A₁ adenosine receptors mediate hypoglycemia-induced neuronal injury

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Abstract

The cellular mechanisms that lead to neuronal death following glucose deprivation are not known, although it is recognized that hypoglycemia can lead to perturbations in intracellular calcium ($[Ca^{2+}]_i$) levels. Recently, activation of A₁ adenosine receptors (A₁AR) has been shown to alter $[Ca^{2+}]_i$ and promote neuronal death. Thus, we examined if A₁AR activation contributes to hypoglycemia-induced neuronal injury using rat cortical neurons. First, we observed that hypoglycemia was associated with large increases in neuronal adenosine release. Next, decreased neuronal viability was seen with progressive reduction in glucose concentration (25, 6, 3, 0.75 and 0 mM). Using the calcium-sensitive dye, Fluo-3, we observed both acute and long-term changes in relative $[Ca^{2+}]_i$ during hypoglycemic conditions. Demonstrating a role for adenosine in this process, both the loss in neuronal viability and the early changes in $[Ca^{2+}]_i$ were reversed by treatment with A₁AR antagonists (8-cyclopentyl, 1,3-dipropylxanthine; 9-chloro-2-(2-furyl)(1,2,4)-triazolo(1,5-c)quinazolin-5-amine; and *N*-cyclopentyl-9-methyladenine). We also found that hypoglycemia induced the expression of the pro-apoptotic enzyme, caspase-3, and that A₁AR antagonism reversed hypoglycemia-induced caspase-3 activity. Collectively, these data show that hypoglycemia induces A₁ARs activation leading to alterations in $[Ca^{2+}]_i$, which plays a prominent role in leading to hypoglycemia-induced neuronal death.

Journal of Molecular Endocrinology (2004) **32**, 129–144

Introduction

Hypoglycemia can injure both the mature and immature nervous system (Agardh *et al.* 1980, Hawdon 1999, Vannucci & Vannucci 2001). However, our understanding of the mechanisms of hypoglycemia-induced neuronal injury is still evolving. The mechanisms that induce neuronal injury following glucose deprivation are more complex than simply depriving neurons of their primary energy source (Auer & Siesjo 1993). Rather, hypoglycemic injury may involve altered neurotransmitter action, resulting from the release of excitatory amino acids, such as glutamate (Wieloch 1985, Butcher *et al.* 1987, McGowan *et al.* 2002) or other neurochemicals, such as adenosine (Fowler 1993, Zhu & Krnjevic 1993, Calabresi *et al.* 1997),

which can influence neuronal function and survival (Dunwiddie & Masino 2001, Turner *et al.* 2002a,b).

Adenosine is usually present in the extracellular space at low concentrations (Fowler 1993, Zhu & Krnjevic 1993, Dunwiddie & Masino 2001). However, under conditions of hypoxia, tissues inflammation or hypoglycemia, local adenosine levels increase markedly (Fredholm *et al.* 1994, Haas & Selbach 2000, Dunwiddie & Masino 2001, Rivkees *et al.* 2001). After its release, adenosine acts via specific G-protein coupled receptors that include the A₁, A_{2A}, A_{2B}, and A₃ adenosine receptors (Fredholm *et al.* 1994, Olah & Stiles 1995). Of these receptor subtypes, A₁ adenosine receptors (A₁ARs) have the highest affinity for adenosine and are activated following increased extracellular adenosine levels (Dunwiddie &

Masino 2001, Rivkees *et al.* 2001). In the brain, A₁ARs are abundantly and widely expressed (Fastbom *et al.* 1987) and mediate many of the important effects of adenosine in the central nervous system (Dunwiddie & Masino 2001, Rivkees *et al.* 2001).

Activation of A₁ARs in the adult brain is reported to protect neurons against excitotoxic injury (von Lubitz & Marangos 1990, Simpson *et al.* 1992). However, recent evidence shows that in young animals, A₁AR activation actually promotes injury in the developing brain (Turner *et al.* 2002*b*) by mechanisms which appear to involve altered intracellular calcium levels ($[Ca^{2+}]_i$) (Turner *et al.* 2002*a*). Thus, adenosine-induced effects and A₁AR action vary throughout development.

Increasing evidence shows that regulation of $[Ca^{2+}]_i$ within a narrow range is critical for neuronal survival, as too much or too little $[Ca^{2+}]_i$ results in neuronal death (Johnson *et al.* 1992, Zipfel *et al.* 2000). Changes in $[Ca^{2+}]_i$ play a role in a number of pathological conditions (Duchen 2000, Zhu *et al.* 2000), and altered $[Ca^{2+}]_i$ is observed following hypoglycemia (Cheng & Mattson 1991, Silver *et al.* 1997). Adenosine, which is released following hypoglycemia, can also influence $[Ca^{2+}]_i$ by activation of A₁ARs (Mynlieff & Beam 1994, Wu & Saggau 1994). Activation of A₁ARs can induce depression of neuronal firing following hypoglycemia (Calabresi *et al.* 1997), suggesting that a relationship between hypoglycemia, altered $[Ca^{2+}]_i$ and A₁ARs may exist.

Considering the above observations, we hypothesized that A₁ARs may mediate hypoglycemia-induced neuronal death via a mechanism that involves changes in $[Ca^{2+}]_i$. To permit direct observations of the effects of glucose deprivation on neurons, we studied isolated neurons in culture. We have also studied changes in relative $[Ca^{2+}]_i$ ($r[Ca^{2+}]_i$) following hypoglycemia and examined the role of A₁ARs in mediating hypoglycemia-induced effects.

Research design and methods

Drugs

8-cyclopentyl, 1,3-dipropylxanthine (DPCPX), 9-chloro-2-(2-furyl)(1,2,4)-triazolo(1,5-c)quinazolin-5-amine (CGS 15943), *N*-cyclopentyl-9-methyladenine (CPMA) and halothane were obtained

from Sigma (St Louis, MO, USA). Ionomycin was obtained from CalBiochem (San Diego, CA, USA). 1,2-bis(O-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-AM (BAPTA-AM) was obtained from Molecular Probes (Portland, OR, USA). Drugs were dissolved in 100% DMSO or 100% ethanol and diluted at least 1000-fold in each study.

