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Inhibition of Mitochondrial Function in Astrocytes: Implications for Neuroprotection

Ludmila A. Voloboueva¹, Sang Won Suh^{2,3}, Raymond A. Swanson^{2,3}, and Rona G. Giffard^{1,4}

¹Department of Anesthesia, Stanford University School of Medicine, Stanford, CA

²Department of Neurology, University of California at San Francisco, San Francisco, CA

³Veterans Affairs Medical Center, San Francisco, CA

⁴Department of Neurosurgery, Stanford University School of Medicine, Stanford, CA

Abstract

Much evidence suggests that astrocytes protect neurons against ischemic injury. Although astrocytes are more resistant to some insults than neurons, few studies offer insight into the real time changes of astrocytic protective functions with stress. Mitochondria are one of the primary targets of ischemic injury in astrocytes. We investigated the time course of changes in astrocytic ATP levels, plasma membrane potential, and glutamate uptake, a key protective function, induced by mitochondrial inhibition. Our results show that significant functional change precedes reduction in astrocytic viability with mitochondrial inhibition. Using the mitochondrial inhibitor fluorocitrate (FC, 0.25 mM) that is preferentially taken by astrocytes we found that inhibition of astrocyte mitochondria increased vulnerability of co-cultured neurons to glutamate toxicity. In our studies the rates of FC-induced astrocytic mitochondrial depolarization were accelerated in mixed astrocyte/neuron cultures. We hypothesized that the more rapid mitochondrial depolarization was promoted by an additional energetic demand imposed by the co-cultured neurons. To test this hypothesis we exposed pure astrocytic cultures to 0.01 – 1 mM aspartate as a metabolic load. Aspartate application accelerated the rates of FC-induced mitochondrial depolarization, and, at 1 mM, induced astrocytic death, suggesting that strong energetic demands during ischemia can compromise astrocytic function and viability.

Keywords

ischemia; mitochondria; astrocyte; neuron; fluorocitrate

Introduction

Increasing evidence shows that astrocytes play a critical role in neuronal protection during ischemic injury (Chen and Swanson 2003; Dienel and Hertz 2005). Astrocytes contain the principal glycogen store in the brain (Dringen et al. 1993) and provide energy substrates like lactate and pyruvate to neurons (Anderson et al. 2003). Astrocytes spatially buffer ions including K⁺ and play a crucial role in regulation of the extracellular ionic environment (Walz 1989; Leis et al. 2005). These astrocytic functions are particularly important within ischemic areas where energy depletion and ionic disruption are severe (Lo et al. 2003).

During ischemic injury astrocytes also provide neurons with antioxidant substrates, such as glutathione and reduced ascorbate (Anderson et al. 2003; Swanson et al. 2004). Massive release of glutamate occurs during ischemia and plays a major role in ischemia-induced cell death (Benveniste 1991; Choi 1994). Astrocyte glutamate uptake is crucial for limiting the excitotoxic effect of glutamate (Rosenberg and Aizenman 1989; Dugan et al. 1995). Downregulation of EAAT1/GLAST or EAAT2/GLT1, astrocytic glutamate transporters, but not EAAT3/EAAC1, the neuronal subtype, has been shown to increase ischemic injury following focal cerebral ischemia in rat (Watase et al. 1998; Fukamachi et al. 2001; Rao et al. 2001).

Mitochondria are one of the primary targets of ischemic injury in astrocytes (Bambrick et al. 2004). Astrocytes have high rates of oxidative metabolism and derive a substantial amount of ATP from mitochondrial production (Hertz et al. 2006). Multiple astrocytic functions, including high affinity glutamate uptake, are energy demanding (Anderson and Swanson 2000), thus mitochondrial dysfunction can seriously impair astrocytic neuroprotective function.

Astrocytes have been shown to be more resistant than neurons to some forms of ischemic insults in vitro (Goldberg and Choi 1993; Xu et al. 2001), and astrocytic markers are better preserved in animal stroke models (Chen et al. 1993; Lee et al. 2003). However, viability studies provide little information about the time course and extent of functional astrocytic impairment that develops before cell death. In this study we used the mitochondrial inhibitor fluorocitrate (FC) to investigate changes in ATP levels, plasma membrane potential, and glutamate uptake, and correlated them with changes in astrocytic viability. FC blocks the activity of Kreb's cycle enzyme aconitase acting as a "suicide" substrate (Clarke 1991). FC preferentially inhibits the Kreb's cycle of astrocytes over neurons, possibly due to its more avid uptake by glial cells, and has been shown, within a certain range of concentrations and exposure times, to produce selective damage to glial cells only (Hassel et al. 1992; Fonnum et al. 1997). Our results indicate that mitochondrial inhibition induced significant functional impairment that occurred before astrocytic death. We also used FC to selectively compromise mitochondrial function in astrocytes co-cultured with neurons to assess whether this could affect neuronal vulnerability to glutamate toxicity.

