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Neuroprotection mediated by glutamate carboxypeptidase II (NAALADase) inhibition requires TGF-β

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Abstract

Inhibition of glutamate carboxypeptidase (GCP) II (EC 3.4.17.21), also termed *N*-acetylated alpha-linked acidic dipeptidase (NAALADase), has been shown to protect against ischemic injury presumably via decreasing glutamate and increasing *N*-acetyl-aspartyl-glutamate (NAAG). NAAG is a potent and selective $mGlu_3$ receptor agonist. Activation of glial $mGlu_3$ receptors has been shown to protect against NMDA toxicity by releasing transforming growth factors, TGF-βs. We hypothesized that GCP II inhibition could be neuroprotective also via TGF-βs, due to increased NAAG. To verify this, Enzyme-Linked Immunosorbent Assays (ELISAs) were performed on media from both control and ischemic cultures treated with the GCP II inhibitor, 2-(phosphonomethyl)-pentanedioic acid (2-PMPA). We found that 2-PMPA attenuated ischemia-induced declines in TGF-β. To further assess the role of TGF-βs in 2-PMPA-mediated neuroprotection, a neutralizing antibody to TGF-β (TGF-β Ab) was used. In both in vitro and in vivo models of cerebral ischemia, TGF-β Ab reversed the neuroprotection by 2-PMPA. Antibodies to other growth factors had no effect. Data suggests that neuroprotection by GCP II inhibition may be partially mediated by promoting TGF-β release.

Keywords: GCP II (glutamate carboxypeptidase); NAALADase (*N*-acetylated alpha-linked acidic dipeptidase); NAAG (*N*-acetyl-aspartyl-glutamate); TGF-β (transforming growth factor-β); Ischemia; Metabotropic glutamate receptor; Glutamate

1. Introduction

Excessive glutamate neurotransmission is hypothesized to be involved in neuronal injury in many disorders of the central nervous system (Choi et al., 1987), including stroke. Extensive research has focused on post synaptic glutamate receptor antagonism as a strategy to attenuate the pathological consequence of this excess. Several antagonists, operating via various receptors and receptor-subtypes, have been shown to reduce extracellular glutamate-mediated injury.

Recently, a novel mechanism of neuroprotection was reported wherein inhibition of glutamate carboxypeptidase (GCP II; EC 3.4.17.21), also known as *N*-acetylated-α-linked acidic dipeptidase (NAALADase), afforded substantial neuroprotection in both in vitro and in vivo models of cerebral ischemia (Slusher et al., 1999). GCP II is an enzyme, found on the external surface of predominantly astrocytic cells (Berger et al., 1999) in the brain and on non-myelinating Schwann cells in the peripheral nervous system (Berger et al., 1995), that readily hydrolyzes the endogenous neuropeptide *N*-acetyl-aspartyl-glutamate (NAAG) to *N*-acetyl-aspartate (NAA) and glutamate (Robinson et al., 1987). We have previously suggested that the underlying mechanism of this novel neuroprotective strategy is likely mediated through both decreased glutamate availability and increased NAAG availability (Slusher et al., 1999). Decreased glutamate availability might be expected to be neuroprotective by limiting toxic glutamate receptor activation (Meldrum, 1990). Neuroprotection mediated via increased NAAG availability is, however, less understood.

NAAG is a dipeptide found in the brain in millimolar concentrations (0.5–2.7 mM) (Pouwels and Frahm, 1997), localized to glutamatergic pathways and reactive microglia (Neale et al., 2000). NAAG is released in a calcium-dependent manner upon synaptic stimulation (Neale et al., 2000) and thus, has been considered to be a candidate neurotrans-

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mitter. Studies have demonstrated effects of NAAG as a partial antagonist/agonist at the NMDA receptor (Sekiguchi et al., 1989; Valivullah et al., 1994) and as an agonist at the group-II metabotropic glutamate receptors (Bruno et al., 1998c; Lu et al., 2000; Thomas et al., 2000; Wroblewska et al., 1997).