Isolation of cortical neurons

The Yale Animal Care and Use Committee approved the use of animals in all experiments. Cortical neurons were prepared according to commonly used methods (Brewer *et al.* 1993). Briefly, embryonic day 18 rats (Sprague–Dawley) were removed from the uteri of anesthetized dams (halothane, 1–2%) and kept on ice in calcium, magnesium-free Hanks' balanced salt solution (containing 10 mM Hepes, 2 mM sodium pyruvate; GIBCO/BRL, Rockville, MD, USA). Whole brains were removed and cortical tissue was isolated and placed into Hibernate E medium (supplemented with B27; GIBCO/BRL). The cortical tissue was pooled, triturated gently and centrifuged (200 *g*, 1 min). The cell pellet was resuspended in neurobasal medium (NBM) (GIBCO/BRL) containing 25 μ M L-glutamate (Sigma), 0.5 mM L-glutamine, penicillin–streptomycin and B27 supplements (GIBCO/BRL). Cells were placed in poly-D-lysine (Sigma) coated, 12-well plates (Costar; Corning, Inc., Corning, NY, USA). Media were changed on the third day after initial dispersion using glutamate-free, NBM (containing 25 mM glucose). Cells were fed with glutamate-free NBM every 4–5 days thereafter or until additional changes were made. The percentage of neurons in these cultures was typically around 98%, as determined by staining for the neuronal marker, NeuN (Wolf *et al.* 1996, Turner *et al.* 2002*a*). A cell density of 0.5×10^6 cells per well was used in all experiments.

Experimental procedures

Neurons were cultured in 25 mM glucose-NBM for 7 days. Some cultures remained in 25 mM glucose-NBM for a further 5 days, while in other cultures the medium was changed to 6 mM glucose-NBM for 5 days. In some experiments, cultures were exposed to Locke's solution (in mM: NaCl, 154; KCl, 5.6; CaCl₂, 2.3; MgCl₂, 1.0;

NaHCO₃, 3·6; Hepes, 5; pH 7·4), within which the glucose concentration was varied from 0 to 6 mM, as described in experiments. Generally, studies were performed 12 days after initial dispersion. Each experiment was performed at least three times using different sets of neuronal cultures isolated from the litters of different dams.

Fluorescein diacetate (FDA) staining

To identify living cells, 2 µg/ml FDA (Sigma) were added to the medium. FDA is esterified and accumulates to its fluorescein derivative only in living cells (Rotman & Papermaster 1966). After incubation with FDA for 5 min, cells were washed three times with PBS and photographed at low magnification under UV fluorescence on an inverted fluorescence microscope (Olympus IX-FLA; Olympus America, Inc., Melville, NY, USA). Each image was analyzed using software that readily distinguishes labeled cells from background (Image Pro software; Image Pro, Inc., Boston, MA, USA). Four separate fields per well were captured digitally and the mean $\pm$ S.E. estimated across four wells per treatment group.

Caspase-3 activity

To determine if neuronal injury involves pathways associated with programmed cell death, neurons were incubated with the fluorescein-conjugated, selective caspase-3 inhibitor, FAM-DEVD-FMK (CaspasTag Assay Kit; Serological, North Cross, GA, USA) for 1 h in a tissue culture incubator (37 °C, 5% CO₂). This irreversible inhibitor binds to the active form of caspase-3. Cells were then washed twice with 1 $\times$ wash buffer (supplied in the CaspasTag kit). Caspase-3-positive cells were easily identified as FAM-DEVD-FMK emits a green fluorescent signal when excited by UV light. Four fields per well were photographed under UV fluorescence with the Olympus IX-FLA fluorescence microscope. Caspase-3-positive cells were counted (Image Pro software) and the mean ($\pm$ S.E.) determined across four wells per treatment group.

Confocal microscopy

Changes in $r[Ca^{2+}]_i$ were determined by confocal microscopy, as described (Turner *et al.* 2002a). Non-ratiometric dyes such as Fluo-3 are better suited to

this experimental goal than ratiometric dyes because they are inherently more sensitive and respond more rapidly to changes in $[Ca^{2+}]_i$ (information from Molecular Probes). Used in this manner, Fluo-3 is well suited to monitor changes in intracellular Ca^{2+} signaling that occur in sub-second and second time frames. Consequently, to indicate altered Ca^{2+} signaling, we studied hypoglycemia-induced changes in $r[Ca^{2+}]_i$ following depolarizing stimuli, using Fluo-3 loaded neurons.

Neurons were plated on poly-D-lysine-coated, 22 $\times$ 40 mm cover slips and grown for 12 days *in vitro*. In 25 mM glucose-NBM, neurons were loaded for 20 min with the Ca^{2+} -sensitive dye, Fluo-3-AM (25 ng/ml) (Molecular Probes), plus Pluronic F127 (50 ng/ml) (Molecular Probes) to facilitate Fluo-3-AM entry into the cells. The acetoxymethyl ester (AM) group is cleaved, trapping Fluo-3 inside the cell (information from Molecular Probes). Coverslips were then mounted into recording chambers that allowed a laminar-flow exchange of medium. NBM was then replaced with Hibernate E/B27 medium, which can maintain neuronal viability outside of the incubator environment for extended periods (Brewer & Price 1996).

Because $[Ca^{2+}]_i$ increases following depolarization (Franklin & Johnson 1992), we examined changes in Fluo-3 intensity in cells stimulated with KCl (50 mM in Hibernate E/B27). After addition of KCl, changes in Fluo-3 intensity were monitored at discrete intervals over time (because the response of the cells to KCl varied between preparations, the scan frequency and total recording period were modified accordingly). Following each recording, cells were washed three times with fresh medium and allowed to recover for 3–5 min. We observed that Fluo-3 intensity returned to baseline values after the first or second wash (5–10 s). Because Fluo-3 is not a ratiometric dye, changes in $[Ca^{2+}]_i$ were relative and are therefore referred to as $r[Ca^{2+}]_i$.

Fluo-3 images were captured as multi-TIFF files and expressed as plots of intensity over time (FluoView; Olympus). Confocal imaging was performed on at least three separate fields of cells for each cover slip, in three separate studies, using neurons isolated from different litters for each study. Cells within each field were randomly selected ($n=15$ per field) for analysis of changes in $r[Ca^{2+}]_i$.

