text{-}1_I$ purified from ischemic or control brain indicates that activation of $PP\text{-}1_I$ probably involves dissociation of 14-3-3γ, a novel inhibitory modulator of PP-1 found to interact with $PP\text{-}1_I$. Altered regulation of PP-1, a multifunctional cell signaling enzyme, represents a potentially critical event in cerebral ischemia. However, the pathophysiological significance of $PP\text{-}1_I$ activation in global cerebral ischemia remains to be tested by the application *in vivo* of membrane-permeable PP-1-specific inhibitors currently under development.

Previous studies have implicated PP-1 in the control of apoptosis in a number of cell types based on the protective effects of non-selective small molecule phosphatase inhibitors that do not distinguish between PP-1 and PP-2A (Morana *et al.* 1996; N'cho and Brahmi 1999;

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Chatfield and Eastman 2004). Both inhibition (Munoz *et al.* 2000) and activation (Yung and Tolkovsky 2003) of PP activity have been reported using *in vitro* models of ischemia induced by oxygen and glucose deprivation in differentiated PC12 cells and astrocytes. Initial inhibition followed by activation of PP-1 and/or PP-2A has also been reported in a rat model of transient global cerebral ischemia (Martin de la Vega *et al.* 2001, 2002). However, in those studies only spontaneously active forms of PP-1 and PP-2A were measured using non-selective inhibitors of PP-1 and PP-2A. Our more focused studies involving partial purification and biochemical characterization of PP-1 from brain subjected to ischemia and reperfusion *in vivo* provide more direct evidence that PP-1_I, a major form of regulated PP-1, is activated following global cerebral ischemia with reperfusion.

Protein phosphatase-1_I purified from brain subjected to global cerebral ischemia contained significantly less of the novel PP-1 inhibitor protein 14-3-3γ compared with control PP-1_I. These findings suggest that dissociation of inhibitory 14-3-3γ is involved in the ischemic activation of PP-1 activity. The reduced association was specific for 14-3-3γ and was evident following 2 h of reperfusion, so it is unlikely to be an artifact of postmortem processing. Whether PP-1_I activation occurs in neurons and/or glia is unknown; this issue demands higher resolution assays of PP-1_I activation under development. The Ca^{2+}-dependent protein kinases Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase Cδ have also been implicated in ischemic neuronal death (Hajimohammaddreza *et al.* 1995; Bright *et al.* 2004), and can phosphorylate 14-3-3 (Ellis *et al.* 2003; Hamaguchi *et al.* 2003). We are exploring the possibility that phosphorylation of 14-3-3γ by Ca^{2+}/calmodulin-dependent protein kinase II and/or protein kinase Cδ induces its dissociation from PP-1_I, thereby activating PP-1_I and the dephosphorylation of downstream substrates involved in the regulation of cell death. Identification of ischemic activation of PP-1_I and its modulation by 14-3-3γ provide the rationale for designing specific inhibitors of PP-1_I activation in order to directly test the pathophysiological significance of PP-1_I activation in ischemia and other neurodegenerative disorders.

Although our results suggest that 14-3-3γ dissociation is involved in PP-1_I activation in brain following ischemia/ reperfusion, the loss of 14-3-3γ by itself might not be the sole mechanism of PP-1_I activation. Potential mechanisms of PP-1_I activation by ischemia/ reperfusion include stimulation of an activating kinase, inhibition of an inhibitory kinase, recruitment of an activator and/or loss of an inhibitor. PP-1_I is activated following phosphorylation of I-2 by an unidentified endogenous protein kinase. Preliminary results indicate that this protein kinase is associated with purified PP-1_I, but is not glycogen synthase kinase-3, cyclin-dependent protein kinase-5, or extracellular regulated protein kinase-1 based on the insensitivity of purified brain PP-1_I activation to selective inhibitors of these kinases (data not shown). We are currently investigating the possibility that the PP-1_I activating kinase is one of the kinases identified in purified PP-1_I.