Recently, agonists of the group-II metabotropic glutamate receptors have been shown to protect in various models of neuronal injury including NMDA- and kainate-induced neurotoxicity (Bruno et al., 1995; Thomas et al., 2000), oxygen-glucose deprivation-induced neuronal death (Buisson and Choi, 1995), traumatic brain injury (Allen et al., 1999) and ischemia (Lu et al., 2000; Thomas et al., 2000). Several mechanisms that underlie the neuroprotection afforded by these agonists have been postulated including reduced glutamate release through pre-synaptic receptor activation (Sanchez-Prieto et al., 1996), inhibition of cAMP formation (Buisson and Choi, 1995) and inactivation of voltage-sensitive calcium channels (Schoepp and Conn, 1993). Recent studies have shown that the neuroprotective activity induced by the activation of group-II metabotropic glutamate receptors also requires the de novo synthesis of a new protein and involves glial-neuronal signaling (Bruno et al., 1997). This protein was subsequently identified to be a transforming growth factor-beta (TGF-β, (Bruno et al., 1998a)).

In this study, we investigated the role of transforming growth factors in neuroprotective mechanisms mediated by inhibition of GCP II.

2. Materials and methods

All procedures reported here have been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and put forth by the National Institutes of Health of the United States.

2.1. Materials

NAAG was obtained from Tocris-Cookson (Ballwin, MO). 2-(Phosphonomethyl)-pentanedioic acid (2-PMPA) was synthesized by SRI International (Menlo Park, CA). All other drugs, chemicals and antibodies were obtained from Sigma (St. Louis, MO). Enzyme-Linked Immunosorbent Assay (ELISA) kits, for the detection of various trophic factors, were obtained from Promega (Madison, WI). Regular HEPES buffered saline (HBS), phosphate-free HEPES buffered saline solution (HBSS) and artificial cerebral spinal fluid (ACSF) were custom made by Paragon Biotech (Baltimore, MD). Dulbecco's Minimum Essential Media (DMEM) purchased from GIBCO (Rockville, MD). The serum used in making up the tissue culture medium was obtained from HyClone Laboratories (Logan, UT). Monofilament sutures were obtained from J.A. Webster (Sterling, MA) and halothane from Penn Veterinary Supply (Lancaster, PA).

Male Sprague–Dawley rats were obtained from Charles River Laboratory (Cambridge, MA) and timed pregnant rats from Harlan Sprague Dawley, (Indianapolis, IN). These animals were housed in a well ventilated vivarium with free access to food and water until completion of the experiments.

2.2. Dissociated cortical cell cultures

As described elsewhere (Thomas et al., 2000), dissociated neuronal/glial cortical cultures were prepared using papain dissociation of cortices removed from embryonic 17-day-old rat fetuses obtained from timed pregnant Harlan Sprague–Dawley rats. Cultures were maintained for approximately 18–20 days before they were used in the experiments.

2.3. Metabolic inhibition in cortical cell cultures

As previously described (Thomas et al., 2000), cultures were washed once with phosphate-free HBSS (143 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 5.0 mM HEPES, 2.0 mM $CaCl_2$, 11.1 mM D-glucose and 0.005% phenol red) and ischemic conditions simulated by a 20-min exposure, at 37 °C, to 10 mM 2-deoxyglucose and 5 mM potassium cyanide in glucose, phosphate and potassium-free HBSS. 2-Deoxyglucose was used to inhibit glycolysis and potassium cyanide was used to inhibit mitochondrial function. In antibody-related experiments, cultures were pre-incubated with the antibody for 30 min prior to the insult. Following exposure, cultures were washed once again with phosphate-free HBSS and allowed to recover for 24 h in phosphate-free DMEM. Unless otherwise specified, antibodies and protective drugs were present during both exposure and throughout recovery.

2.4. Measurement of growth factors in cortical cell cultures

Media was collected from both control and ischemic cultures at the end of the 20-min exposure. To measure the amount of biologically active TGF-βs, brain derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) released during exposure, ELISAs were performed using the respective Emax Immunoassay Systems obtained from Promega.

