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Neuroprotective signaling pathways are modulated by adenosine in the anoxia tolerant turtle

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Cumulative evidence shows a protective role for adenosine A1 receptors (A1R) in hypoxia/ischemia; A1R stimulation reduces neuronal damage, whereas blockade exacerbates damage. The signal transduction pathways may involve the mitogen-activated protein kinase (MAPK) pathways and serine/threonine kinase (AKT), with cell survival depending on the timing and degree of upregulation of these cascades as well as the balance between pro-survival and pro-death pathways. Here, we show *in vitro* that extracellular signal-regulated kinase (ERK1/2) and phosphatidylinositol 3-kinase (PI3-K/AKT) activation is dependent on A1R stimulation, with further downstream effects that promote neuronal survival. Phosphorylated ERK1/2 (p-ERK) and AKT (p-AKT) as well as Bcl-2 are upregulated in anoxic neuronally enriched primary cultures from turtle brain. This native upregulation is further increased by the selective A1R agonist 2-chloro-*N*-cyclopentyladenosine (CCPA), whereas the selective antagonist 8-cyclopentyl-1,3-dihydropylxanthine (DPCPX) decreases p-ERK and p-AKT expression. Conversely, A1R antagonism resulted in increases in phosphorylated JNK (p-JNK), p38 (p-p38), and Bax. As pathological and adaptive changes occur simultaneously during anoxia/ischemia in mammalian neurons, the turtle provides an alternative model to analyze protective mechanisms in the absence of evident pathologies.
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Introduction

Owing to their high basal oxygen requirements, neurons are extremely sensitive to ischemic insult. Any interruption in blood flow to the brain results in a rapid depletion of oxygen, which severely compromises ATP production and leads to failure of ionic conductance and excitotoxicity (Lee *et al*, 2000). Neurons respond to compromised oxygen availability by triggering a dynamic network of signaling pathways including the mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3-K/AKT). The MAPKs constitute a superfamily of proline-directed serine/threonine kinases that includes the extracellular signal-regulated kinases (ERK) and the stress-activated protein kinases (such as JNK/SAPK1 and p38/SAPK2). The MAPKs control numerous intracellular events in response to external stimuli, ultimately changing cell function through altered gene expression or the posttranslational modification of proteins (Martindale and Holbrook, 2002). Activation of the MAPKs and PI3-K/AKT involves phosphorylation and takes place in response to a variety of stressors including oxidative stress and anoxia (Clerk *et al*, 1998; Kamada *et al*, 2007).

The ERK signaling cascade has been extensively studied in several cell types; activation of ERK has been found to be protective in PC12 cell and rat ischemic models (Xia *et al*, 1995; Irving *et al*, 2000), though others have found ERK activation promotes cell death after ischemia (Wang *et al*, 2003; Zhuang and Schnellmann, 2006; Sawe *et al*, 2008). Activation of the ERK pathway is thus implicated in both neuronal survival and death and differences may be linked to the trigger, dynamics, and duration of its activation (Martindale and Holbrook, 2002; Ho *et al*, 2007). Rapid and transient activation of ERK seems to be pro-survival (Li *et al*, 2002), whereas long-term activation apparently promotes cell death (Wang *et al*, 2003). The role of other MAPK pathways is also under debate, with investigations reporting JNK and p38 as apoptosis inducers (Guan *et al*, 2006),

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whereas others showed protective effects of JNK and p38 in different mammalian ischemic models (Dougherty *et al*, 2002; Beguin *et al*, 2007).

The PI3-K/AKT is generally reported to be anti-apoptotic (Zhao *et al*, 2006); in part through its interactions with the Bcl-2 family of proteins. Activation of the AKT pathway phosphorylates Bad and suppresses its pro-apoptotic activity in both *in vivo* ischemic models (Miyawaki *et al*, 2008) and *in vitro* (Datta *et al*, 1997). Studies reveal that a high degree of cross-talk exists between the signal transduction pathways, however, and alteration of one pathway will affect other pathways and thus influence the outcome after ischemia/reperfusion.

Determining whether a particular pathway is pro-survival or pro-apoptotic in mammalian models, however, is difficult, in part because they simultaneously exhibit both physiological and pathological responses to such stressors as hypoxia, ischemia, or oxidative stress. But such events can be dissected and analyzed in the anoxia tolerant freshwater turtle, which survives extended anoxia and postanoxic reoxygenation without brain damage (Lutz and Milton, 2004; Kesaraju *et al*, 2009). It is thus likely in such a model that modulation of a particular molecular signaling cascade is adaptive rather than pathological. Determining survival mechanisms in such an alternative animal might help to disentangle the complexities of vulnerable mammalian neurons and provide insights into the mechanisms of mammalian anoxic brain damage and survival, including critical upstream signals that regulate intracellular events.