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Abbreviations used

I-2	inhibitor-2
PKA	cAMP-dependent protein kinase
DARPP	dopamine- and cyclic AMP-regulated neuronal phosphoprotein Mr 32 kDa

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PMSF	phenylmethylsulfonyl fluoride
PP	protein phosphatase
PP-1$_{cat}$	catalytic subunit of PP-1
PP-1α$_{cat}$	PP-1 catalytic subunit α

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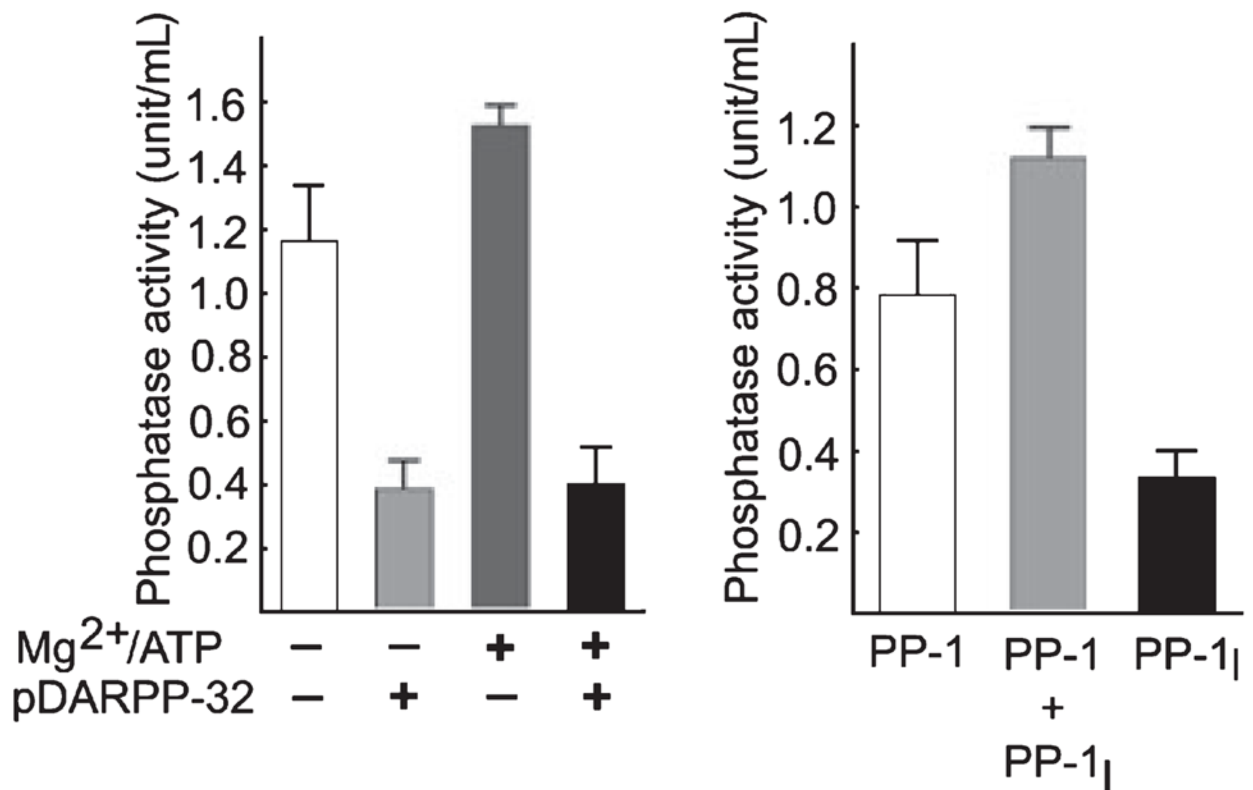

Fig. 1.

Identification of protein phosphatase (PP)-1_I activity in pig brain. (a) Phosphorylase phosphatase activity was determined in soluble pig brain extracts in the absence or presence of Mg^{2+}/ATP and phospho-T34-DARPP-32, as indicated. PP-1 activity is defined as phospho-T34-DARPP-32-sensitive phosphorylase phosphatase activity; the residual activity is primarily because of PP-2A. (b) The activity of PP-1_I is defined as Mg^{2+}/ATP-dependent PP-1 activity, or the difference between PP-1 activity measured in the absence (basal PP-1) or presence (basal PP-1 + PP-1_I) of Mg^{2+}/ATP. Mean ± SD (n = 3).