2.5. Lactate dehydrogenase measurements in cortical cell cultures

Cellular injury was assessed by measuring the accumulation of lactate dehydrogenase (LDH; Koh and Choi,

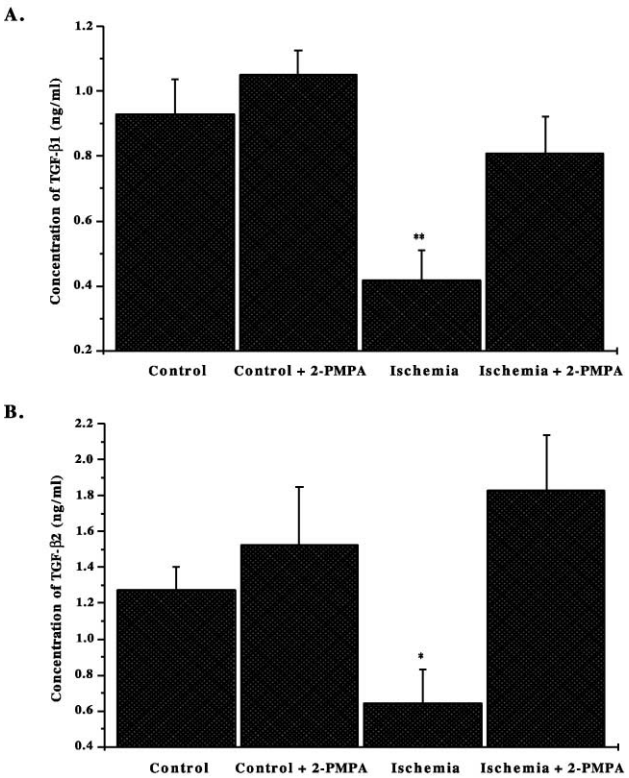

Fig. 1. TGF-β1, -β2 levels are increased by GCP II inhibition in ischemic cultures. To simulate ischemic injury, rat cortical cultures were exposed to 10 mM 2-deoxyglucose/5 mM potassium cyanide for 20 min. Cultures were also exposed to the GCP II inhibitor, 2-PMPA (10 μM), either with or without the metabolic inhibitors. In order to detect TGF-β1, -β2 levels, ELISAs were performed on media collected from control and ischemic cultures using the Emax Immunoassay Systems obtained from Promega. Statistical analyses were performed using Student's two-population *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$ versus control). (A) 2-PMPA significantly increases TGF-β1 levels in ischemic cultures. (B) 2-PMPA also significantly increases TGF-β2 levels in ischemic cultures.

1987), in the culture media at the end of the 24-h recovery period. Each experiment contained an independent control without metabolic inhibitors and an independent measurement of maximal injury. Maximal injury was defined as that caused by a 20-min exposure to 10 mM 2-deoxyglucose and 5 mM potassium cyanide. Data were then normalized within each experiment as a percent of maximal injury and control ((condition − control)/(max. injury − control) × 100). All data is presented as mean ± S.E.M values of three determinations in three or more independent experiments. The values were compared by Student's two population *t*-test.

2.6. Middle cerebral artery occlusion

Male Sprague Dawley rats, weighing approximately 260–290 g were used in these experiments. Animals were randomly assigned to four treatment groups (10–17 animals per group) prior to the experiment. Each animal received an intracerebral injection of either the neutralizing antibody to TGF-β (TGF-β Ab) or ACSF in addition to an intravenous injection of either 2-PMPA or HBS (50 mM HEPES and 168 mM NaCL). TGF-β Ab or ACSF was delivered 20 min prior to the middle cerebral artery occlusion surgery and 2-PMPA or HBS was administered immediately after middle cerebral artery occlusion surgery.

TGF-β Ab was dissolved in phosphate-free ACSF and administered stereotaxically into the right hemisphere of the rat at various injection sites. Each injection was a slow infusion of 25 μg of TGF-β Ab in a volume of 1.25 μl. A total of 325 μg of TGF-β Ab was injected into 13 brain sites. Equal volumes of ACSF were injected in the control animals at the same stereotaxic sites.