One signaling molecule thought to affect AKT and the MAPK pathways is adenosine (AD), a neuroprotectant rapidly formed during ischemia as a result of the intracellular breakdown of ATP (Dunwiddie and Masino, 2001). AD has a role in preconditioning in the brain (Ciccarelli *et al*, 2007) and heart (Downey *et al*, 2007). Recent studies have shown that activation of AKT confers protection during adenosine mediated and myocardial ischemic preconditioning (Ban *et al*, 2008); however, the intracellular signaling pathways mediating the protective effects of adenosine are still under investigation (Brust *et al*, 2006). In turtles, it has been shown that AD is critical at the physiological level to anoxic neuronal survival, acting as a 'retaliatory metabolite' to balance energy supply and demand (Nilsson and Lutz, 1991). The AD levels increase as much as 12-fold over the initial 1 to 2 hours anoxia (Nilsson and Lutz, 1991) accompanied by an increase in A1 receptor (A1R) sensitivity (Lutz and Manuel, 1999). The ADR activation modulates extracellular levels of excitotoxins (Milton *et al*, 2002; Milton and Lutz, 2005) and is a key element of channel arrest (Pek and Lutz, 1997). We have also recently reported that AD affects MAPK and AKT levels in the turtle brain *in vivo* where an initial upregulation of phosphorylated ERK1/2 (p-ERK) and p-AKT and suppression of p-p38MAPK were blocked by the general AD antagonist aminophylline (Milton *et al*, 2008), whereas blockade of the AD A1R increased cell death and reactive oxygen species (ROS) release in cultured turtle neurons (Milton *et al*, 2007). Similarly, investigations in mammalian models reported that purinergic activation of ERK and AKT pathways mediate ischemic preconditioning in heart (Germack *et al*, 2004), whereas AD-mediated suppression of p38 and Bad was reported in astrocytes exposed to oxygen glucose deprivation (Ciccarelli *et al*, 2007). By contrast, Brust *et al* (2006) showed that A1R stimulation inhibits presynaptic neurotransmission through activation of p38MAPK. We hypothesize that adenosine activates the pro-survival MAPK (ERK) and AKT pathways while suppressing the pro-death MAPK pathways (JNK and p38) in isolated anoxic turtle neurons. Using phospho-protein-specific antibodies, we measured changes in the levels of these activated proteins during anoxia and in the presence of A1R modulators. In this study, we expand our earlier studies of general AD-linked protective mechanisms (Milton *et al*, 2007, 2008) by investigating the effects of A1R-specific agonist and antagonist administration on the MAPKs, AKT, and the primary modulators of stress-related apoptosis.

Materials and methods

All experiments were approved by the Florida Atlantic University Institutional Animal Care and Use Committee. Juvenile freshwater turtles (4 to 6 inch carapace) were obtained from a commercial supplier (Clive Longdon, Tallahassee, FL, USA). Turtles were maintained in freshwater aquaria on a 12-hour light–dark cycle and were fed three times weekly. Animals are killed for cell culture harvest by decapitation in accordance with the animal care standards established by the American Veterinary Medical Association for reptiles.

Cell Culture Preparation

We have successfully isolated and maintained turtle primary neuronally enriched cell cultures using a density centrifugation method adopted from Brewer (1997) (Milton *et al*, 2007). Briefly, brain tissue was minced aseptically and digested in a cocktail of enzymes (collagenase (25 U/mL), dispase (0.32 U/mL), hyaluronidase (1300 U/mL) in 4 mL of minimal essential media containing 10% fetal bovine serum (FBS), 56 units each of penicillin and streptomycin with gentle rocking for 4 hours before centrifugation at 750 *g* for 15 minutes in a density gradient at room temperature (25°C) using Optiprep medium (Sigma-Aldrich, St Louis, MO, USA). The neuronal layer was then plated in culture dishes with minimal essential media medium containing FBS and penicillin maintained at 30°C in 5% CO_2 incubator (Kendro Laboratory Products, Asheville, NC, USA). Survival rates were ~75% beyond 4 days *in vitro* (DIV). Immunostaining with the neuronal markers NeuN and NCAM and the glial marker GFAP confirmed that cultures are consistently >85% neuronal cells.

Immunostaining

To confirm the neuronal phenotype, cultured neurons were double immunostained with primary antimouse NeuN antibody, antirabbit neurofilament and antirabbit cysteine string protein neuronal markers. Alexa Fluor 568 goat antimouse or antirabbit antibodies (Invitrogen, Molecular probes, Eugene, OR, USA) were used as secondary antibodies. Briefly, cells were fixed with 1 to 2 mL of freshly prepared 4% paraformaldehyde for 10 minutes. Cells were washed three times with phosphate-buffered saline and further permeabilized with 0.1% Triton X-100 in phosphate-buffered saline at room temperature. Cells were then washed with 0.1% Triton X-100 and nonspecific binding was blocked with blocking solution (0.5% bovine serum albumin in phosphate-buffered saline for 15 minutes. Cells were incubated overnight at 4°C with primary antimouse NeuN and antirabbit neurofilament/cysteine string protein antibodies (Chemicon, Billerica, MA, USA). After washings, this was followed by Alexa Fluor 568 goat antimouse/rabbit-secondary antibodies used for 1 hour in a 1:1000 dilution. After further washings, cells were prepared with Vectashield mounting medium (Vector laboratories, Inc., Burlingame, CA, USA) and coverslipped. Cells were observed using a confocal microscope equipped for epifluorescence.