Adenosine measurements

Aliquots of media were collected and stored at -70°C until processed. Nucleosides were extracted and quantified as described (Knudsen *et al.* 1992, Blackburn *et al.* 1998). For extraction of nucleosides, media samples were thawed on ice in the presence of perchloric acid at a final concentration of 0.4 M. Samples were vortexed and placed on wet ice for 15 min. Samples were then neutralized with 0.6 M KHCO_3 and 0.72 M KOH. After clarification by centrifugation, samples were analyzed by reversed phase HPLC as described (Blackburn *et al.* 1998). Adenosine peaks were verified by enzymatic shift assay using adenosine deaminase (Knudsen *et al.* 1992) and adenosine levels were quantified by comparison with known amounts of adenosine resolved by the same protocol.

Statistical analysis

For cell viability assays, the mean number ($\pm$ S.E.) of FDA-positive cells was determined per treatment group and data were normalized to percent of control values (control values represent 100% viability). Statistical differences were determined by ANOVA using a Bonferroni post-test comparison of means (GraphPad Prism v3.0; GraphPad Software, Inc., San Diego, CA, USA). For confocal studies, changes in Fluo-3 intensity over time for each cell were normalized to the baseline intensity of that cell, averaged for all cells sampled ($n=15$ per field) and the mean intensity at each time point determined. The transformed data were then fitted to the general equation: $Y = \text{bottom} + (\text{max} - \text{min}) / (1 + \exp((T_{50} - X) / \text{slope}))$, where Y = percent baseline intensity, X = time, max = maximum, min = minimum (GraphPad Prism v3.0). From these curves the max and T_{50} (time to reach 50% of maximum) were derived. Differences in the max and T_{50} values were compared by ANOVA using a Bonferroni post-test comparison of means (GraphPad Prism v3.0).

Results

Hypoglycemia-induced cell death in NBM

We first examined effects of hypoglycemia on neurons cultured in the commonly used NBM (Brewer *et al.* 1993). Cells were initially cultured in

25 mM (450 mg/dl) glucose in NBM (25 mM glucose-NBM) for 7 days, as this is the standard glucose concentration for NBM. Because 25 mM glucose is considerably higher than physiological levels (80–150 mg/dl or 4.4–8.3 mM), the media were replaced with 6 mM glucose-NBM, which represents a mid-range physiological glucose concentration, for an additional 5 days. For comparison, some cultures remained in 25 mM glucose-NBM for the duration of the experiment. Both sets of cultures were then subjected to progressively lower concentrations of glucose for 24 h. Cell viability was then assessed by FDA labeling.

When neurons that were cultured in 25 mM glucose-NBM were exposed to progressively lower concentrations of glucose (6, 3, 0.75, 0 mM), we observed a modest loss in FDA-positive cells, decreasing to 85% through to 57% of control values (25 mM glucose control = 100% viability, indicated by black bar; Fig. 1A). When cells that were exposed to 6 mM glucose-NBM for 5 days were then exposed to progressively lower concentrations of glucose (3, 0.75, 0 mM), a similar loss of FDA-positive cells was observed, decreasing to 86% through to 49% of control values (6 mM glucose control = 100% viability, indicated by black bar; Fig. 1B).

These observations show that exposing neurons to low concentrations of glucose for 24 h resulted in limited neuronal loss regardless of whether cells were initially cultured in either 6 or 25 mM glucose-NBM. Thus, because 6 mM glucose represents a more physiological concentration a number of the studies that follow were performed after neurons were exposed to 6 mM glucose.

NBM vs Locke's medium

NBM is a complex mixture of organic molecules that have been shown to provide alternative energy sources in the absence of glucose (Honegger *et al.* 2002). Thus, to examine the influence of hypoglycemia in the absence of other energy sources, we next assessed cell viability using Locke's solution, in which glucose is the only energy source (see Methods).

As above, neurons were cultured in 25 mM glucose-NBM for 12 days. Cultures were then continued in 25 mM glucose-NBM, or in NBM containing different concentrations of glucose for 24 h. In parallel, studies were performed in which neurons were treated with decreasing glucose

Figure 1 Cell viability in NBM vs Locke's solution. After 7 days in 25 mM glucose, neurons were incubated in either 25 or 6 mM glucose for 5 days and then exposed to decreasing amounts of glucose for 24 h. (A) FDA labeling of neurons maintained in 25 mM glucose (black bar) and then exposed to reductions in glucose. (B) FDA labeling of neurons maintained in 6 mM glucose (black bar) and then exposed to reductions in glucose. (C) FDA labeling of neurons maintained at 25 mM glucose (black bar) and exposed to decreasing glucose concentrations for 24 h in either NBM (white bars) or Locke's solution (gray bars). (D) Photomicrographs of FDA-labeled cells in cultures exposed for 24 h to 25, 6, 0.75 or 0 mM glucose-NBM (1–4), or 0 mM glucose-Locke's solution (5). Scale in D5 is 50 μ m. In (A–C), statistical differences determined by ANOVA using a Bonferroni post-test comparison of means. Data represent means $\pm$ SE of four duplicate studies.

concentrations in Locke's solution for 24 h, after which FDA staining was performed and cell viability assessed.

Similarly to that observed above, reducing glucose concentration in NBM (white bars Fig. 1C), decreased the number of remaining live cells to 84% through to 48% of control values (25 mM control=100% viability, indicated by black bar; Fig. 1C). However, when glucose concentrations were lowered in the same manner in Locke's solution (gray bars, Fig. 1C), the number of remaining live cells declined to 42% through to 15% of that seen in control wells (Fig. 1C). These observations show that hypoglycemia-induced neuronal death is more pronounced in Locke's solution than in NBM.

Hypoglycemia induces increased adenosine levels

We next assessed whether hypoglycemia influences adenosine released from neurons. Cells were kept in 25 mM glucose-NBM for 7 days and then exposed to 0.75 mM glucose-Locke's solution for 0, 4, 8 and 24 h. We chose 0.75 mM glucose-Locke's solution because greater cell death was observed in Locke's solution at this glucose concentration compared with 0.75 mM glucose-NBM.