A pilot study to examine the extent of penetration of TGF-β Ab in the normal rat brain tissue had been performed earlier. Immunohistochemistry results revealed that the amount of TGF-β Ab used in this experiment produced

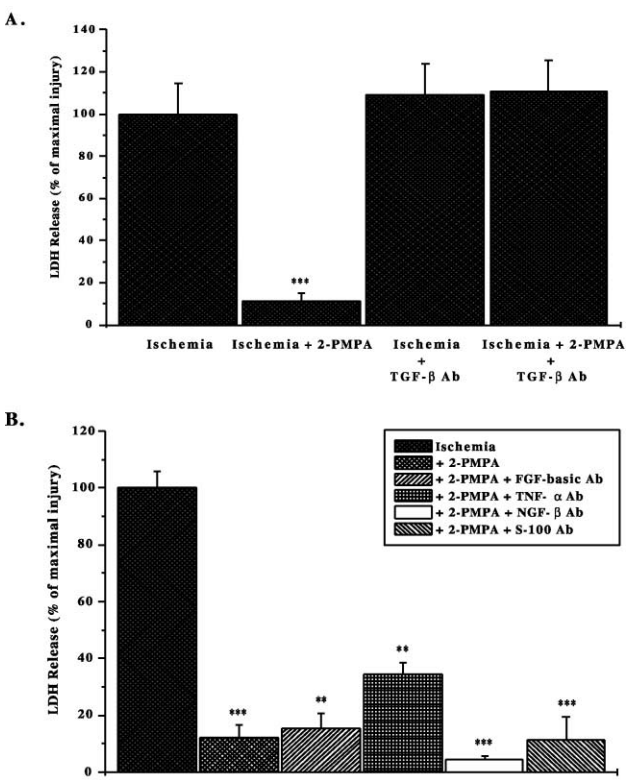

Fig. 2. Neutralizing antibody to TGF-β reverses protective effect of GCP II inhibition in rat cortical cultures. Cultures were exposed to 2-deoxyglucose/potassium cyanide with either 2-PMPA (10 μM), or TGF-β Ab, (final concentration of 10 μg/ml) or a combination of both. At the end of the exposure, cultures were washed and allowed to recover in DMEM for 24 h. 2-PMPA or TGF-β Ab or a combination of both were present during the 24-h recovery as well. Cellular injury was quantified by measuring LDH released into the media during the recovery period. Data were normalized within each experiment as a percent of maximal injury and control, as detailed in Materials and methods, and presented as mean ± S.E.M values. ($^{**}P < 0.01$, $^{***}P < 0.001$ versus ischemia). (A) TGF-β Ab reverses the neuroprotection afforded by 2-PMPA against ischemia. (B) Other trophic factors have no effect on the neuroprotection of 2-PMPA.

complete coverage of the middle cerebral artery territory within a 2 h period.

2-PMPA was dissolved in HBS and the pH was adjusted to 7.4 before administration. 2-PMPA was administered intravenously at a bolus dose of 100 mg/kg followed immediately by an infusion dose of 20 mg/kg/h for 4 h, as this was previously shown to be maximally neuroprotective (Slusher et al., 1999). HBS was used as the vehicle control for the 2-PMPA experiments.

As described previously (Lu et al., 2000; Slusher et al., 1999), the intraluminal filament model of transient middle cerebral artery occlusion was used to induce ischemic injury. Briefly, under 1.5% halothane anesthesia, the common carotid artery was exposed at the level of external and internal carotid artery bifurcation. The external carotid artery and its branches were cauterized and cut. A piece of 3-0 monofilament nylon suture with a blunted tip was introduced into the internal carotid artery via the proximal end of the external carotid artery stump. The suture was advanced through the carotid canal to the origin of the MCA where it blocked the blood flow to its entire territory. At the end of the 2-h occlusion period, the rat was re-anesthetized and the suture carefully pulled back to the external carotid artery stump to allow for reperfusion. During the surgery, the animal's body temperature was maintained at 37 °C using a heating blanket.

2.7. In vivo data analysis

After 22 h of reperfusion the rats were sacrificed. The brain was cut into seven 2-mm-thick coronal slices, stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC), and subsequently imaged using a computer-assisted digital imaging analysis system. Ischemic injury was quantified based on the volume of tissue completely lacking TTC staining. Total infarct volume of each rat was used for statistical analysis. One-way analysis of variance (ANOVA) test was used for comparison of the effects of treatment. Differences between groups were considered significant at $P < 0.05$.