Experimental Treatments

Cell cultures were used at ~14 DIV and existing media was replaced with minimal essential media containing the specific A1R blocker 8-cyclopentyl-1,3-dihydropyl-xanthine (DPCPX, 1 nmol/L) or the A1R agonist 2-chloro-*N*-cyclopentyladenosine (CCPA, 0.6 nmol/L). Cells were then subjected to experimental conditions including normoxia (maintained at room temperature), or 1, 4, or 12 hours anoxia in an anoxia chamber (Bactron Environmental Hypoxia Chamber, Sheldon Manufacturing, Cornelius, OR, USA) under anoxic gas mixture (90% N_2, 5% H_2, 5% CO_2, AirGas, Miami, FL, USA). Continuous monitoring of oxygen is performed with an OM-4 oxygen meter (Microelectrodes Inc., Bedford, NH, USA), which ensures that the chamber is anoxic. Culture plates established from the same turtles and exposed simultaneously to experimental conditions without the drugs were used as controls.

Protein Extractions

For protein extraction, neurons were washed with ice-cold phosphate-buffered saline. Cells were further treated with 50 μL of RIPA lysis buffer (5 mmol/L EDTA, pH 8.0, 0.15 mol/L NaCl, 1% Triton X-100, 10 mmol/L Tris-Cl, 2 mmol/L protease inhibitor pH 7.4) and scraped into individual tubes on ice for 10 minutes. The samples were triturated and centrifuged for 10 minutes at 18,500 *g* at 4°C. The supernatant was then frozen at −80°C until use. The protein concentration was determined by standard BCA protein assay (Pierce Biotechnology, Rockford, IL, USA).

Immunodetection

Proteins were detected by standard Western blot as described earlier, using horseradish peroxidase (HRP)-conjugated secondary antibodies (Milton *et al*, 2008). The rabbit polyclonal antibodies for the nonphosphorylated MAPKs and rabbit Bax polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). These included ERK1/2 (SC-93), JNK1 (SC-571), p38MAPK (SC-535), and Bax (SC-526). Rabbit polyclonal antibody to AKT (#9272) and phosphorylated antibodies phospho-AKT (#9271), phospho-p38MAPK (#4631), phospho-JNK (#4671), phospho-ERK1/2 (#4376), all were from Cell Signaling Technology (Danvers, MA, USA). Antirabbit Bcl-2 antibody was from Chemicon (Temecula, CA, USA).

The protein-secondary antibody complexes were identified using a chemiluminescent system (GE Healthcare, Piscataway, NJ, USA). Densitometric analysis was conducted using National Institute of Health Image J 1.60 software and band densities expressed as a percentage of the β-actin signal. Actin was used as a loading control, and its expression remained unchanged in all experimental conditions. Earlier studies in our laboratory have also indicated that β-actin levels do not change with anoxia or reoxygenation in this model (Milton *et al*, 2008). Data are expressed as percent change of the control group.

Statistically significant differences between treatments were determined by analysis of variance with *post hoc* analysis using the SPSS statistical package. Differences were considered statistically significant at $P < 0.05$.

Results

We performed double-labeled immunostaining using NeuN, neurofilament, and cysteine string protein neuronal markers. Figure 1A clearly indicates the neuronal nuclei (green) and localization of the neuronal filaments in cytoplasm (red), and Figure 1B shows neuronal nuclei (green) and cysteine string protein (red) in the neuronal cytoplasm thus confirming the neuronal identity of our cultures.

Alterations in Mitogen-Activated Protein Kinase/AKT Protein Levels in Normoxia and Anoxia Without Adenosine Receptor Agonists and Antagonists

Baseline: We observed significant changes in all the activated/phosphorylated MAPKs/AKT in turtle neuronally enriched cell cultures, though the changes in cell culture occurred more slowly and to a lesser degree than was reported in the whole brain (Milton *et al*, 2008). Increases in the protein levels of presumed pro-survival pathways p-ERK and p-AKT (Figures 3A and 4A) were higher than those thought to be primarily pro-death: p-p38 and p-JNK (Figures 5A and 6A). Basal anoxic levels of the nonphosphorylated MAPKs and AKT in cell culture were generally not different from normoxia in untreated controls; the levels of actin also remained unchanged in all experimental conditions, indicating that the increases in protein levels were not a generalized stress effect on the cells (Figure 2).

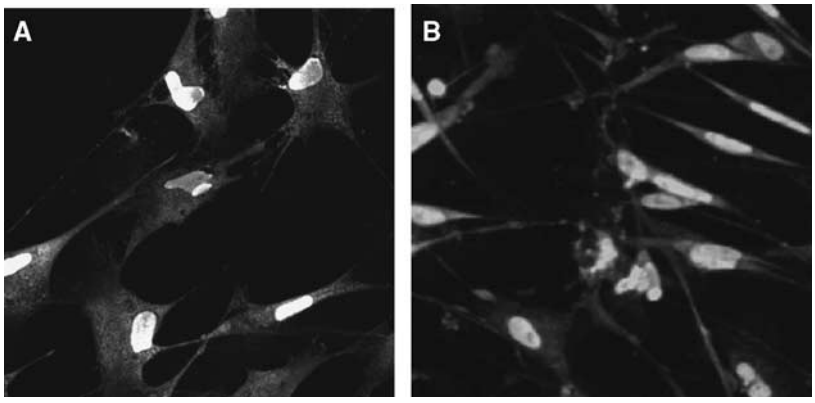

Figure 1 (**A**) Merged image (× 40) of double-labeled NeuN (green) and neurofilament (red) immunostaining showing the NeuN-positive neuronal nuclei and neuronal filaments in the cytoplasm. Scale bar = 25 μm. (**B**) Merged image (× 40) of double-labeled NeuN (green) and cysteine string protein (red) in the neuronal cytoplasm showing NeuN-positive neuronal nuclei and cysteine string proteins. Scale bar = 25 μm. The color reproduction of this figure is available on the html full text version of the manuscript.