We found that in 25 mM glucose-NBM, the adenosine concentration was 1.85 ± 0.12 nM (Fig. 2). Immediately after switching from 25 mM glucose-NBM to 0.75 mM glucose-Locke's (0 h) adenosine levels were determined to be 0.40 ± 0.07 nM. This decrease was most likely due to a washout effect following the change in medium from NBM to Locke's solution. After 4 h in 0.75 mM glucose-Locke's solution, adenosine levels increased to 4.35 ± 0.36 nM and continued to increase by 8 h to 5.75 ± 0.41 nM, reflecting a 10- to 14-fold increase compared with the 0 h time point (Fig. 2). At 24 h, the adenosine concentration had decreased to 3.13 ± 0.41 nM, perhaps reflecting the greater loss of cells at this time point (Fig. 2). These data show that hypoglycemia can trigger increased adenosine release from neurons.

Changes in calcium signaling after acute (15 min) hypoglycemia

After observing that reduction in glucose concentration results in adenosine release and neuronal death, we next examined if reductions in glucose

Figure 2 Hypoglycemia induces release of adenosine from neurons. Neurons were cultured for 12 days in 25 mM glucose-NBM. One group of cells remained in 25 mM glucose-NBM. A parallel set of neurons were exposed to 0.75 mM glucose-Locke's solution for either 0, 4, 8, or 24 h. Medium was collected from each well and processed for measurement of adenosine (see Methods). Data represent means $\pm$ S.E.M. of quadruplicate wells for each treatment group. Differences in the means determined by ANOVA using a Bonferroni post-test comparison of means.

concentration alter $[Ca^{2+}]_i$. Other studies have observed changes in absolute $[Ca^{2+}]_i$ following hypoglycemia but whether neurons were still functional in these studies was unclear (Mattson *et al.* 1993, Silver *et al.* 1997). Depolarizing stimuli can evoke increases in $[Ca^{2+}]_i$, indicating normal cell functioning (Franklin & Johnson 1992). Thus, we examined changes in $r[Ca^{2+}]_i$ following varying degrees of hypoglycemia, under depolarizing conditions, as previously described (Turner *et al.* 2002a).

We first examined acute changes in $r[Ca^{2+}]_i$ 15 min after the onset of hypoglycemia. Neurons were kept in 25 mM glucose-NBM for 12 days and parallel cultures were then exposed to 6, 0.75 or 0 mM glucose for 15 min. After 15 min, changes in Fluo-3 intensity were recorded following depolarization with 50 mM KCl. Because of the need for viable neurons, NBM was used rather than Locke's solution, as the latter medium is associated with greater neuronal toxicity when glucose concentrations are reduced.

For each treatment, all cells in the field studied were responsive to KCl addition, and 15 randomly chosen cells from each field were sampled for changes in Fluo-3 over time. In 25 mM glucose, cells responded to KCl addition with a sharp rise in Fluo-3 intensity that was followed by a steady

Figure 3 Hypoglycemia induces acute and chronic changes in relative $[Ca^{2+}]_i$. Neurons were maintained in 25 mM glucose-NBM for 12 days. Cells were then loaded with Fluo-3-AM, media were changed to 0–25 mM glucose-Hibernate E/B27 media, and changes in Fluo-3 intensity monitored over time following KCl-evoked depolarization. Sigmoidal plots were derived from normalized data of 15 cells per field studied. (A) KCl-evoked changes in Fluo-3 intensity in cells exposed to 25, 6, 0.75 or 0 mM glucose for 15 min. (B) KCl-evoked changes in Fluo-3 intensity in cells exposed to 25, 6, 0.75 or 0 mM glucose for 24 h. Arrowheads in (A) and (B) show the time at which KCl was added. Beneath curves in (A) and (B), maximum at plateau (Max) (expressed as percent of baseline intensity) and T_{50} (time to reach 50% of maximum) are shown for comparison (expressed as the means $\pm$ S.E.). In (A) and (B), statistical differences determined by ANOVA, using a Bonferroni post-test comparison of means.

plateau (Fig. 3A). The maximum at plateau (max) was 281% of baseline intensity and the time to reach 50% of maximum (T_{50}) was 19.7 s. In 6 mM glucose, the maximum was slightly lower (252% of baseline intensity; Fig. 3A) but the response time was essentially unchanged (T_{50} = 20.2 s; Fig. 2A). In 0.75 mM glucose, there was a substantial reduction in responsiveness following KCl stimulation (max = 151% of baseline intensity, T_{50} = 33 s; Fig. 3A). In 0 mM glucose, cells responded poorly following KCl addition (max = 143% of baseline intensity; T_{50} = 50.7 s; Fig. 3A). These data show that acute hypoglycemia results in substantial reductions in depolarization-induced changes in $r[Ca^{2+}]_i$.

Changes in calcium signaling after hypoglycemia for 24 h

After examining changes in $r[Ca^{2+}]_i$ 15 min after hypoglycemia, we next examined if lowering

glucose levels for 24 h influenced KCl-evoked changes in $r[Ca^{2+}]_i$. Neurons were kept in 25 mM glucose medium for 12 days. Cells were then exposed to 25, 6, 0.75 or 0 mM glucose. After 24 h, cells were loaded with Fluo-3-AM and responses to KCl depolarization observed.

For each treatment, all cells in the field studied were responsive to KCl addition, and 15 randomly chosen cells from each field were sampled for changes in Fluo-3 over time. Following addition of 50 mM KCl, cells maintained in 25 mM glucose responded robustly (max = 279% of baseline intensity; T_{50} = 18.2 s; Fig. 3B). Cells exposed to 6 mM glucose for 24 h displayed a similar response (max = 308% of baseline intensity; T_{50} = 15.1 s; Fig. 3B). However, cells exposed to 0.75 mM glucose for 24 h displayed increased responsiveness following KCl stimulation (max = 353% of baseline intensity; T_{50} = 11.9 s; Fig. 3B). When cells were exposed to 0 mM glucose for 24 h, an even greater

response to KCl depolarization was observed, with a much larger maximum at the plateau (403% of baseline intensity; Fig. 3B) and a steeper rise in Fluo-3 intensity ($T_{50}=11.6$ s; Fig. 3B). These data show that long-term exposure to hypoglycemia is associated with an exaggerated response to depolarizing stimuli.

$[Ca^{2+}]_i$ determines the degree of hypoglycemic injury

Having observed reduced cell viability and altered depolarization-induced changes in $r[Ca^{2+}]_i$ following hypoglycemia, we next assessed if modifying $[Ca^{2+}]_i$ following hypoglycemia influences neuronal survival. Thus, we examined neuronal injury under conditions of hypoglycemia while increasing or decreasing $[Ca^{2+}]_i$.