3. Results

3.1. Inhibition of GCP II prevents the ischemia-induced decline in TGF-β levels, in vitro

To examine the effect of 2-PMPA on TGF-β release, ELISAs were performed on media collected from both control and ischemic cortical cultures treated with 2-PMPA

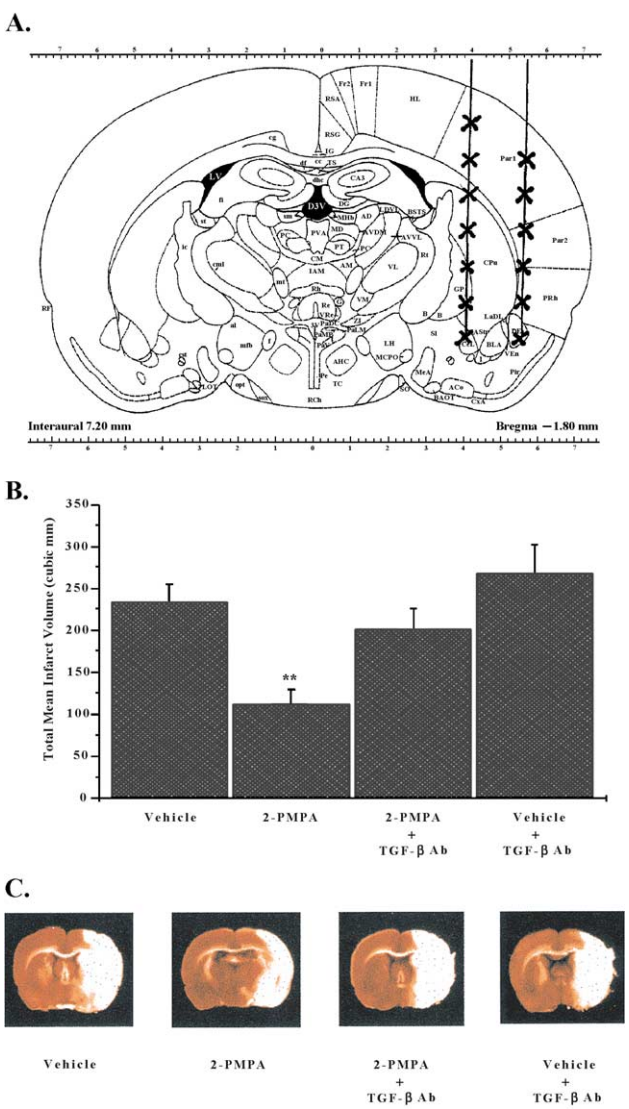

Fig. 3. Neutralizing antibody to TGF-β reverses protective effects of GCP II inhibition in rat middle cerebral artery occlusion. Rats were randomly assigned to four treatment groups and subjected to 2 h of transient middle cerebral artery occlusion. 2-PMPA was administered intravenously at a bolus dose of 100 mg/kg followed immediately by an infusion dose of 20 mg/kg/h for 4 h. HBS was used as the vehicle control for the 2-PMPA experiments. TGF-β Ab or ACSF was delivered 20 min prior to the middle cerebral artery occlusion surgery and 2-PMPA or HBS administered immediately after middle cerebral artery occlusion surgery. After 22 h of reperfusion the rats were sacrificed, the brains removed and stained with TTC and ischemic injury quantified as a function of infarct tissue volume. All data are expressed as mean ± S.E.M. Comparison of the effects of 2-PMPA treatment versus each of the other groups was performed using one-way ANOVA. ($^{**}P < 0.01$). (A) Intracerebral injection sites of TGF-β Ab. The injection sites were in the area of the right hemisphere covered by middle cerebral artery. The medial injections were aimed at Bregma AP −1.8, ML −4, and DV −8 to −2, in mm increments. The lateral injections were aimed at Bregma AP −1.8, ML −5.5, and DV −8 to −3, also in mm increments. The injections were initiated ventrally. Each injection site received an infusion of 25 μg of TGF-β Ab in a volume of 1.25 μl over 2 min. (B) TGF-β Ab reverses the neuroprotection afforded by 2-PMPA against middle cerebral artery occlusion-induced injury. (C) Representative TTC stained brain slice images selected from each of the four treatment groups. While viable tissue appears red as a result of the reduction of the dye by functional mitochondrial enzymes, infarcted tissue appears white.