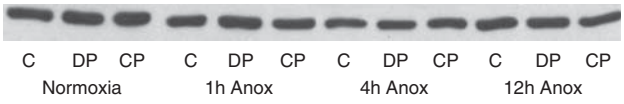

Figure 2 Representative Western blot showing expression of β-actin in turtle neuronally enriched cell cultures. Actin expression is unchanged in all experimental conditions. C, controls; CCPA, 2-chloro-*N*-cyclopentyladenosine; CP, CCPA treated; DP, DPCPX treated; DPCPX, 8-cyclopentyl-1,3-dihydropylxanthine.

ERK/p-ERK: Changes in p-ERK were rapid, increasing significantly to 374% ± 11% of normoxic controls by 1 hour anoxia. The p-ERK levels reached 440% ± 9% by 4 hours anoxia before decreasing to 308% ± 18% by 12 hours anoxia (Figure 3). Nonphosphorylated ERK expression in anoxia did not change significantly from normoxic controls.

AKT/p-AKT: Levels of the presumed pro-survival p-AKT protein levels also increased rapidly in anoxia, more than doubling in the first hour of anoxia and increasing further to 318% ± 11% by 4 hours of anoxia (Figure 4). The levels dropped slightly but were still elevated after 12 hours of anoxia. Levels of nonphosphorylated AKT did not change significantly in any experimental conditions.

p38/p-p38: Active p-p38 levels increased to 178% ± 11% of basal by 4 hours anoxia, with a slight decrease by 12 hours anoxia to 161% ± 7% of normoxic control (Figure 5). Levels of nonphosphorylated p38MAPK also increased significantly (about twofold) by 4 hours of anoxia, but decreased toward basal by 12 hours of anoxia.

JNK/p-JNK: As with p-p38, we observed a significant increase to 194% ± 6% over basal in p-JNK by 4 hours anoxia, which also decreased by 12 hours anoxia to only 154% ± 10% of normoxic levels (Figure 6).

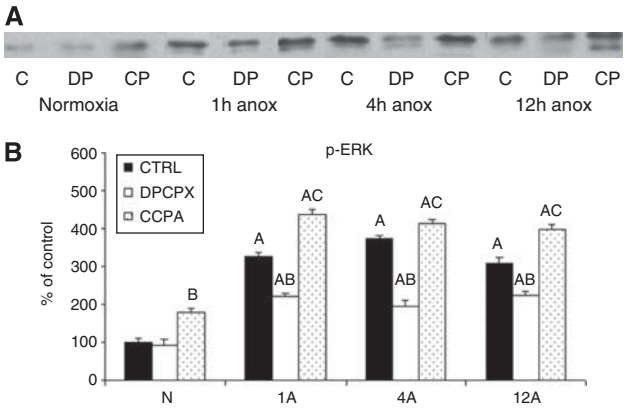

Figure 3 Representative Western blot of phosphorylated extracellular signal-regulated kinase (p-ERK) (**A**) and densitometric analyses (**B**) of p-ERK1/2 levels under control and experimental conditions (treated with ADR agonist and antagonist) in turtle neuronally enriched cell cultures. All normoxia, 1, 4, and 12 hours anoxia all on a single gel C, controls; DP, DPCPX treated; CP, CCPA treated. Data are mean ± s.e.m., $N = 5$ independent experiments/group. A = significant difference from normoxia control for the same treatment, $P < 0.05$; B = significant difference from controls at the same time point; C = significant difference from controls at that point and DPCPX, $P < 0.05$. CCPA, 2-chloro-*N*-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dihydropylxanthine.

Levels of nonphosphorylated JNK did not change significantly in anoxia.

Bcl-2/Bax: The Bcl-2 to Bax ratio is a critical factor whose balance determines cell survival or death through apoptosis. In this study, we observed significant increases in both Bcl-2 and Bax levels when subjected to anoxic conditions. The increase in Bcl-2 was relatively higher (182% ± 12%; Figure 7B) compared with Bax levels, which increased only to 142% ± 10% of normoxia by 4 hours anoxia (Figure 7D).

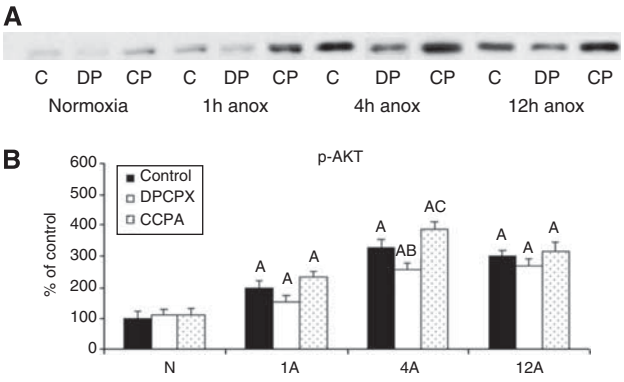

Figure 4 Representative Western blot of p-AKT (**A**) and densitometric analyses (**B**) of p-AKT levels under control and experimental conditions (treated with ADR agonist and antagonist) in turtle neuronally enriched cell cultures. Normoxia, 1, 4, and 12 hours anoxia all on different gels. C, controls; DP, DPCPX treated; CP, CCPA treated. Data are mean ± s.e.m., $N = 5$ independent experiments/group. A = significant difference from normoxia control for the same treatment, $P < 0.05$; B = significant difference from controls at the same time point; C = significant difference from controls at that point and DPCPX, $P < 0.05$. CCPA, 2-chloro-*N*-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dihydropylxanthine.