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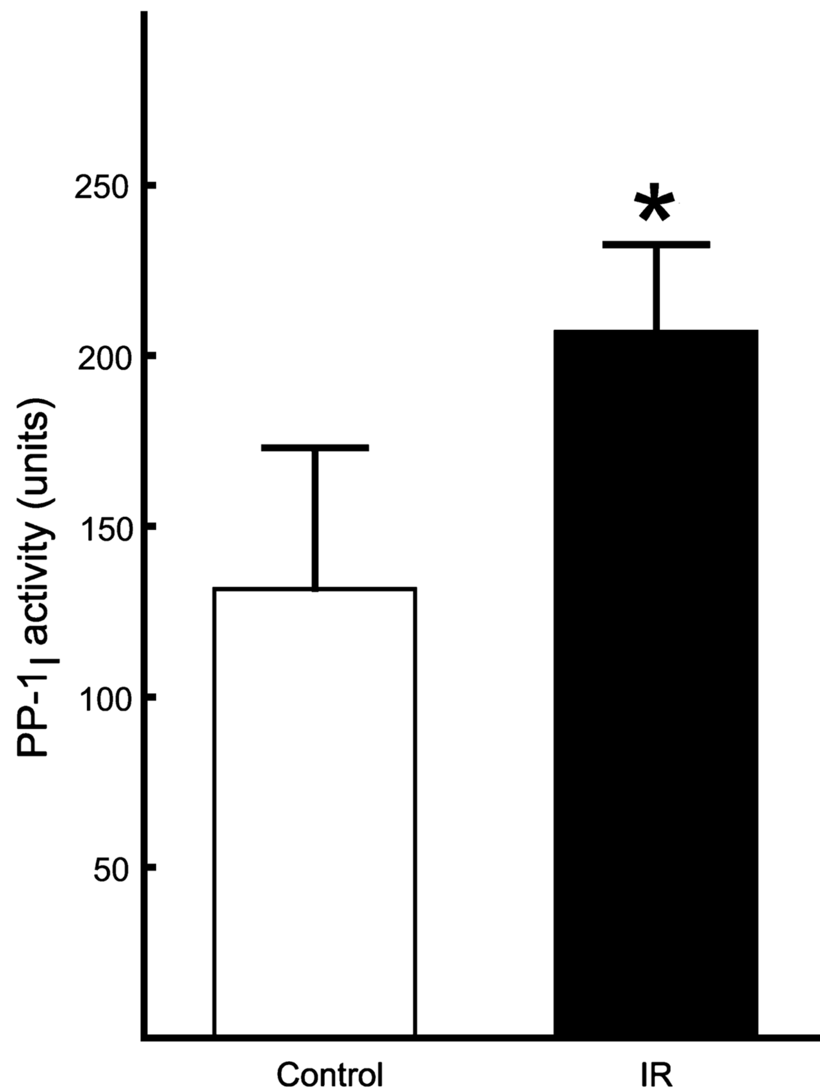

Fig. 2.

Activation of brain protein phosphatase (PP)-1_I by global cerebral ischemia. Total activity of PP-1_I was determined in soluble brain extracts from control pigs and pigs subjected to 10 min of cardiac arrest followed by resuscitation and reperfusion for 2 h. PP-1_I activity was quantified as described in Fig. 1. Mean ± SD ($n = 3$). *$p < 0.05$ versus control (Student *t*-test).