Cells were initially cultured in 25 mM glucose-NBM for 7 days and the medium was changed to 6 mM glucose-NBM for a further 5 days. Cells were then maintained in either 6 or 0.75 mM glucose-NBM for a further 24 h.

To reduce $[Ca^{2+}]_i$, some cells were treated with the intracellular calcium chelator BAPTA-AM (2 μ g/ml) while being exposed to 0.75 mM glucose-NBM. BAPTA-AM can readily penetrate the cell membrane but is cleaved at the AM group, trapping BAPTA inside the cell. At the concentration and conditions used, BAPTA markedly inhibits KCl-stimulated intracellular Ca^{2+} accumulation (Collatz *et al.* 1997, Kress & Guenther 1999, Nonner *et al.* 2000). To test for time-dependency of effects, we added BAPTA-AM at 5 min or at 8 h after hypoglycemia, and stained neurons with FDA 24 h after exposure to low glucose.

In 6 mM glucose-NBM, BAPTA-AM (horizontal striped bar, Fig. 4) reduced cell viability to 55% of the control wells (indicated by black bar; Fig. 4). To study the effects of profound hypoglycemia, we exposed neurons to the lowest glucose concentration used in the studies above (0.75 mM). Changing from 6 to 0.75 mM glucose reduced cell viability to 47% of the control wells (white bar, Fig. 4). When neurons were treated with 0.75 mM glucose plus BAPTA-AM, cell viability was reduced to 17% of the control wells if BAPTA-AM was added in the first 5 min (first light gray bar, Fig. 4). In contrast, if BAPTA-AM was added at 8 h following initial hypoglycemic insult, cell viability was 73% of the control wells (second light gray bar, Fig. 4).

Figure 4 Neuronal injury following combined hypoglycemia and reduced $[Ca^{2+}]_i$. Neurons were maintained for 7 days in 25 mM glucose and then media changed to 6 mM glucose for 5 days (black bar). Cells were then exposed to 0.75 mM glucose in the absence (white bar) or presence of BAPTA-AM (2 μ g/ml; light gray bars) or ionomycin (0.1 μ g/ml, for 5 min; dark gray bars). BAPTA-AM (B) or ionomycin (Iono) were added either 5 min (5 m) or 8 h after initiation of hypoglycemia (0.75 mM glucose). After 24 h of hypoglycemia, neurons were stained with FDA to assess cell viability. To determine effects of changes in $[Ca^{2+}]_i$ in the absence of severe hypoglycemia, BAPTA-AM (horizontal line bar) or ionomycin (vertical line bar) were added to media containing 6 mM glucose-NBM with no other changes. Each set of data represents the mean of quadruplicate wells (sampled four times per well). Statistical differences were determined by ANOVA using a Bonferroni post-test comparison of means. Data shown are means $\pm$ S.E.

bar, Fig. 4). Importantly, cell viability in cultures exposed to 0.75 mM glucose-NBM plus BAPTA-AM (at 5 min or 8 h) was significantly different from that found in 0.75 mM glucose-NBM without BAPTA-AM ($P<0.001$, ANOVA).

In a parallel set of cell cultures, we added the Ca^{2+} ionophore ionomycin to neurons exposed to 0.75 mM glucose. Ionomycin was used at 0.1 μ M for 5 min, after which media were exchanged for ionomycin-free media. Used in this manner, ionomycin displays little or no neuronal toxicity (Turner *et al.* 2002a). To test for any time-dependency of effects, we added ionomycin at 5 min or at 8 h after hypoglycemia and stained neurons with FDA 24 h after exposure to low glucose.

By itself, ionomycin did not influence cell viability (vertical striped bar, Fig. 4) compared with the control wells (indicated by black bar; Fig. 4).

However, in the presence of 0.75 mM glucose-NBM, ionomycin increased cell viability to 65% of control wells if added in the first 5 min (first dark gray bar, Fig. 4) but severely decreased cell viability to 9% of control wells (second dark gray bar, Fig. 4) if added at 8 h after initial hypoglycemic insult (Fig. 4). Importantly, cell viability of cultures exposed to 0.75 mM glucose-NBM plus ionomycin (5 min or 8 h) were significantly different from that found for 0.75 mM glucose-NBM without ionomycin ($P < 0.001$, ANOVA).

These results are consistent with the hypothesis that changes in $r[Ca^{2+}]_i$ may play a role in hypoglycemia-induced cell death. In addition, the time at which changes in $[Ca^{2+}]_i$ are initiated following glucose deprivation appears to influence the degree of hypoglycemia-induced cell death.

A₁AR antagonism reverses hypoglycemic cell death

Because A₁AR activation can result in injury in isolated neurons (Turner *et al.* 2002a), and we have shown here that hypoglycemia can induce adenosine release from neurons, we next assessed if A₁ARs mediate the observed effects of hypoglycemia. To examine this issue, we first exposed neurons to hypoglycemia in the absence or presence of the selective A₁AR antagonist, DPCPX (Lohse *et al.* 1987). We compared NBM with Locke's solution to determine if the effect of blocking A₁ARs was influenced by the presence or absence of alternative energy sources during hypoglycemia.

Neurons were kept in 25 mM glucose-NBM for 7 days and then in 6 mM glucose-NBM for a further 5 days. Neurons either remained in 6 mM glucose-NBM or in medium where this was replaced with 0.75 mM glucose-NBM for 24 h. A parallel set of neurons was exposed to 0.75 mM glucose-Locke's solution for 24 h. Neurons were exposed to low glucose in the absence or presence of 100 nM DPCPX, which is a concentration that blocks A₁ARs (Fredholm *et al.* 1994, Turner *et al.* 2002a). After 24 h, neurons were labeled with FDA and cell viability assessed.