We performed mitochondrial inhibition studies in glucose-free medium, with similarly treated cultures in glucose-free medium without FC serving as a control. We chose these conditions based on our observations and earlier reports (Pauwels et al. 1985; Marrif and Juurlink 1999; Almeida et al. 2001) indicating that astrocytes readily compensate for decreased rates of mitochondrial ATP production by strong upregulation of glycolysis and increased glucose consumption from the medium. Because glucose depletion is characteristic of ischemia, glucose-free medium might also have physiological relevance to ischemic conditions.

A growing body of studies indicates the reciprocal nature of neuron-astrocyte interactions (Magistretti et al. 1993; Reichert et al. 2001; Chen and Swanson 2003), but the mechanisms by which neurons might influence astrocyte response and survival remain practically unknown. In this study we observed that the addition of neurons to astrocyte cultures influenced the rates of astrocytic mitochondrial depolarization, and identified one mechanism that can be partially responsible for this phenomenon.

Methods

Cell culture

Primary cultures of cortical astrocytes and neurons were prepared as previously described (Dugan et al. 1995) using a protocol approved by the Stanford Animal Care and Use Committee. In brief, for astrocyte cultures cerebral cortices from 1–2 day old mouse pups were dissociated in trypsin (0.25%), followed by trituration. The cells were suspended in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 10% equine serum (ES) (HyClone, Logan, UT), 2 mM glutamine and 10 ng/ml EGF (Sigma) and plated at density 2 hemispheres per 24 well plate. The cultures were fed biweekly with astrocyte growth medium (GM) (MEM supplemented with 10% ES and 2 mM glutamine) and used on d.i.v. 22–30. Some astrocytic cultures were exposed upon confluence (days in vitro (DIV) 12–16) to 3 μ M cytosine arabinoside for 48 h to inhibit growth of other cell types. Neuronal cultures were prepared by suspending dissociated E14–15 cortices in MEM with 2mM glutamine, 5% FBS, 5% ES and plating on poly-D-lysine (100 ng/ml) and laminin (4 ng/ml) coated wells at a density of 3 hemispheres/24 well plate. Twenty four hours after plating half of the culture medium was replaced with glial conditioned medium (GCM, collected from confluent astrocyte cultures in flasks that had been fed with MEM containing 5% FBS for at least 3 days) and supplemented with 2% B-27 supplement (Gibco, Grand Island, NY) and 3 μ M cytosine arabinoside. Half of the medium was replaced with GCM on DIV 5–7, and the cells were used on DIV 10–12. For mixed astrocytic/neuronal cultures dissociated E14–15 cortices were plated at 3 hemispheres/plate on a confluent astrocyte (DIV 12–16) monolayer. One day after plating the cells were treated with 3 μ M cytosine arabinoside. After 5–6 days half of the medium was exchanged with GM, the cultures were used for experiments 9–11 days after neuronal cell plating.

Fluorocitrate (FC) and glutamate treatments

FC was purchased as the Ba^{2+} salt (Sigma). 8 mg of FC Ba^{2+} salt was dissolved in 100 μ L of 1 mM HCL. 600 μ L of 0.1 mM Na_2SO_4 was added, and the suspension was centrifuged at 4,000 g for 2 min. The supernatant was mixed with BSS₀ to a final volume of 16 mL, and the pH adjusted to 7.4. All treatments, unless indicated, were performed under the condition of glucose deprivation. Balanced salt solution (BSS₀: 116 mM NaCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , 5.4 mM KCl, 1 mM NaH_2PO_4 , 14.7 mM NaHCO_3 , 10 mM HEPES, pH 7.4) was added to each well to bring the total well volume to 1 mL. This was followed by 3 washes during which 0.8 mL medium was removed and replaced by 0.8 mL BSS₀. After the final wash the volume was adjusted to 0.5 mL, and the cells were kept in a humidified 5% incubator for 1 h. After 1 h 400 μ L of medium was removed and replaced with BSS₀ for control wells or by FC diluted in BSS₀ to a final FC concentration of 0.25 mM. We used 0.25 mM FC in our experiments because at that concentration FC induced relatively quick changes in astrocytic physiology but did not increase neuronal death in pure neuronal cultures (see Results). At the end of FC treatment the medium was exchanged for BSS_{5.5}. BSS_{5.5} is identical to BSS₀ but also contains 5.5 mM glucose. For glutamate exposure, cells were additionally treated with 10–1000 μ M glutamate for 10 min, as previously described (Dugan et al. 1995), at the end of a 2 h FC treatment.