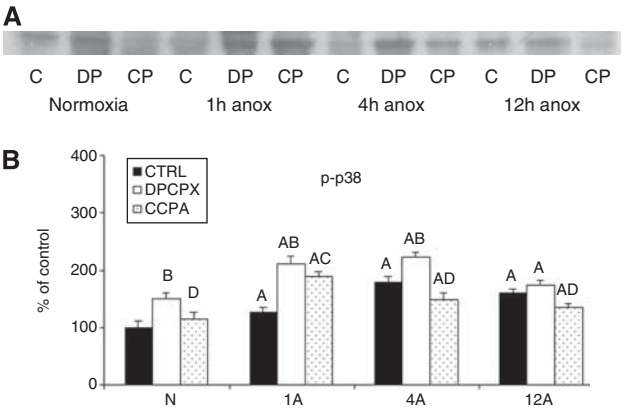

Figure 5 Representative Western blot of p-p38 (**A**) and densitometric analyses (**B**) of p-p38MAPK levels under control and anoxic experimental conditions (treated with ADR agonist and antagonist) in turtle neuronally enriched cell cultures. Normoxia, 1, 4, and 12 hours anoxia samples all on a single gel. C, controls; DP, DPCPX treated; CP, CCPA treated. Data are mean ± s.e.m., $N = 5$ independent experiments/group. A = significant difference from normoxia control for the same treatment, $P < 0.05$; B = significant difference from controls at the same time point; C = significant difference from controls at that point and DPCPX, $P < 0.05$; and D = significant difference from DPCPX, $P < 0.05$. CCPA, 2-chloro-*N*-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dihydropylxanthine; MAPK, mitogen-activated protein kinase.

Alterations in Mitogen-Activated Protein Kinase/AKT Pathway Components After Exposure to Adenosine Receptor Agonists and Antagonists

2-Chloro-N-Cyclopentyladenosine/8-Cyclopentyl-1,3-Dihydropylxanthine exposures: Inhibition of adenosine receptors with the specific adenosine A1R antagonist DPCPX significantly suppressed the activation of the presumed pro-survival signaling p-ERK cascade and moderately decreased p-AKT levels compared with untreated anoxic controls, and resulted in the dramatic suppression of Bcl-2 protein levels (Figure 7). The suppression of p-ERK and p-AKT occurred simultaneously with moderately increased activation of the pro-death p38MAPK cascade and also increased the levels of the pro-apoptotic Bax. The A1R blockade, however, had no significant effect on p-JNK levels. Conversely, p-AKT and p-ERK levels were greater in cells treated with CCPA than anoxic controls, whereas p-p38 and p-JNK levels were lower. Neither drug treatment significantly affected the levels of nonphosphorylated Akt or the MAPK in general, except for an increase in nonphosphorylated JNK with DPCPX exposure at 12 hours anoxia. The levels of actin also remained unchanged in all experimental conditions, indicating that the increases in protein levels were not a generalized treatment effect on the cells (Figure 2).

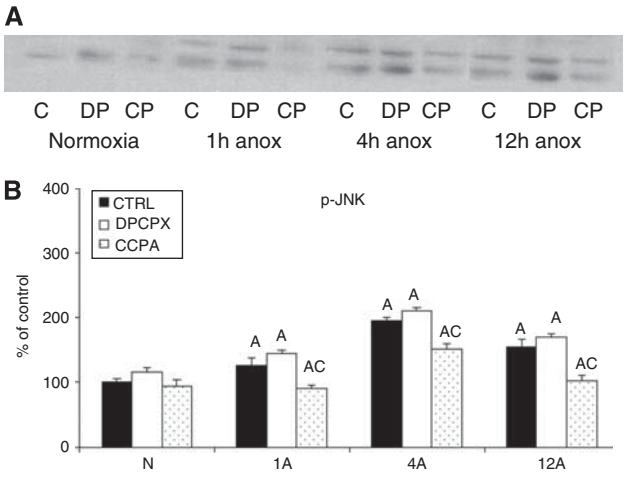

Figure 6 Representative Western blot of p-JNK (**A**) and densitometric analyses (**B**) of p-JNK levels under control and experimental conditions (treated with ADR agonist and antagonist) in turtle neuronally enriched cell cultures. Normoxia, 1, 4, and 12 hours anoxia samples all on a single gel. C, controls; DP, DPCPX treated; CP, CCPA treated. Data are mean ± s.e.m., $N = 5$ independent experiments/group. A = significant difference from normoxia control for the same treatment, $P < 0.05$; B = significant difference from controls at the same time point; C = significant difference from controls at that point and DPCPX, $P < 0.05$. CCPA, 2-chloro-*N*-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dihydropylxanthine.