In NBM, cell viability in 0.75 mM glucose (first white bar) was 45% of that found in control wells (indicated by black bar; Fig. 5A). However, cell viability in 0.75 mM glucose plus DPCPX (second white bar) was 71% of that seen in control wells

Figure 5 Reversal of hypoglycemic damage by DPCPX. (A) Cells were maintained in 25 and then 6 mM glucose-NBM (7 and 5 days respectively). Cells were kept in 6 mM glucose-NBM (black bar) or the medium was changed to 0.75 mM glucose in NBM (white bars) or in Locke's solution (light gray bars), in the absence or presence of 100 nM DPCPX. After 24 h, cell viability was assessed by FDA staining. In a parallel experiment, cells were exposed to 0.75 mM glucose-NBM in the absence or presence of 100 nM DPCPX (added 8 h after hypoglycemia started; dark gray bar). (B–D) Neurons were initially kept in 25 followed by 6 mM glucose-NBM and then exposed to 0.75 mM glucose-NBM in the absence or presence of CGS 15934 (B) or CPMA (C), or exposed to 0.75 mM-Locke's solution in the absence or presence of CGS 15934 (D) or CPMA (E). After 24 h, cell viability was assessed by FDA staining. Differences assessed by ANOVA, using a Bonferroni post-test comparison of the means. Data shown are means $\pm$ S.E.

(Fig. 5A). In Locke's solution, cell viability in 0.75 mM glucose (first gray bar) was 15% of that seen in control wells (Fig. 5A). However, in 0.75 mM glucose plus DPCPX (second gray bar), cell viability was 41% of that observed in control wells (Fig. 5A).

We also examined neuronal viability when DPCPX was added to neurons 8 h after initial exposure to 0.75 mM glucose-NBM. In neurons exposed to 0.75 mM glucose-NBM in the absence of DPCPX (third white bar) we observed a decline in cell viability to 51%, compared with the control wells (Fig. 5A). When neurons were exposed to 0.75 mM glucose-NBM in the presence of DPCPX (dark gray bar), cell viability was 60% compared with controls. However, this was not significantly different from that seen in 0.75 mM glucose-NBM without DPCPX (Fig. 4A).

Thus, DPCPX can increase neuronal survival following hypoglycemia in NBM or Locke's solution. These data also show that early addition of DPCPX is more effective in reducing hypoglycemic injury than later addition of the antagonist.

Next, to confirm if the ability of DPCPX effects were specific to A₁AR antagonism, we examined the influence of the non-xanthine A₁AR antagonists CGS 15943 and CPMA on hypoglycemic injury (Ukena *et al.* 1987, Merkel *et al.* 1993). Neurons were kept in 25 mM followed by 6 mM glucose-NBM, as described above. Hypoglycemia was then induced by exposing neurons to 0.75 mM glucose-NBM or 0.75 mM glucose-Locke's solution in the presence or absence of CGS 15943 or CPMA (0.1, 1 and 10 µM, for each drug). After 24 h, neurons were stained with FDA.

Both CGS 15943 and CPMA reduced hypoglycemia-induced cell death in a concentration-dependent manner (Fig. 5B–E). Following exposure to 0.75 mM glucose-NBM, both drugs were able to increase FDA-positive neurons to numbers observed at 6 mM glucose-NBM (Fig. 5B and C). When neurons were exposed to 0.75 mM glucose-Locke's solution, CGS 15943 and CPMA also reversed cell death (Fig. 5D and E), but not to the same degree observed when neurons were exposed to 0.75 mM glucose-NBM. These data confirm that A₁AR antagonism can protect neurons from hypoglycemia-induced injury.

A₁AR antagonism reverses hypoglycemia-induced changes in [Ca²⁺]_i

Because A₁AR activation decreases [Ca²⁺]_i (Mynlieff & Beam 1994, Wu & Saggau 1994), and the above results suggest a role for A₁ARs in mediating the early events of hypoglycemic

neuronal injury, we next assessed if the changes in r[Ca²⁺]_i following acute hypoglycemia involve A₁AR activation. Changes in KCl-evoked Fluo-3 intensity were initially assessed in 25 mM glucose. Neurons were then exposed to 0.75 mM glucose-NBM for 15 min, in the absence or presence of 100 nM DPCPX, and changes in KCl-evoked Fluo-3 intensity were monitored.

For each treatment, all cells in the field studied were responsive to KCl addition, and 15 randomly chosen cells from each field were sampled for changes in Fluo-3 over time. Following KCl addition, changes in r[Ca²⁺]_i in neurons kept in 25 mM glucose-NBM increased over time (max=318% of baseline intensity; Fig. 6A). In 0.75 mM glucose-NBM, neurons responded with much less intensity (max=179% baseline intensity). However, in the presence of 0.75 mM glucose-NBM plus DPCPX, cell responsiveness had partially recovered (max=237% of baseline intensity; Fig. 6A). Suggesting that DPCPX preserves intrinsic mechanisms that trigger changes in r[Ca²⁺]_i, the dynamic response of the neurons in 0.75 mM glucose+DPCPX ($T_{50}=10.7$ s) was closer to that observed in 25 mM glucose ($T_{50}=13.5$ s). In contrast, neurons exposed to 0.75 mM glucose in the absence of DPCPX were much more sluggish in their response time ($T_{50}=30.1$ s). These data show that A₁ARs may play a role in mediating hypoglycemic changes in r[Ca²⁺]_i.

A₁AR antagonism, hypoglycemic cell death and reduced [Ca²⁺]_i

Having demonstrated that the combination of reduced [Ca²⁺]_i and hypoglycemia was more injurious to neurons than hypoglycemia alone, we next tested the involvement of A₁ARs in this combined toxicity. Neurons were exposed to 0.75 mM glucose-NBM plus BAPTA-AM in the absence or presence of 100 nM DPCPX. Whereas 0.75 mM glucose plus BAPTA-AM (2 µg/ml) reduced cell viability to 20% of the control wells (indicated by black bar; Fig. 6B), in the presence of DPCPX, 0.75 mM glucose-NBM plus BAPTA-AM reduced cell viability to 54% of the control wells (Fig. 6B). These studies suggest that A₁AR activation may mediate or enhance the neurotoxic effect of hypoglycemia by altering [Ca²⁺]_i, a finding consistent with previously reported effects of A₁AR activation (Turner *et al.* 2002a).

Figure 6 Hypoglycemia-induced changes in $[Ca^{2+}]_i$: protection by A_1 AR antagonism. (A) KCl-evoked changes in Fluo-3 intensity monitored over time (every 3–2 s, for 60 s) in neurons exposed to 25 mM glucose, or 15 min of hypoglycemia (0.75 mM glucose) in the absence or presence of 100 nM DPCPX. Sigmoidal plots were derived from normalized data of 15 cells per field studied. Maximum at plateau (Max) (percent baseline intensity) and T_{50} (time to reach 50% of maximum) values are also shown (means±S.E.). Arrowhead shows the time when KCl added. (B) After exposure to 25 followed by 6 mM glucose-NBM (7 and 5 days respectively), cells were then exposed to 0.75 mM glucose-NBM plus BAPTA-AM (2 µg/ml) in the absence or presence of 100 nM DPCPX for 24 h. Cell viability was then assessed by FDA staining. Statistical differences were determined by ANOVA using a Bonferroni post-test comparison of means. Data shown are means±S.E. of four duplicate studies.