Measurements of ATP levels

Cellular ATP concentrations were measured using the CellTiter-Glo luminescent ATP assay kit, based on the luciferase/luciferin reaction, from Promega (Madison, WI) according to the manufacturer's instructions. A Veritas luminescence counter (Turner BioSystems) was used to measure the luminescence signal of the samples in opaque white 96-well plates. ATP standards (Sigma) were used to create an ATP calibration curve for the assay. Protein

concentrations were measured using bicinchoninic acid (BCA) protein assay reagent kit from Pierce (Rockford, IL) per manufacturer's instructions.

Fluorescence measurements of mitochondrial and plasma membrane potential

For assessment of changes in mitochondrial membrane potential, astrocytes were loaded for 30 min prior to measurements with 50 nM tetramethylrhodamine ethyl ester (TMRE). TMRE fluorescence (excitation 535 nm, emission 590 nm) was visualized on an Axiovert 200M fluorescence microscope (Carl Zeiss, Germany). Several fields per dish were selected at random by phase-contrast optics, and the intensity of six 100×150 μm areas of confluent cells (~30 cell/each area) was analyzed to produce the average intensity reading for each well. The average background intensity of a cell-free region was measured in each well and the background signal was subtracted. The plasma membrane sensitive dye DiBAC4(3) (excitation 488 nm, emission 530 nm, 1 μM) was used to investigate changes in astrocyte plasma membrane potential. The imaging protocol was the same as that described for TMRE. The presented fluorescence data were normalized by setting the control conditions to a value of 1.0.

Glutamate uptake

Glutamate uptake was measured as described previously (Swanson 1992). Both control and FC treated cultures had 300 μL of 1.67 μCi/mL L-[¹⁴C(U)] glutamate plus unlabeled glutamate added to a total glutamate concentration of 100 μM. Uptake was terminated after a 7 min incubation at 37°C by two washes in ice-cold Hank's balanced salt solution followed immediately by cell lysis in 0.1 M NaOH. Aliquots were taken for scintillation counting and for protein assay using BCA method.

Cell viability assays

Cell death was quantified 24 h after 10 min of glutamate exposure or indicated FC treatments by measuring lactate dehydrogenase (LDH) efflux as previously described (Koh and Choi 1987). LDH values for pure cultures were expressed as a fraction of freeze-thaw LDH release for maximum LDH release. LDH values for mixed cultures were expressed as a fraction of the maximal neuronal LDH released after 18–24 h exposure to 500 μM NMDA or, alternatively, to 10 min acute 1 mM glutamate exposure. In some instances, the cell death was evaluated by microscopic evaluation of Hoechst 33342 (5 μM) and propidium iodide (PI, 5 μM) labeled cells. PI readily penetrates cells with compromised plasma membranes but does not cross the intact membranes of live cells. Hoechst is a cell-permeant nucleic acid stain that labels cells of both live and dead nuclei.

Statistics

Statistical differences between two treatment groups were determined using unpaired two-tailed Student's t-test with Welch's correction. Comparisons between multiple groups were performed with ANOVA followed by Bonferroni test for selected groups. Data in all plots are presented as mean±SD.

Results

Mitochondrial inhibition promotes marked physiological and functional changes in astrocytes preceding astrocytic death

Changes in intracellular ATP levels—FC-induced mitochondrial inhibition in glucose-free BSS₀ medium resulted in a rapid decrease of astrocytic ATP levels while control astrocytes treated with BSS₀ alone did not demonstrate significant changes in ATP levels over the same time course. As shown in Fig. 1A, FC treatment for 2 and 3 h significantly

decreased by 53 % and 84 %, respectively, cellular ATP content compared to control. Longer (4 h and 5 h) FC treatments promoted practically complete depletion of cellular ATP levels. In comparison, FC treatment in glucose-replete BSS_{5.5} medium induced slow and relatively small changes in astrocytic ATP levels (Fig. 1B). Because of the stronger effect of FC mitochondrial inhibition in glucose-free medium, and because glucose-free conditions are relevant to ischemic conditions of substrate deprivation, all subsequent experiments were performed in BSS₀ medium.