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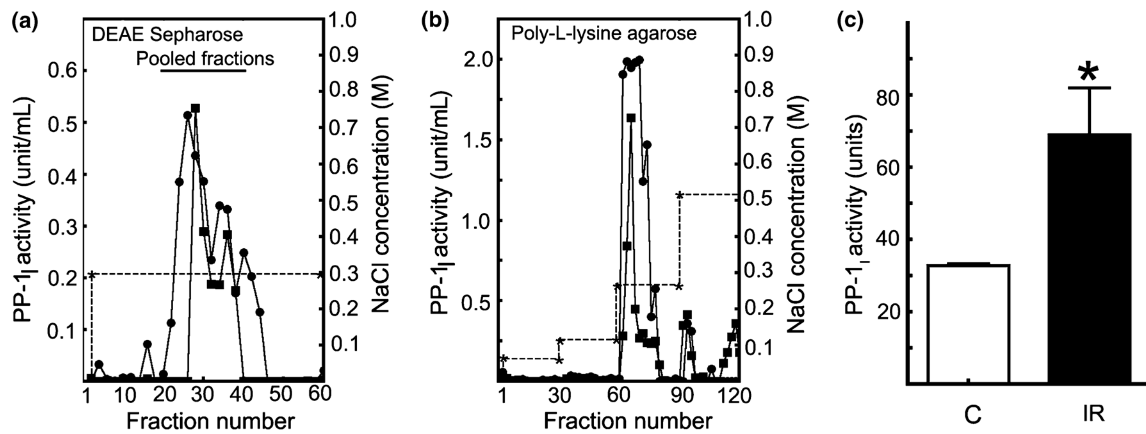
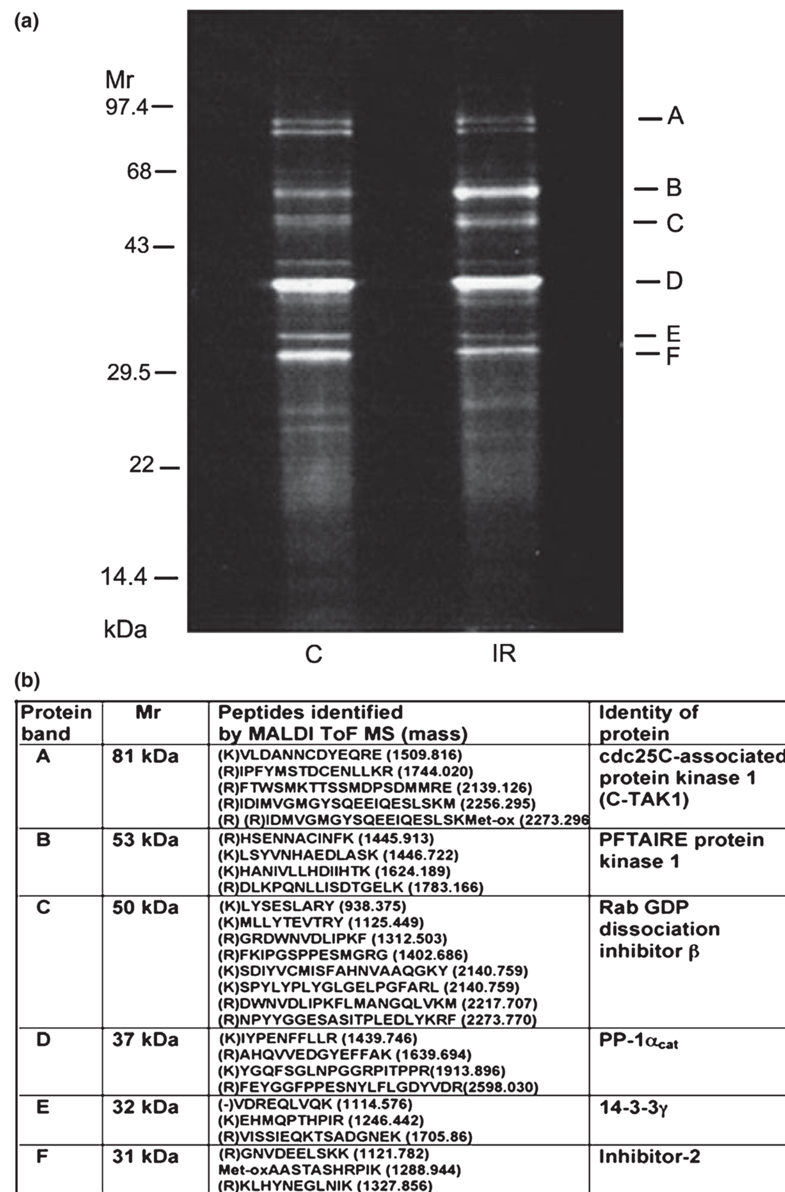


Fig. 3.

Quantification of protein phosphatase (PP)-1_I following partial purification of brain extracts from control and ischemic brain. The activity of PP-1_I is defined as the activity in the presence of Mg^{2+}/ATP minus activity in the absence of Mg^{2+}/ATP. (a) Soluble brain extracts from a control pig and from a pig subjected to 10 min of cardiac arrest followed by resuscitation and reperfusion for 2 h were prepared as described in Experimental procedures and loaded onto a DEAE Sepharose column. PP-1_I activity from control (■) and ischemic (●) brain was determined. Data are shown for a single representative experiment (n = 3). (b) Active fractions from the DEAE Sepharose column were pooled as indicated by the bar in (a) and loaded onto a poly-L-lysine agarose column. PP-1_I activity from control (■) and ischemic (●) brain was determined. (c) Total PP-1_I activity from the poly-L-lysine agarose chromatography step from control or ischemic brain was quantified by determining the area under the peak of activity. Mean ± SD (n = 3). *$p < 0.05$ versus control (Student t-test).