(10 μM). As shown in Fig. 1A and B, 2-PMPA preserved both TGF-β1 and -β2 levels in metabolically inhibited cultures. In contrast, the TGF-β levels in control cultures were unaffected by 2-PMPA.

3.2. Inhibition of GCP II provides neuroprotection against metabolic inhibition, in vitro

As was previously shown (Slusher et al., 1999), 2-PMPA provided substantial neuroprotection ($> 85\%$, $P < 0.001$, Fig. 2) in a tissue culture model of cerebral ischemia, at the maximally protective dose of 10 μM.

3.3. Neuroprotection mediated by inhibition of GCP II is reversed by neutralizing antibody to TGF-β, in vitro

To examine whether the neuroprotection afforded by inhibition of GCP II was mediated by the normalized TGF-β levels, cultures were co-treated with a non-selective neutralizing antibody to TGF-β Ab and 2-PMPA. While 2-PMPA (10 μM) robustly protected primary cortical cells from ischemic injury, cultures pre-treated with TGF-β Ab (10 μg/ml) could not be rescued with 2-PMPA (Fig. 2A). Pre-treatments with neutralizing antibodies to other growth factors, such as fibroblast growth factor (FGF-basic; 10 μg/ml), tumor necrosis factor (TNF-α; 0.1 μg/ml), nerve growth factor (NGF-β; 10 μg/ml) and S-100 (8.1 μg/ml), were ineffective in reversing the neuroprotection provided by 2-PMPA against ischemia (Fig. 2B). Doses for the neutralizing antibodies to these growth factors, except TNF-α, were chosen based on that for TGF-β Ab. For TNF-α neutralizing antibody, 0.1 μg/ml dose was chosen since preliminary experiments revealed higher doses of the antibody to be toxic.

3.4. Inhibition of GCP II provides neuroprotection against middle cerebral artery occlusion, in vivo

2-PMPA (100 mg/kg i.v. bolus followed by 20 mg/kg/h i.v. infusion for 4 h) produced significant neuroprotection ($> 50\%$, $P < 0.001$, Fig. 3B) against middle cerebral artery occlusion induced injury compared to the vehicle-treated groups.

3.5. Neuroprotection mediated by inhibition of GCP II is reversed by neutralizing antibody to TGF-β, in vivo

Whereas 2-PMPA robustly protected against middle cerebral artery occlusion induced injury, administration of TGF-β Ab (325 μg via intra-cerebral injection, Fig. 3A) prior to the middle cerebral artery occlusion insult blocked the neuroprotective effect of 2-PMPA (Fig. 3B). Additionally, as was observed in the in vitro model of cerebral ischemia, TGF-β Ab alone did not significantly induce or alter the ischemic injury (Fig. 3B). Representative brain slice images from each treatment group are shown in Fig. 3C.

4. Discussion

Transforming growth factors are ubiquitous cytokines that affect the biological activity of a variety of cell types including neurons, astrocytes and microglia (Flanders et al., 1998; Henrich-Noack et al., 1996; Unsicker et al., 1991). In addition to the role they play as regulators of normal cellular physiology, such as cell growth and differentiation (Flanders et al., 1998), TGF-βs also play an important role as mediators of pathophysiological processes, such as inflammation and tissue repair (Roberts and Sporn, 1996). While TGF-βs are minimally expressed in the normal intact brain (Flanders et al., 1998; Henrich-Noack et al., 1996; Unsicker et al., 1991), trauma or excitotoxic events markedly increase the expression of TGF-βs (Henrich-Noack et al., 1996). Recently, increased expression of TGF-βs was also reported in human stroke patients and in animal models of cerebral ischemia (Ata et al., 1999; Krupinski et al., 1996). Though the functional role of TGF-βs in response to cerebral ischemia has not been fully elucidated, reports have shown that TGF-βs, in particular TGF-β1, have the capacity to rescue neurons from excitotoxic or ischemia/hypoxia-induced injury both in vitro and in vivo (Henrich-Noack et al., 1996; Prehn et al., 1993). Therefore, agonists promoting the release of endogenous TGF-βs may provide additional methods of therapeutic intervention during excitotoxic events.