FC-induced plasma membrane depolarization—Because astrocyte plasma membrane potential is maintained by a powerful Na⁺/K⁺ ATPase that consumes about 20% of cellular ATP during non-stimulated conditions (Silver and Erecinska 1997), we investigated changes in astrocyte plasma membrane potential accompanying FC-induced changes in ATP levels. Fig. 2 shows that a significant 1.5 fold increase in DiBAC4(3) fluorescence developed after 2h of FC treatment reflecting astrocytic plasma membrane depolarization. A significant increase in DiBAC4(3) signal was also observed after 3 and 4 h of FC treatment. Observations were not extended for more than 4 h because plasma membrane integrity was strongly compromised beyond that time point. Exposure to high K⁺ extracellular buffer induced plasma membrane depolarization and was used to confirm DiBAC4(3) response (Fig. 2A, B).

The effect of FC treatment on mitochondrial membrane potential—We next investigated changes in mitochondrial membrane potential (Ψ_m) promoted by FC inhibition. As demonstrated in Fig. 3 no changes in Ψ_m were observed during the first 2 h of FC exposure, with a significant ($p=0.003$) Ψ_m decline developing after 3 h of FC treatment. Because the rapid decline in ATP levels in the first 2 h (Fig. 1A) was not accompanied by changes in Ψ_m , we investigated the possibility that Ψ_m was maintained by mitochondrial ATPase reversal. In this case Ψ_m is maintained by hydrolysis of glycolytically produced ATP at mitochondrial complex V. As demonstrated in Fig. 3, application of 1 μ M oligomycin, a specific complex V inhibitor at that concentration, promoted quick Ψ_m depolarization in FC treated astrocytes. This indicates that during mitochondrial inhibition with FC, ATP is consumed rather than produced by astrocyte mitochondria. It has been shown that TMRE staining can be influenced by plasma membrane potential according to the Nernst equation (Ward et al. 2000). In our experiments we observed a mild non-significant increase in TMRE staining at 2 h, despite the significant plasma membrane depolarization at this time point (Fig. 2). Mitochondrial inhibition and subsequent F₁F₀-ATPase reversal have been shown to lead to mitochondrial hyperpolarization in different cell types including astrocytes (Hortelano et al. 1999; Almeida et al. 2001; Beltran et al. 2002). This phenomenon can be responsible for the slight increase in TMRE staining at 2 h time point notwithstanding the apparent plasma membrane depolarization. We also investigated the effect of high K⁺- induced plasma membrane depolarization on TMRE signal. In our experiments strong high K⁺-induced plasma membrane depolarization induced by high K⁺ exposure did not promote changes in TMRE staining of astrocytes (Fig. 3C).

Cell viability—Before proceeding to measure changes in glutamate uptake we assessed whether FC treatment reduced astrocyte viability. As indicated in Fig. 4, control treatment with glucose deprivation alone did not reduce astrocyte viability during the observation time of 5 h. FC treatment induced a trend to increased astrocytic death after 3 h that did not reach significance. A 74% increase in cell death was observed after 4 h. 5 h FC exposure resulted in complete astrocytic death.

Glutamate uptake—Because astrocyte glutamate uptake is energy demanding, and because the cell plasma membrane potential contributes to the driving force for glutamate

uptake we investigated changes in glutamate uptake with FC treatment. As shown in Fig. 5, the time course of changes in glutamate uptake correlated with that of ATP decline (Fig. 1A). There was a significant 58% and 74% reduction in the levels of glutamate uptake after 2 and 3 h of FC exposure, respectively. The measurements were not extended beyond 3 h when significant changes in astrocytic viability were observed (Fig. 4). The results indicate that the important function of glutamate uptake was markedly compromised before any significant increase in astrocyte death.