Protein band	Mr	Peptides identified by MALDI ToF MS (mass)	Identity of protein
A	81 kDa	(K)VLDANNCDYEQRE (1509.816) (R)IPFYMSTDCENLLKR (1744.020) (R)FTWSMKTTSSMDPSDMMRE (2139.126) (R)IDIMVGMGYSQEEIQESLSKM (2256.295) (R) (R)IDMVGMGYSQEEIQESLSKMet-ox (2273.296	cdc25C-associated protein kinase 1 (C-TAK1)
B	53 kDa	(R)HSENNACINFK (1445.913) (K)LSYVNHAEDLASK (1446.722) (K)HANIVLLHDIIHTK (1624.189) (R)DLKPQNLLISDTGELK (1783.166)	PFTAIRE protein kinase 1
C	50 kDa	(K)LYSESLARY (938.375) (K)MLLYTEVTRY (1125.449) (R)GRDWNVDLIPKF (1312.503) (R)FKIPGSPPESMGRG (1402.686) (K)SDIYVCMISFAHNVAAQGKY (2140.759) (K)SPYLYPLYGLGELPGFARL (2140.759) (R)DWNVDLIPKFLMANGQLVKM (2217.707) (R)NPYYGGESASITPLEDLYKRF (2273.770)	Rab GDP dissociation inhibitor β
D	37 kDa	(K)IYPENFFLLR (1439.746) (R)AHQVVEDGYEFFAK (1639.694) (K)YGQFSGLNPGGRPITPPR(1913.896) (R)FEYGGFPPESNYLFLGDYVDR(2598.030)	PP-1α_{cat}
E	32 kDa	(-)VDREQLVQK (1114.576) (K)EHMQPTHPIR (1246.442) (R)VISSIEQKTSADGNEK (1705.86)	14-3-3γ
F	31 kDa	(R)GNVDEELSKK (1121.782) Met-oxAASTASHRPIK (1288.944) (R)KLHYNEGLNIK (1327.856)	Inhibitor-2

Fig. 4.

Identification of protein components of purified protein phosphatase (PP)-1_I by mass spectrometry. (a) Purified PP-1_I from control (C) and ischemic/reperfused (IR) pig brain was subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The bars on the left indicate the migration of marker proteins. The bars on the right indicate the migration of the major proteins present in purified PP-1_I. (b) Protein bands were excised from the gel and digested with trypsin, and tryptic peptides were analyzed by matrix-assisted laser desorption time of flight mass spectrometry (MALDI ToF MS). The molecular masses and amino acid sequences of the tryptic peptides identified for each major protein are shown. (a and b) migrated as a doublet; analysis of both bands yielded peptides derived from C-TAK1.

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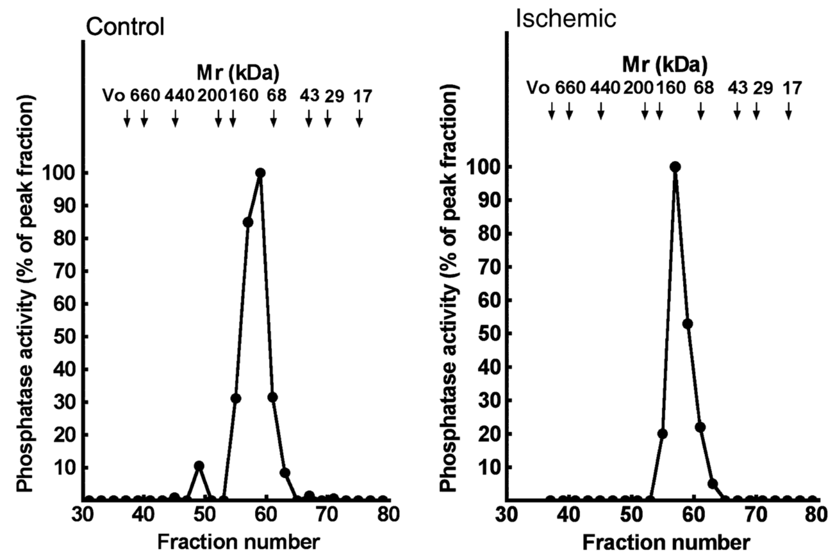

Fig. 5.

Analysis of purified protein phosphatase (PP)-1_I by gel filtration. Purified PP-1_I from control (left) and ischemic (right) brain were analyzed on a Superdex 200 column (1.0 × 60 cm) at a flow rate of 0.75 mL/min with collection of 1.25 mL fractions. The arrows indicate the void volume (*V*o) and the elution positions of the marker proteins.