In earlier experiments we had shown that, under pathological conditions, inhibition of GCP II increased the availability of NAAG (Slusher et al., 1999). NAAG is an endogenous agonist at the group-II metabotropic glutamate receptors with specificity for the $mGlu_3$ receptors (Wroblewska et al., 1997), localized on both neurons and glia (Petralia et al., 1996; Pin and Duvoisin, 1995). While activation of presynaptic $mGlu_3$ receptors has been shown to be neuroprotective via reduced glutamate release (Sanchez-Prieto et al., 1996), activation of glial $mGlu_3$ receptors has also been shown to be neuroprotective via the de novo synthesis of TGF-β (Bruno et al., 1997, 1998a). Since NAAG was shown to protect mouse cortical cells against NMDA-induced toxicity via the release of TGF-βs (Bruno et al., 1998c), we predicted that inhibition of GCP II and the resultant increase of extracellular NAAG could also regulate the release of endogenous TGF-β and protect cortical neurons against ischemic injury. Our results demonstrate a role for TGF-βs in neuroprotective mechanisms mediated by inhibition of GCP II in both in vitro and in vivo models of cerebral ischemia.

In our initial experiments, the contribution of growth factors in neuroprotection mediated by inhibition of GCP II was examined by treating both control and metabolically

inhibited cultures with the potent and selective GCP II inhibitor, 2-PMPA (10 μM). While this dose of 2-PMPA is higher than its K_i for GCP II inhibition (the K_i of 2-PMPA for GCP II is approximately 1 nM), this dose of 2-PMPA was selected based on previous observations for maximal neuroprotection in an identical in vitro model of cerebral ischemia (> 85%, Slusher et al., 1999). Additionally, we previously reported 10 μM 2-PMPA to be inactive in more than 100 different receptor, transporter, ion channel and enzyme assays including several glutamatergic sites such as NMDA, AMPA and glutamate transporters (Slusher et al., 1999). In these experiments, TGF-β levels were measured at various time points during and following metabolic inhibition. ELISAs revealed significant decline in TGF-β levels during the exposure phase (10 and 20 min; approximately 40% of control) while TGF-β levels were below the sensitivity of detection during the recovery phase (15, 30, 45, 60, 120, 240, and 1440 min; data not shown). Treatment of both control and ischemic neuronal cultures with 2-PMPA revealed that whereas 2-PMPA had no effect on the TGF-β levels in control cultures, 2-PMPA preserved both TGF-β1 and -β2 levels in metabolically inhibited cultures during the exposure phase (Fig. 1). Levels of the other trophic factors (BDNF, CNTF), known to be neuroprotective in various models of neuronal injury (Connor and Dragunow, 1998), were unaltered by 2-PMPA (data not shown), suggesting a specific role for transforming growth factors in GCP II-mediated mechanisms.

Earlier reports have shown that exogenous application of TGF-βs is neuroprotective in several neuronal injury models (Flanders et al., 1998; Henrich-Noack et al., 1996; Pratt and McPherson, 1997; Prehn et al., 1993). Since 2-PMPA prevented the decline of endogenous TGF-β levels induced by ischemia, we next examined, in vitro, the role of TGF-βs in neuroprotective mechanisms mediated by inhibition of GCP II using a non-specific neutralizing antibody to TGF-β. Whereas 2-PMPA protected against metabolic inhibition, TGF-β Ab completely reversed the neuroprotection afforded by 2-PMPA (Fig. 2). Neutralization of the endogenous TGF-βs, in the absence of 2-PMPA, did not increase the severity of ischemic damage (Fig. 2). Our data are consistent with previously published in vitro results wherein exogenous application of TGF-β1 and TGF-β2 antibodies did not increase the severity of damage induced by a 10-min exposure to 100-μM NMDA (Bruno et al., 1998a). Additionally, since a 20-min exposure to the metabolic inhibitors, 2-deoxyglucose (10 mM) and potassium cyanide (5 mM), causes complete morphological damage of the cortical cultures, small changes in injury, induced upon the addition of TGF-β Ab, may be difficult to determine.