Astrocyte mitochondrial dysfunction increases glutamate toxicity for co-cultured neurons

Earlier studies demonstrated that astrocytes protect neurons against glutamate toxicity in mixed astrocyte/neuron cultures (Rosenberg and Aizenman 1989; Dugan et al. 1995). Because astrocytic glutamate uptake was significantly decreased after 2 h of 0.25 mM FC treatment (Fig. 5), we investigated whether 2 h FC treatment would impair the ability of astrocytes to protect neurons against glutamate toxicity. Astrocytic/neuronal co-cultures were exposed to different concentrations of glutamate at the end of 2 h FC treatment. As shown in Fig. 6A, FC pretreatment potentiated glutamate-induced neuronal death. FC pretreatment and subsequent 10 and 100 μ M glutamate exposure resulted in 27% and 70% neuronal death, respectively, compared to 5% and 20% neuronal death in control cultures not treated with FC. To exclude a possible direct effect of FC treatment on neuronal survival, we studied the effect of 2 h FC pretreatment in pure neuronal cultures. As shown in Fig. 6B, FC-pretreated neuronal cultures demonstrated similar levels of neuronal death compared to control cultures exposed to the same conditions of glucose deprivation and subsequent glutamate treatment. Thus FC-induced astrocyte mitochondrial inhibition compromised neuronal survival during glutamate challenge. Morphological observations showed that the cell death in mixed cell cultures was limited to neurons at all glutamate concentrations tested. The top panels of Fig. 6C show representative images of control and FC-exposed mixed cultures that were exposed to 100 μ M glutamate. The phase contrast images of the same areas on the bottom panels of Fig. 6C demonstrate that glutamate-induced death is limited to neurons.

Energetic challenge promotes faster mitochondrial depolarization in FC-treated astrocytes

Though co-culturing with neurons did not change astrocyte viability, we observed faster rates of astrocytic mitochondrial depolarization in mixed cultures. As shown in Fig. 3, 2 h FC treatment didn't affect TMRE fluorescence of pure astrocytic cultures. In contrast, a significant decline in TMRE intensity in astrocytes was commonly observed after 2 h FC treatment in neuron/astrocyte co-cultures (Fig 7). While different mechanisms could be responsible for the change in astrocyte behavior, we hypothesized that co-culturing with neurons imposed additional energetic demands on astrocytes. An energetic load would induce a decline in cellular ATP levels that might otherwise be used to maintain mitochondrial Ψ_m (by reversal of the mitochondrial ATPase). In order to test our assumption we exposed astrocytes in pure cultures to different concentrations of aspartate during 0.25 mM FC treatment. Aspartate is taken up by the same transporters, with a similar K_d and energy requirements, as glutamate (Drejer et al. 1983), but, unlike glutamate, cannot be used as a mitochondrial fuel during FC inhibition in glucose-free medium. As shown in Fig. 8, co-exposure of astrocytes to different concentrations of aspartate and 0.25 mM FC for 2 h resulted in significant mitochondrial depolarization compared to control cells or astrocytes treated with FC alone (Fig. 3). Treatment with 100 μ M and 1 mM aspartate resulted in a 69% and 77% decrease in TMRE fluorescence, respectively. We also investigated whether a decrease in plasma membrane potential contributed to the decrease in TMRE fluorescence induced by FC and aspartate co-treatment. As shown in Fig. 8C, FC +100 μ M aspartate treatment caused no significant change in plasma membrane potential compared to FC treatment alone, thus indicating that the decrease in TMRE staining was not

due to plasma membrane depolarization in those experiments. Fig 8D demonstrates the effects of the increased metabolic stress induced by exposure to different aspartate concentrations on the rates of ATP depletion in astrocyte cultures (as compared to Fig. 1A).

Because mitochondrial depolarization is known to be associated with astrocyte cell death (Dienel and Hertz 2005) we investigated the changes in astrocyte viability induced by co-exposure to FC and aspartate. Fig 9A shows that 1 mM aspartate applied during FC inhibition induced a significant 30% decrease in astrocyte viability. In contrast, 100 μ M aspartate + FC treatment was not accompanied by significant astrocyte death, despite the profound loss of mitochondrial membrane potential. This is in striking contrast with the commonly observed coincidence of mitochondrial membrane depolarization and loss of astrocytic viability ((Juurlink and Hertz 1993) and Fig 9B).