ERK/p-ERK: Compared with the other MAPKs and AKT, the greatest effects from the manipulation of A1Rs with DPCPX and CCPA were on p-ERK levels. The p-ERK levels increased to 440% ± 10% by 1 hour anoxia in CCPA-treated cells (Figure 4B) and remained elevated through 12 hours anoxia. The native increases in p-ERK activation were significantly suppressed by treatment with DPCPX, such

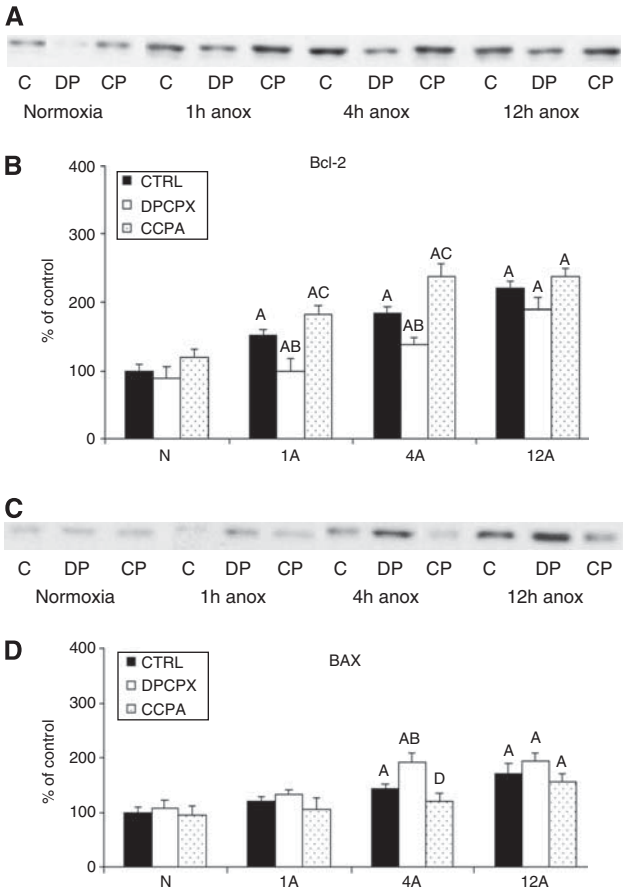

Figure 7 Representative Western blots of Bcl-2 and Bax (**A**, **C**) and densitometric analyses (**B**, **D**) of Bcl-2 and Bax levels under control and experimental conditions (treated with ADR agonist and antagonist) in turtle neuronally enriched cell cultures. Normoxia, 1, 4, and 12 hours anoxia samples all on a single gel. C, controls; DP, DPCPX treated; CP, CCPA treated. Data are mean ± s.e.m., $N = 5$ independent experiments/group. A = significant difference from normoxia control for the same treatment, $P < 0.05$; B = significant difference from controls at the same time point; C = significant difference from controls at that point and DPCPX, $P < 0.05$; and D = significant difference from DPCPX, $P < 0.05$. CCPA, 2-chloro-*N*-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dihydropylxanthine.

that p-ERK increases were only twofold above normoxic levels at 1, 4, and 12 hours anoxia (Figure 3). Treatment with CCPA and DPCPX did not alter the nonphosphorylated levels of ERK.

AKT/p-AKT: Treatment with CCPA over 1 hour anoxia increased the activated AKT levels slightly compared with untreated controls; this increase was not significant until 4 hours anoxia. Treatment with DPCPX decreased native p-AKT increases only slightly, to 154% ± 15% and 261% ± 7% of untreated controls at 1 and 4 hours anoxia, respectively. The trend was similar at 12 hours anoxia; however, the differences in treated cells were not significantly different from untreated anoxic controls (Figure 4). Levels of the nonphosphorylated AKT remained unchanged in cultures treated with DPCPX or CCPA.

p38/p-p38: Manipulation of the A1R resulted in relatively small effects on p38MAPK compared with p-ERK or p-AKT. Treatment with CCPA decreased p-p38 levels below untreated anoxic controls at 4 and 12 hours anoxia, but had no effect in normoxic cells or at 1 hour anoxia (Figure 5). Treatment with DPCPX significantly increased the p-p38 levels by 1 and 4 hours anoxia (210% ± 15% and 224% ± 7%, respectively) compared with their respective controls. The p-p38 levels at 12 hours anoxia were still elevated, but the differences were not significant from the controls at the same time point.

JNK/p-JNK: As was seen in the whole brain (Milton *et al*, 2008), manipulation of A1R had the least effects on p-JNK levels. The activated levels of JNK were only modestly but nonetheless significantly suppressed by CCPA at 4 and 12 hours anoxia (Figure 6), to 78% and 65% of anoxic controls, respectively, whereas increases in p-JNK in cells treated with A1R antagonist DPCPX were not significant at any time point. Nonphosphorylated JNK levels were unaltered by treatment with DPCPX in 1 and 4 hours anoxia, though a significant increase of about twofold was observed by 12 hours anoxia compared with untreated levels at the same time point. The JNK levels were reduced by pretreatment with CCPA; however, the suppression was not significant compared with the controls.