Specificity for TGF-βs in GCP II-mediated mechanisms was ascertained by pre-treating cultures with neutralizing antibodies to FGF-basic, NGF-β, TNF-α and S-100 protein, factors thought to be major players in the regulation of ontogenetic and/or lesion evoked neuronal death in the central nervous system (Ciccarelli et al., 1999; Connor and Dragunow, 1998). In contrast to the effects of TGF-β Ab on 2-PMPA-mediated neuroprotection, neutralizing antibodies to FGF-basic, NGF-β, TNF-α and S-100 were ineffective in reversing the neuroprotection provided by 2-PMPA (Fig. 2B). These data suggest a specific effect of TGF-βs and, therefore, may be the necessary factors involved in neuroprotective mechanisms mediated by inhibition of GCP II.

We also examined the contribution of TGF-βs, in neuroprotective mechanisms mediated by inhibition of GCP II, using an in vivo model of cerebral ischemia. Our in vivo results are consistent with our in vitro observations. While intravenous administration of 2-PMPA reduced the ischemic injury induced by middle cerebral artery occlusion, concomitant intracerebral injections TGF-β Ab with 2-PMPA attenuated the neuroprotective effects of GCP II inhibition against middle cerebral artery occlusion (Fig. 3). As with the in vitro experiments, TGF-β Ab, acting on its own, did not worsen middle cerebral artery occlusion-induced ischemic injury (Fig. 3).

In summary, our results provide evidence suggesting that neuroprotection afforded by inhibition of GCP II may be mediated by endogenous TGF-β release, possibly via NAAG's activation of $mGlu_3$ receptors. These results are consistent with earlier findings wherein 2-PMPA was neuroprotective through decreased glutamate and increased NAAG (Slusher et al., 1999), and NAAG itself was neuroprotective through both mGluR3 activation (Bruno et al., 1998c; Lu et al., 2000; Thomas et al., 2000) and TGF-β release (Bruno et al., 1998c). While activation of glial $mGlu_3$ receptors was not specifically confirmed in these experiments, recently presented evidence suggests that activation of glial mGluR3 is necessary in neuroprotective mechanisms mediated by NAAG and GCP II inhibition (Thomas et al., 2001). Also noteworthy is the fact that in in vivo ischemia experiments, NAAG demonstrates strong neuroprotection in subcortical regions of the brain (Lu et al., 2000) rich in glial $mGlu_3$ expression (Testa et al., 1994).

Regardless of the precise nature of the mechanisms involved, these experiments demonstrate that GCP II inhibition may be involved in regulating endogenous TGF-β release, possibly via increasing the availability of the endogenous mGluR3 ligand, NAAG (Slusher et al., 1999). Both TGF-β and $mGlu_3$ receptor agonists have been shown to protect against different forms of excitotoxicity and neuronal apoptosis (Bruno et al., 1998b; Flanders et al., 1998; Nicoletti et al., 1996; Pratt and McPherson, 1997). Consequently, GCP II inhibitors could find potential therapeutic uses in various diseases wherein neuronal degeneration is marked by excitotoxicity and neuronal apoptosis. Additionally, unlike NMDA or AMPA receptors antagonists, which have been known to cause sedation, impairment of synaptic plasticity, ataxia or psychotomimetic

effects, GCP II inhibitors do not cause similar acute behavioral effects in normal animals (Slusher et al., 1999).

Taken together, GCP II inhibitors are a promising approach to neuroprotection because these drugs may not only decrease glutamate but also increase NAAG, thereby providing protection via pre-synaptic metabotropic receptor activation and through TGF-β release via post-synaptic glial metabotropic receptor activation.

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