Discussion

In this paper we investigated the time course of changes in astrocyte physiology promoted by mitochondrial inhibition under the conditions of glucose deprivation. Several interrelated factors contribute to mitochondrial impairment during ischemia. An increase in cytoplasmic free Ca^{2+} is characteristic of the pathogenesis of brain ischemia (Silver and Erecinska 1992). Ca^{2+} sequestration in mitochondria (Dux et al. 1987; Zaidan and Sims 1994) triggers mitochondrial dysfunction (Morley et al. 1994; Siesjo et al. 1995). Following arterial occlusion in brain, production of reactive oxygen species (ROS) is increased in ischemic areas during early reperfusion (Chan 1996; Piantadosi and Zhang 1996; Kuroda and Siesjo 1997). Exposure to elevated ROS levels results in oxidation of mitochondrial lipids, sulfhydryl groups and iron sulfur complexes of mitochondrial respiratory enzymes (Wagner et al. 1990; Gilboe et al. 1991; Nakahara et al. 1992; Liu et al. 1993) leading to impairment of mitochondrial oxidative phosphorylation. Different isoforms of nitric oxide synthase are activated during cerebral ischemia (Endoh et al. 1994; Nakashima et al. 1995; Bolanos et al. 1997; Wiencken and Casagrande 1999) leading to excessive production of nitric oxide (NO). NO is a potent competitive inhibitor of mitochondrial cytochrome oxidase (complex IV) (Brown 1995; Giuffre et al. 1996). In addition, both extracellular and intracellular pH drops significantly in ischemic tissue (Siesjo 1988; Kintner et al. 2000). Because mitochondrial respiration is sensitive to inhibition by acidosis (Hillered et al. 1984), systemic pH decrease during ischemia has been associated with mitochondrial impairment (Rehncrona et al. 1979; Kim et al. 1996).

Recent *in vivo* studies indicated that astrocytic oxidative metabolism could be significantly impaired in parts of the postischemic brain apparently without astrocytic loss (Thoren et al. 2005). Our studies showed that mitochondrial inhibition in glucose-free medium induced a rapid decrease in cellular ATP levels, depolarized plasma membrane, and decreased the rates of glutamate uptake before inducing significant loss of astrocyte viability. In our experiments astrocytes were able to maintain Ψ_m during mitochondrial inhibition by F_1F_0 -ATPase hydrolysis of cellular ATP (Akerman and Jarvisalo 1980; Buchet and Godinot 1998). This hydrolysis is apparently important for astrocytic viability because Ψ_m collapse is associated with release of proapoptotic factors such as cytochrome c, apoptosis-inducing factors, and some procaspases (Liu et al. 1996; Susin et al. 1999). On the other hand, the reversal of the mitochondrial ATPase promotes higher rates of cellular ATP depletion and thus faster deterioration of energy requiring astrocytic protective functions. During the pathological conditions, such as ischemia, astrocytes have the potential to ameliorate damage to neurons, depending on the extent to which key functions are preserved. Using astrocyte/neuron co-cultures we confirmed that specific inhibition of astrocytic mitochondria impaired the well-known ability of astrocytes to protect co-cultured neurons against glutamate toxicity (Rosenberg and Aizenman 1989; Dugan et al. 1995). Our results suggest

that astrocytic mitochondrial dysfunction, though still not extensively studied, may be an important early event in the development of ischemic injury.

While the ability of astrocytes to influence neuronal responses to ischemic injury is well known (Swanson et al. 2004), few studies have investigated the effect of neurons on astrocyte vulnerability. *In vivo* studies demonstrate that therapeutic agents that are beneficial for neuronal survival also decrease the extent of astrocytic death in ischemia (Chen and Swanson 2003). In our study the astrocytes were able to maintain mitochondrial membrane potential during FC inhibition for 2 h by ATPase reversal in pure cultures but not in the presence of co-cultured neurons (Fig 2 and 6). This is in agreement with previous studies that report the failure of astrocytes to use the compensatory mechanism of ATPase reversal in mixed neuron/astrocyte co-cultures (Reichert et al. 2001).