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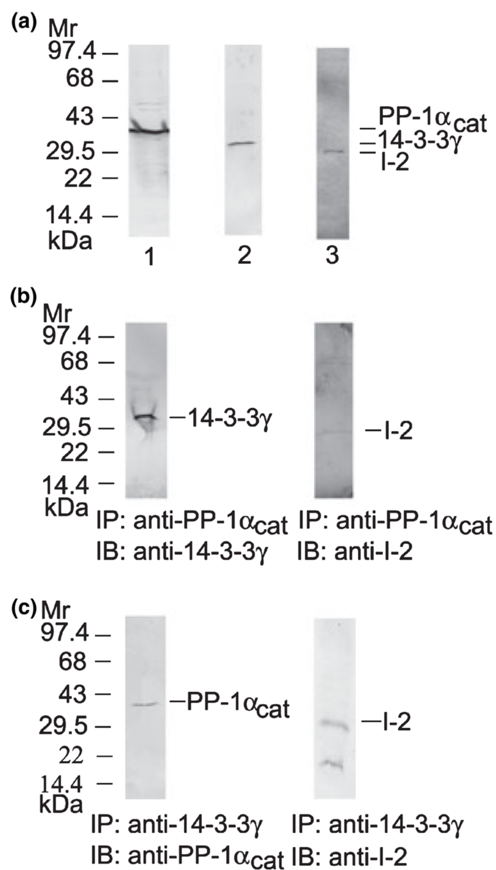

Fig. 6.

Identification of a protein phosphatase (PP)-1_I : inhibitor-2 (I-2) : 14-3-3γ complex in control brain. (a) Purified control brain PP-1_I was immunoblotted with anti-PP-$1\alpha_{cat}$ (lane 1), anti-14-3-3γ (lane 2), or anti-I-2 (lane 3). (b) Purified control brain PP-1_I was immunoprecipitated with anti-PP-$1\alpha_{cat}$ followed by immunoblotting with anti-14-3-3γ (left) or anti-I-2 (right). (c) Purified control brain PP-1_I was immunoprecipitated (IP) with anti-14-3-3γ followed by immunoblotting (IB) with anti-PP-$1\alpha_{cat}$ (left) or anti-I-2 (right).

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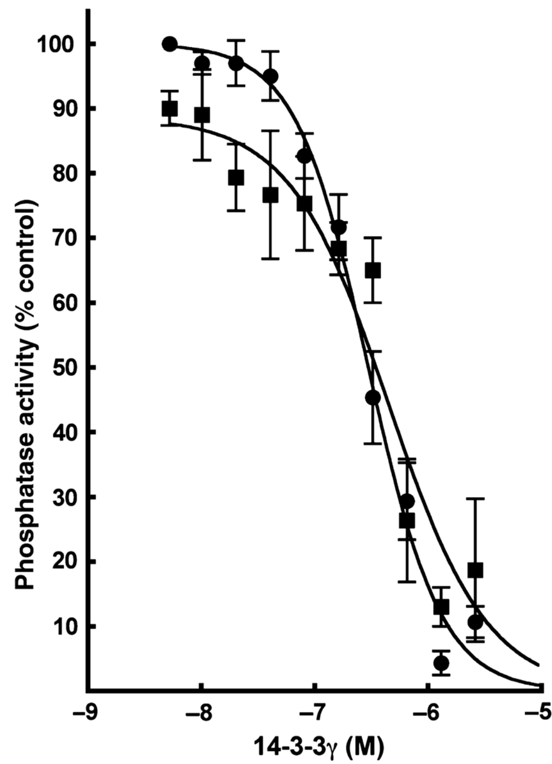

Fig. 7.

Inhibition of protein phosphatase (PP)-1 catalytic subunit α (PP-1α_{cat}) and reconstituted PP-1α_{cat} : inhibitor-2 (I-2) by 14-3-3γ. Purified PP-1α_{cat} (■) and reconstituted PP-1α_{cat} : I-2 (●) were pre-incubated with various amounts of 14-3-3γ for 10 min and then assayed using [^{32}P]phospho-Bad as substrate.