Alterations in Pro and Antiapoptotic Pathway Components After Exposure to Adenosine Receptor Agonists and Antagonists

Bcl-2/Bax: We observed an increase in antiapoptotic Bcl-2 levels in cells pretreated with CCPA at all time points. This increase was significant by 1 hour anoxia (152% ± 18%) and increased to 185% ± 12% by 4 hours anoxia; these levels remained elevated at 12 hours anoxia (Figure 7B). Conversely, exposing the cells to DPCPX decreased Bcl-2 levels to 75% of untreated controls by 4 hours anoxia. The CCPA and DPCPX had the opposite effects on Bax expression: pretreatment with CCPA decreased Bax levels, whereas DPCPX increased Bax by 4 and 12 hours anoxia to 193% ± 11% and 200% ± 15%, respectively (Figure 7D) compared with their controls at that time point. Thus, in cultures treated with the A1R antagonist, the Bcl-2/Bax ratio decreased from 1.28 in untreated anoxic controls to 0.71 at 4 hours anoxia and 0.95 in extended anoxia, whereas CCPA increased the ratio to 2.0 at 4 hours anoxia and 1.5 by 12 hours anoxia.

Discussion

Although the physiology of anoxic survival in freshwater turtles such as *Trachemys scripta* and

Chrysemys picta has been widely examined, fewer studies have looked at molecular adaptations in the turtle brain. *In vivo* activation of MAPKs (Greenway and Storey, 2000), and the suppression of Bax (Haddad, 2007; Kesaraju *et al*, 2009) in hypoxia have been reported but links between the physiology and molecular pathways are even less well studied. We previously reported that initial *in vivo* increases in p-ERK and p-AKT activation, and the suppression of p-p38, could be abrogated by the general AD receptor blocker aminophylline (Milton *et al*, 2008). We have also reported that the AD agonist CCPA protects neuronally enriched cultures, whereas the AD blocker DPCPX increases cell death under conditions of oxidative stress (Milton *et al*, 2007). With treatments similar to those presented in this paper, cell death increased from ~10% in normoxic cells (control and drug treatments) to 25% in untreated cells after 12 hours anoxia/reoxygenation, but was >50% in DPCPX-treated cells (Milton *et al*, 2007). In this study, we expanded on those findings using neuronally enriched primary cell cultures to examine interactions between the specific adenosine A1R and the MAPK pathways, as well as the downstream regulators of apoptosis, Bcl-2 and Bax. In general, we found that the MAPK and AKT pathways *in vitro* responded similarly to the anoxic brain *in vivo*, though the changes were slower and less dramatic than in the whole brain, and were sustained over the long term rather than elevated only temporarily. The ERK and AKT pathways increased significantly in the initial hour of anoxia, and these levels were further increased threefold to fourfold over normoxic levels after 4 hours of anoxia. By contrast, we observed a sixfold increase in p-ERK in the first hour of anoxia *in vivo*, which returned to normoxic levels by 4 hours anoxia (Milton *et al*, 2008). Changes in p-AKT *in vitro* were in line with whole brain induction (AKT *in vivo* increased to 323% of basal by 1 hour anoxia (Milton *et al*, 2008), though again in cell culture this elevation was sustained rather than temporary.

In contrast to the whole brain, we also observed a moderate increase in p38 and JNK activation in the cell cultures, though the increases were less than for the presumed pro-survival kinases. These results are similar to those observed in mammalian models where hypoxia/ischemia leads to the activation of all the MAPKs (Irving *et al*, 2000; Li *et al*, 2002; Wang *et al*, 2003), and together with the sustained MAPK activation suggests that the cells in culture are under more physiological stress during anoxia than is the intact brain, most likely due to the lack of glial support or such whole body adaptations as buffering capacity.

Mammalian hypoxia/ischemia experimental models to date have examined the role of MAPK and AKT signaling cascades in determining the outcome of cerebral ischemia. However, the triggers to the activation of these complex cascades in the brain have not been widely investigated. Fewer studies have focused on the immediate effects of adenosine on preventing neuronal cell death after ischemia in mammals, though these studies support our results showing A1R protection occurring through the upregulation of ERK and AKT and the suppression of p38MAPK (Gervitz *et al*, 2002; Ciccarelli *et al*, 2007; Ban *et al*, 2008). A reduction in blood flow to the mammalian brain during hypoxia/ischemia causes a steep elevation in the extracellular levels of adenosine as ATP breaks down; adenosine in turn acts as a neuroprotectant (Canals *et al*, 2005; Dunwiddie and Masino, 2001). Unlike mammals, however, the adenosine increase in the turtle brain is temporary (Nilsson and Lutz, 1991). By 4 hours anoxia, the turtle has entered a fully hypometabolic state, in which energy consumption is suppressed to match anaerobic energy production, allowing ATP levels to return to normal (see reviews Lutz and Milton, 2004; Milton and Prentice, 2007). Thus, in the turtle, the initial 1 to 2 hours anoxia is a crucial period of physiological stress, which it survives by applying a suite of adaptations, and it is during this transition phase that we see significant molecular changes, including increases in a variety of heat shock proteins (Kesaraju *et al*, 2009) and the MAPK reported here. The initial *in vivo* increases in p-ERK and p-AKT and the suppression of p-p38 in the first hour of anoxia in the turtle are linked to AD, and can be blocked with the general AD receptor antagonist aminophylline (Milton *et al*, 2008). A similarly rapid activation of ERK and AKT in the mammal has a key role in neuroprotection (Li *et al*, 2002), but it is reported that prolonged elevation of ERK increases cell death in an *in vivo* rat ischemic model (Wang *et al*, 2003). By contrast, the early and prolonged activation of the PI3-K-AKT pathway leads to neuronal survival in the rat ischemic model (Shibata *et al*, 2002). In contrast to our *in vivo* work (Milton *et al*, 2008), p-ERK activation is sustained in the primary neuronal cultures, although cell death still averaged <22% over 4 hours anoxia versus <10% basal cell death in normoxia (Milton *et al*, 2007). High survival with sustained elevation of p-ERK may result from the other accompanying changes we observed, including increases in p-AKT and Bcl-2, which overshadowed more modest increases in p-JNK, p-p38MAPK, and Bax. Alternatively, it may be that the sustained activation of ERK in mammalian systems is not a trigger for further cell damage but rather a symptom of additional stress that culminates in cell death.