In order to investigate whether energetic requirements imposed by neurons were partially responsible for astrocyte Ψ_m depolarization, we initially stimulated FC-treated astrocytes with different concentrations of glutamate. We observed that an initial decline in mitochondrial potential upon glutamate addition was commonly followed by complete restoration of Ψ_m in a fraction of astrocytes (data not shown). This could be attributed to the intracellular conversion of glutamate to α -ketoglutarate that can enter the Krebs' cycle downstream of FC inhibition of aconitase. We therefore conducted the additional experiments described above using aspartate. While aspartate is taken up by the same glutamate transporters (Drejer et al. 1983) unlike glutamate, it cannot be metabolized and enter the Krebs' cycle downstream of FC inhibition. Because glutamate is metabolized oxidatively, it also cannot be used as a fuel under ischemic conditions of oxygen and glucose deprivation. For these reasons aspartate exposure in our experiments provided an adequate model of glutamate-induced energetic load during ischemia. Our results showed that FC-treated astrocytes exposed to 100 μ M and 1 mM aspartate for 2h demonstrated significantly decreased TMRE staining (Fig. 8). These results suggest that the additional energetic load imposed by co-cultured neurons releasing glutamate is a potential mechanism that increases astrocyte mitochondrial depolarization during FC treatment. In our studies the addition of aspartate to FC-treated astrocytes did not promote any changes in plasma membrane potential, in a sharp contrast to FC-only treatment that demonstrated no changes in mitochondrial membrane potential but significant plasma membrane depolarization at 2 h time point (Fig. 2,3). The regulatory mechanisms that control preservation of either mitochondrial or plasma membrane potential under the conditions of energy deprivation remain to be investigated. Of note, failure of astrocytes to maintain mitochondrial membrane potential by F_1F_0 -ATPase reversal when co-cultured with neurons, without changes in astrocytic plasma membrane potential, was also reported by another group in a previous study (Reichert et al. 2001).

Our studies also showed that the profound loss of mitochondrial potential induced by 2 h co-exposure to 100 μ M aspartate + FC in glucose-free medium was not accompanied by astrocytic death. The ability of astrocytes to recover from a profound loss of mitochondrial potential without loss of viability, is in sharp contrast with commonly observed astrocyte behavior (Fig. 9) (Dienel and Hertz 2005), though it has been observed previously (Reichert et al. 2001). Studies of this phenomenon are still very limited.

Exposure of astrocytes to higher aspartate concentration of 1 mM in the presence of mitochondrial inhibition promoted significant increase in astrocytic death. As described above, the energetic requirements of aspartate uptake are equivalent to those for glutamate uptake. During ischemic episodes astrocytes *in vivo* can be exposed to glutamate concentrations in the millimolar range. Cellular glutamate ranges from 1 to 10 mM in different brain cell types (Erecinska and Silver 1990). Ischemic injury can result in release

of intracellular glutamate into the extracellular space. Because the extracellular space comprises only 15–20% of brain volume *in vivo* (Dienel and Hertz 2005), this would result in local increases of glutamate concentrations in the millimolar range, which have been observed (Benveniste 1991). While astrocytes in primary cultures are relatively resistant to fuel and oxygen restriction, astrocyte death is strongly amplified by acidosis (Goldman et al. 1989; Giffard et al. 1990; Swanson et al. 1997). Extracellular ion shifts, combined with low pH, promote very rapid loss of astrocyte viability under ischemic conditions (Bondarenko and Chesler 2001). Our results indicate that high energetic demands encountered by astrocytes in the ischemic brain, but not commonly present in cell culture experiments, can be an additional source of astrocyte vulnerability *in vivo*. Indeed, several *in vivo* studies indicate that astrocytes can be equally or even more rapidly injured than neurons (Liu et al. 1999; Lukaszewicz et al. 2002; Zhao et al. 2003). Because the ischemic infarct area is defined by pan-necrosis of both glial and neuronal cells, the extent of astrocyte death can influence the amount of brain that suffers infarction. The results of our and previous studies indicate that, in addition to severe substrate and oxygen restriction, multiple environmental factors can influence the extent of astrocyte death and thus infarct size.

In summary, we demonstrated that inhibition of astrocyte mitochondria promoted functional changes in astrocytes and compromised their ability to protect neurons against excitotoxicity. Neurons, in turn, influenced the rates of astrocyte mitochondrial depolarization promoted by FC inhibition. In our studies an energy-demanding process such as aspartate uptake at the plasma membrane compromised astrocytes' ability to maintain mitochondrial membrane potential during mitochondrial inhibition, and ultimately induced astrocyte death. Our results emphasize dynamic and highly complex interactions between neurons and astrocytes during stress that affect the ability of astrocytes to maintain mitochondrial membrane potential and survive energy deprivation as well as maintain brain protective functions during ischemic injury. The strategies aimed at the protection of astrocytic mitochondria, both by supply of mitochondrial metabolites or preservation of mitochondrial membrane integrity, might be useful in developing therapies to attenuate ischemic injury.

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Fig. 1.

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