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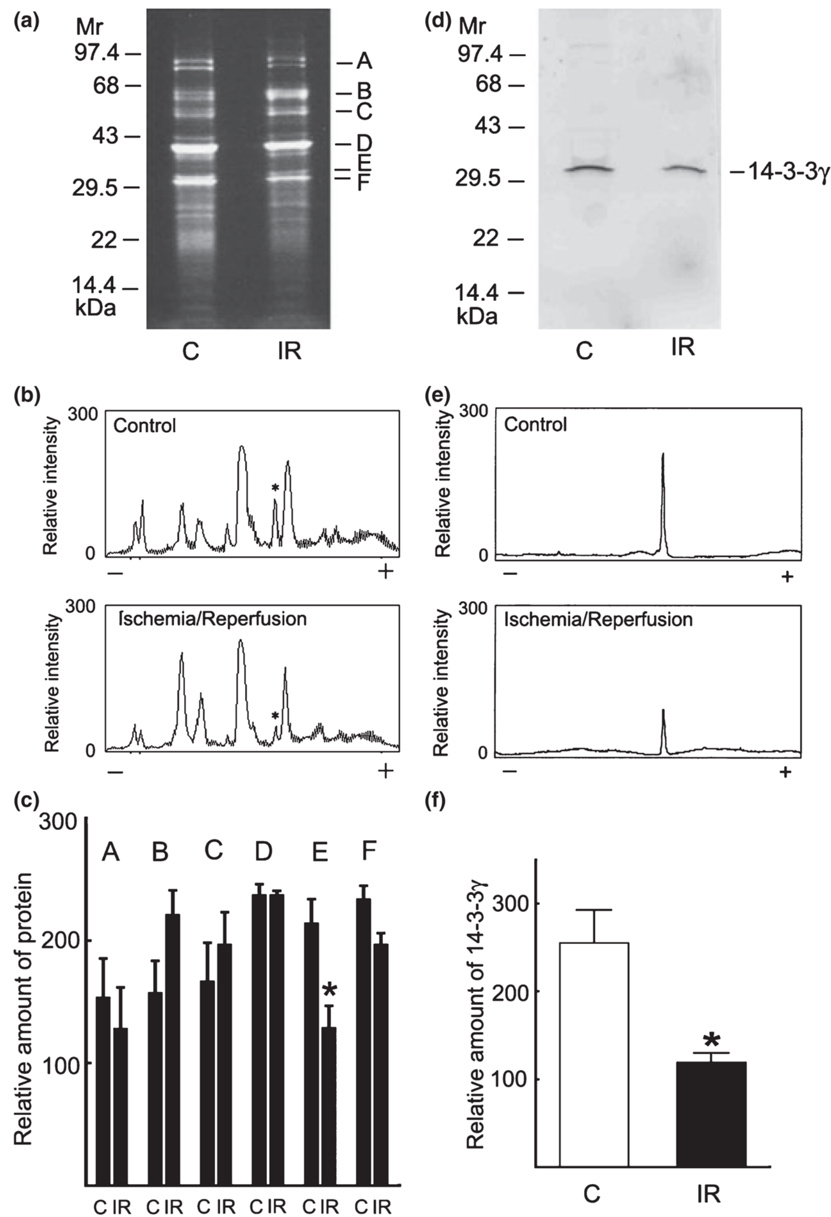

Fig. 8.

Quantification of 14-3-3γ in purified protein phosphatase (PP)-1_I from control and ischemic brain. (a) PP-1_I was purified to near homogeneity from control (C) and ischemic/reperfused (IR) pig brain as described in Experimental procedures, analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and stained with Sypro Ruby which identified six major bands (a–f). (b) Densitometric scans of gels obtained from PP-1_I purified from control (top) or ischemic (bottom) pig brain. The migration of 14-3-3γ is indicated by an asterisk. (c) Quantification of proteins present in PP-1_I purified from control (C) and ischemic brain (IR) pig brain. Relative protein amounts were quantified as peak area obtained by densitometric scans of stained gels. Mean ± SD (n = 3). *$p < 0.05$ versus control (Student t-test). (d) Immunoblot analysis of 14-3-3γ in purified PP-1_I from control (C) and ischemic (IR) brain. (e) Densitometric scans of immunoblots of purified PP-1_I from control (top) or ischemic (bottom) pig brain. (f) Quantification of immunoreactive 14-3-3γ in purified PP-1_I from

control (C) or ischemic (IR) pig brain as peak area. Mean ± SD ($n = 3$). *$p < 0.05$ versus control (Student t-test).

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