As previously reported *in vivo*, AD is critical to survival in cultured neurons; AD1R blockade increases ROS release and cell death, whereas treatment with A1R agonist decreases ROS production and increased survival in anoxia and anoxia/reoxygenation (Milton *et al*, 2007). Similarly, studies in mammalian systems have shown that AD is critical to ischemic survival; AD decreases neurological damage after ischemia/reperfusion injuries, whereas AD blockade increases neurological and behavioral deficits in ischemic rats (Gupta *et al*, 2002).

In mammals, several studies have shown that neuroprotective AD effects even in acute ischemia result from downstream molecular events triggered by ADR activation, including activation of AKT (Gervitz *et al*, 2002) ERK1/2, and other MAP kinases (Brust *et al*, 2006). Activation of the ERK pathway leads to the phosphorylation of a wide range of cellular substrates and activates transcription of cAMP response element binding protein, which activates the pro-survival Bcl-2 and suppresses apoptotic proteins such as BAD, Bim, and the caspases (Chan, 2004; Sawe *et al*, 2008) and prevent apoptosis. Similarly, the protection offered by AKT is thought to be in part through its interactions with the Bcl-2 family of proteins. The AKT targets cAMP response element binding protein to activate pro-survival mechanisms (Shibata *et al*, 2002) and also phosphorylates BAD (Datta *et al*, 1997; Chan, 2004; Kamada *et al*, 2007), thus preventing its translocation to the mitochondria, inhibiting the release of apoptogenic proteins (Wang *et al*, 2007), and decreasing other stress pathways (Kamada *et al*, 2007; Wang *et al*, 2007). Ciccarelli *et al* (2007) have shown that AD1R reduces apoptosis by suppressing JNK and p38MAPK, simultaneously activating ERK and AKT, and decreasing the levels of pro-apoptotic proteins like Bad while elevating levels of antiapoptotic Bcl-X(L). The results of this study suggest that the links between AD and cell survival in anoxia/ischemia in turtles also occur through the MAPKs and their downstream effects. In the turtle cultures, both Bcl-2 and Bax increased along with ERK and AKT, but with Bcl-2 more strongly upregulated than Bax, suggesting that the molecular pathways are tilted towards cell survival even with accompanying (but smaller) increases in p38MAPK and JNK.

In addition, Bcl-2 has been shown to have a major role in suppressing cell death during apoptotic and oxidative stress cell injury by enhancing levels of antioxidants and suppressing the generation of free radicals (Lee *et al*, 2001). In this study, the anoxic increases in Bcl-2 expression above Bax were reversed by ADR blockade; ADR blockade is accompanied by an increase in ROS production and doubling of cell death (Milton *et al*, 2007), implicating that Bcl-2 protein levels can be altered by adenosine receptor activation, which might be directly linked to ROS suppression in species. These studies suggest that the pathway of ROS suppression in turtles is similar to that in mammals, but is expressed more robustly and hence is more successful in protecting turtle neurons against anoxia and reoxygenation damage.

Thus, in addition to its other myriad neuroprotective effects in the anoxic turtle brain; we have shown that activation of ADR receptor also increases cell survival by modulating molecular pathways in favor of cell survival. Pretreatment with CCPA increased activation of ERK and AKT, which further lead to elevation of Bcl-2, inhibition of Bax, and reduced activation of p-p38; these changes in turn are thought to stabilize the mitochondria and reduce ROS release (Gervitz *et al*, 2002; Brust *et al*, 2006). The overall observations from earlier investigations and this current study clearly indicate a link between the physiological mechanisms of survival (elevated AD, low ROS) and molecular adaptations in this anoxia tolerant model. Through utilization of an animal model of anoxia tolerance, honed for survival by millions of years of adaptation, and investigating the differences between *in vivo* and *in vitro* survival, it is clear that increases in p-ERK and p-AKT (pro-survival pathways), and a reduction of activated p38 (pro-death pathway) through ADR stimulation, promote neuronal survival, and that continued high levels of ERK is not necessarily a trigger of cell death if other molecular changes are sufficient to promote survival. The use of an alternative animal model, able to survive extended anoxia and reoxygenation with minimal cell death, allows us to more clearly distinguish between pro-survival and pathological responses in anoxic neurons.

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