Caspase-3 activity

Following glucose deprivation, changes in $[Ca^{2+}]_i$ are known to activate mediators of programmed cell death (Kluck *et al.* 1994, Berridge *et al.* 2000, Zhu *et al.* 2000, Han *et al.* 2001). To examine potential mechanisms of cell injury we first exposed

Figure 7 Hypoglycemic injury induces caspase-3 activity; protection by A_1 AR antagonism. Neurons were cultured in 25 followed by 6 mM glucose-NBM (7 and 5 days respectively) and then exposed to: (A) BAPTA-AM (10 µg/ml) for various times, after which caspase-3 activity was estimated, or (B) 0.75 mM glucose (in NBM or Locke's solution) for 4 h in the absence or presence of DPCPX (100 nM), after which caspase-3 activity was measured. Quadruplicate wells were used for each treatment and data expressed as means±S.E. Differences were assessed by ANOVA, using a Bonferroni post-test comparison of means.

neurons to the calcium chelator, BAPTA-AM (using a concentration of 2 µg/ml), which is known to induce apoptosis (Han *et al.* 2001) and monitored changes in the activity of the pro-apoptotic enzyme, caspase-3 (Yuan *et al.* 1993, Moran *et al.* 1999, Nicholson 1999). At various times after, neurons were incubated with the fluorescein-tagged, irreversible inhibitor of caspase-3, FAM-DEVD-FMK for 1 h, at 37 °C/5% CO₂ (CaspaTag Assay Kit; Serological) and the number of fluorescent neurons counted. An increase in caspase-3 activity over time was clearly evident, peaking at 4 h and declining thereafter (Fig. 7A).

Next, neurons were kept in 25 mM glucose-NBM for 7 days followed by 6 mM glucose-NBM for a further 5 days. Neurons were then exposed to 0.75 mM glucose in NBM or in Locke's solution, in the absence or presence of DPCPX (100 nM). After 4 h, neurons were then assayed for caspase-3 activity. In both 0.75 mM glucose-NBM and 0.75 mM glucose-Locke's solution, caspase-3

activity was much greater than that found for 6 mM glucose-NBM. However, when neurons were exposed to 0.75 mM glucose-NBM or 0.75 mM glucose-Locke's solution in the presence of DPCPX, caspase-3 activity was significantly less than that found in the absence of DPCPX (Fig. 7B).

Collectively these data demonstrate that changes in $[Ca^{2+}]_i$ can promote programmed cell death in neurons. We also observed that hypoglycemia-induced apoptosis can be reversed by blocking A_1 ARs.

Discussion

Our observations suggest that neuronal death from hypoglycemia is influenced by the availability of alternative energy sources and by changes in $[Ca^{2+}]_i$. In addition, hypoglycemia-induced neuronal injury appears to involve both adenosine release and activation of A_1 ARs.

In vitro, energy sources for neurons come directly from the medium in which the cells are cultured. NBM is frequently used to culture neurons for extended periods and contains a complex mixture of organic and inorganic substances (Brewer *et al.* 1993). Substances found in NBM have been shown to support neuronal viability in the absence of glucose (Cox & Bachelard 1988, Izumi *et al.* 1994, Honegger *et al.* 2002) and neurons exposed to hypoglycemia *in vivo* appear able to use alternative sources of energy to glucose (Cooper & Plum 1987, Hertz *et al.* 2000). In support of this, we found that lowering glucose in Locke's solution, in which glucose is the sole source of energy, produced a greater loss in cell viability than that observed in NBM. However, even though our *in vitro* observations are consistent with the findings of others, we cannot rule out the possibility that the absence of trophic substances in Locke's solution could have contributed to cell death (Mattson *et al.* 1993).

Although hypoglycemia can significantly curtail neuronal excitability (Mobbs *et al.* 2001), it is probably uncommon for *in vivo* glucose values to fall to 0 mM even in pathological states. In addition, following mild hypoglycemia, neurons are still functional and can readily depolarize (Mobbs *et al.* 2001). Neuronal depolarization is associated with changes in $[Ca^{2+}]_i$ and several studies show altered absolute $[Ca^{2+}]_i$ following hypoglycemia (Cheng &

Mattson 1991, Silver *et al.* 1997). However, although changes in absolute $[Ca^{2+}]_i$ have been linked to altered neuronal viability, it is unclear if depolarization-induced increases in $[Ca^{2+}]_i$ are affected by glucose deprivation.

To address the effects of hypoglycemia on neuronal function, we studied real-time, dynamic changes in relative $[Ca^{2+}]_i$, using Fluo-3-loaded neurons, following hypoglycemia under depolarizing conditions to allow us to assess rapid changes in $[Ca^{2+}]_i$.

Neurons exposed to 0 or 0.75 mM glucose for 15 min displayed sluggish and reduced Fluo-3 responses following KCl-evoked depolarization, compared with 25 mM glucose. In contrast, after 24 h of hypoglycemia, KCl-evoked Fluo-3 responses in cells exposed to 0 or 0.75 mM glucose were more robust than that found in 25 mM glucose. Whereas we studied real-time, dynamic changes in $r[Ca^{2+}]_i$, our observations are very consistent with studies that measured absolute $[Ca^{2+}]_i$ at fixed intervals, using Fura-2 (Cheng *et al.* 1993, Mattson *et al.* 1993).

In the first 15 min after hypoglycemia, we show that reducing $[Ca^{2+}]_i$ enhances, whereas increasing $[Ca^{2+}]_i$ reduces, hypoglycemic neuronal death. At 8 h after initial hypoglycemia, we show that lowering $[Ca^{2+}]_i$ reduces, whereas increasing $[Ca^{2+}]_i$ enhances, hypoglycemic neuronal death. These observations are in general agreement with studies by others (Mattson *et al.* 1993) and suggest that cell death is a function of $[Ca^{2+}]_i$ relative to an optimal concentration, in agreement with previous findings (Turner *et al.* 2002a) and those of other workers (Johnson *et al.* 1992, Zipfel *et al.* 2000).

Reductions in brain glucose levels trigger a release of excitotoxins (Wieloch 1985, Butcher *et al.* 1987, McGowan *et al.* 2002) and can also induce the release of adenosine (Fowler 1993, Zhu & Krnjevic 1993, Calabresi *et al.* 1997). In our studies we also observed increased adenosine concentrations following hypoglycemia. We have previously shown that direct activation of A_1 ARs with the selective agonist N^6 -cyclopentyladenosine can promote neuronal death both *in vitro* and *in vivo* (Turner *et al.* 2002b). It has been suggested that adenosine can exert toxic effects that are not mediated by cell surface receptors (Koshiba *et al.* 2002). However, because we observed protection from hypoglycemia-induced neuronal injury with DPCPX, CGS 15943 or CPMA, it is most likely

that adenosine, acting via A_1 ARs, mediated mediates cell injury.

It is possible that other adenosine receptors may be activated by the observed increases in adenosine following hypoglycemia. We obtained isolated neurons from embryonic rat cerebral cortex where A_1 ARs are expressed at high levels (Fastbom *et al.* 1987, Reppert *et al.* 1991, Rivkees *et al.* 1995, 2001, Dunwiddie & Masino 2001). Expression of A_{2A} ARs is generally restricted to the striatum and is considerably less than A_1 AR expression in other regions of the brain (Fink *et al.* 1992). In contrast, evidence of A_{2B} AR or A_3 AR expression in the brain remains unconvincing (Dixon *et al.* 1996, Rivkees *et al.* 2000). Thus, the reversal of hypoglycemia-induced neuronal death by the three different adenosine antagonists used in this study most likely involves blockade of A_1 ARs.

The observation that A_1 AR activation promotes neuronal injury appears to be inconsistent with contemporary opinion indicating a neuroprotective role for adenosine in the mature brain (von Lubitz 1999, Dunwiddie & Masino 2001). However, recent evidence indicates that in contrast to that which occurs in mature brains, A_1 AR activation during early postnatal development actually leads to neuronal injury (Turner *et al.* 2002a,b, 2003). Similarly, blockade of N-methyl D-aspartate receptor promotes neuroprotection in adult brains, but induces neuronal injury in neonatal brains (Ikonomidou *et al.* 1999). Thus, influences of neurotransmitter action of neuronal viability depend on the neurochemical and age.

Because most studies involving hypoglycemia use glucose deprivation in combination with other insults (Vannucci & Yager 1992, Yager *et al.* 1992, Ouanonou *et al.* 1999, Breder *et al.* 2000, Tekkok *et al.* 2002), it is difficult to determine direct and indirect effects. In contrast, the data we present here represent effects of hypoglycemia only, using a single population of neurons, wherein direct action of glucose deprivation can more readily be observed.

Hypoglycemia is thought to display a unique distribution of neuronal damage *in vivo* compared with other types of brain injury (hypoxia, ischemia, trauma), suggesting mechanistic differences (Auer 1986). We present data that suggest that glucose deprivation promotes neuronal injury that is mediated by A_1 ARs. In contrast, direct A_1 AR antagonism, using 8-cyclopentyltheophylline, has

been shown to increase neuronal death following exposure of mixed neuron–glia cultures to combined hypoxia/hypoglycemia (Lobner & Choi 1994). However, in the same study, the adenosine uptake inhibitor, dipyrindamole, also enhanced hypoxia/hypoglycemia-induced neuronal death. In a related study, release of adenosine in brain slices has been found to promote neuronal death following combined hypoxia/hypoglycemia (Barth *et al.* 1997). Thus, when considering the influence of adenosine release or A_1 AR antagonism on neuronal injury, the outcome may be inherently dependent on mechanistic differences between hypoglycemia alone or combined hypoglycemia plus hypoxia.

We also observed that A_1 AR antagonism was only effective if added to neurons at the same time that hypoglycemia was initiated and was unable to protect neurons if added to cultures 8 h after lowering glucose. Supporting a role for early activation of A_1 ARs, the decline in $r[Ca^{2+}]_i$ we observed following acute hypoglycemia (first 15 min) was reversed by DPCPX. These data indicate factors other than A_1 AR antagonism may be involved in mediating hypoglycemic injury, particularly at later times (Cheng *et al.* 1993, Mattson *et al.* 1993).

We observed that caspase-3 is activated following altered $[Ca^{2+}]_i$ or hypoglycemia, in agreement with other workers (Cheng *et al.* 1993, Moran *et al.* 1999, Ouyang *et al.* 2000, Han *et al.* 2001). Caspase-3 is an enzyme thought to be key to a cell's final commitment to programmed cell death (Yuan *et al.* 1993, Nicholson 1999). Importantly, we also observed that hypoglycemia-induced increases in caspase-3 activity were reversed by the A_1 AR antagonist, DPCPX. These data indicate that hypoglycemia induces programmed cell death by altering $[Ca^{2+}]_i$ and these events may be triggered by activation of A_1 ARs.

Earlier work suggested that activation of A_1 ARs triggers apoptotic death in neonatal brains and isolated neurons by a mechanism that involves thapsigargin-sensitive decreases in $[Ca^{2+}]_i$ (Turner *et al.* 2002a), indicating that A_1 AR-mediated changes in $[Ca^{2+}]_i$ are probably due to the altered release of calcium from endoplasmic stores (Thastrup *et al.* 1990). Yet, using electrophysiological approaches it will be important to identify that cellular basis of changing calcium dynamics to assess if this is indeed the case and to examine the

cellular current and channels that are influenced by A₁AR activation and hypoglycemia.

In conclusion, we find that alterations in [Ca²⁺]_i play a role in hypoglycemia-induced neuronal injury. We also find that when neurons are exposed to low glucose concentrations, there are both early and long-term alterations in relative [Ca²⁺]_i. Our results also suggest that release of adenosine and activation of A₁ARs plays a prominent role in mediating hypoglycemia-induced neuronal injury.

Acknowledgements

This work was funded by NIH grant RO1-NS33439 to S A R and HL70952 to M R B, the Donaghue Medical Research Foundation and the Fannie F Ripple Foundation Cell Imaging Facility.

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Received in final form 20 September 2003

Accepted 29 October 2003