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An Obligate Role for Oxygen in the Early Stages of Glutamate-Induced, Delayed Neuronal Death

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***In vitro* models of hypoxic/hypoglycemic injury reveal common mechanisms with glutamate excitotoxicity, but glutamate-induced toxicity in the absence of oxygen has never been directly addressed. Therefore, we assessed neuronal survival and intracellular calcium concentrations ($[Ca^{2+}]_i$) in neonatal hippocampal cultures in response to glutamate in the presence and absence of oxygen. Twenty-four hours of hypoxia alone killed 40% of the initial population, attributable to glutamate receptor-stimulated osmotic lysis. A 5 min glutamate exposure in ambient air killed 80% of the initial population by 24 hr later. When cultures were deprived of oxygen during and for 2–24 hr after excitotoxin exposure, glutamate did not cause additional neuronal death beyond that induced by oxygen depletion alone. Toxicities caused by activation of NMDA, AMPA, or kainate receptors were each ameliorated by oxygen depletion. In the absence of oxygen, glutamate evoked normal increases in $[Ca^{2+}]_i$, indicating that glutamate receptors functioned normally. The glutamate-induced increases in $[Ca^{2+}]_i$ were not toxic in the absence of oxygen. In a similar manner, oxygen-depletion prevented neuronal killing by the calcium ionophore, ionomycin. Neuronal death produced by hydrogen peroxide or iron sulfate was not ameliorated by oxygen removal. These oxidants maximally produced only a slow increase in $[Ca^{2+}]_i$ as the plasma membrane permeability increased nonspecifically. Therefore, oxygen-based reactions were an essential component of calcium-mediated, delayed neuronal death.**

[Key words: excitotoxicity, neuronal death, glutamate, hypoxia, ischemia, glutamate receptors, oxygen]

Glutamate toxicity has been proposed as an underlying cause of neuronal death following stroke, ischemia, or traumatic brain injury (Tecoma et al., 1989; Lipton and Rosenberg, 1994). Microdialysis measurements detect elevated glutamate levels in the extracellular space during ischemia (Benveniste et al., 1984). *In vitro*, experimental hypoxic/hypoglycemic injury also produces elevated extracellular glutamate (Monyer et al., 1992) and can be prevented by antagonism of glutamate receptors (Rothman et al., 1987b; Sheardown et al., 1990; Kaku et al., 1991). Under *in vitro* conditions, overstimulation of glutamate receptors permits a massive influx of extracellular calcium that mediates a slowly progressive, delayed neurodegeneration (Choi, 1987; Rothman et al., 1987a).

However, *in vitro* glutamate toxicity has been studied under normal atmospheric oxygen, unlike the hypoxic environment present during the high glutamate associated with ischemia. Indeed, incomplete ischemia has been reported to be more damaging than complete ischemia *in vivo* (Hossmann and Kleihues, 1973), reducing measures of cerebral energy metabolism (Siesjo, 1981). This has led to the hypothesis that hypoxia may be protective against glutamate–receptor overstimulation (Choi, 1990). We have directly tested this hypothesis by examining the effects of oxygen deprivation on glutamate toxicity of cultured hippocampal neurons.

Materials and Methods

Tissue culture. Cultures of neonatal hippocampal neurons were prepared as described previously (Dubinsky, 1993). Cultures were maintained for two weeks in MEM without glutamine containing 27.75 mM glucose and 10% NuSerum (Collaborative Research). The high level of glucose used in these cultures was chosen (1) to provide sufficient glucose for extended culture periods without media replacement (Lopes-Cardozo et al., 1986), (2) to minimize the damaging effects of hypoxia itself (Tombaugh and Sapolsky, 1990), and (3) to possibly stimulate glycolysis via the Crabtree effect (Crabtree, 1929) so that hypoxia would not produce major shifts in energy metabolism.

Toxicity experiments. Unless otherwise specified, cultures were challenged by exposure to 500 μM glutamate for 5 min, rinsed in Earle's Balanced Salt Solution, and incubated at 37°C for 24 hr. Cell survival was assessed by counting preselected fields after trypan blue exclusion (Dubinsky, 1993). Cells were bathed in the following solutions: during the five minutes of glutamate exposure, Earle's MEM supplemented with 26 mM $NaHCO_3$, 100 μM glutamine, 10 μM glycine, 27.75 mM glucose, 35 mM sucrose, pH 7.4, 352 mOsm; during the 24 hr incubation period, Earle's balanced salt solution containing 116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 M $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 27.75 mM glucose, 35 mM sucrose, pH 7.4, 354 mOsm. Addition of 100 μM glutamine during the 24 hr incubation did not increase hypoxia-induced neuronal killing (Goldberg et al., 1988). Experiments minimizing osmotic lysis were performed in a solution composed of (in mM): 116 NaGluconate, 5.4 KGluconate, 5.3 $CaCl_2$, 1.3 $MgSO_4$, 27.7 glucose, 35 sucrose, 26.2 $NaHCO_3$, 1 NaH_2PO_4. The added calcium and magnesium concentrations were adjusted to theoretically yield 1.8 mM free calcium and 0.8 mM free magnesium concentrations in the presence of gluconate. These were calculated using gluconate log K' for Ca^{2+} and Mg^{2+} of 1.21 and 0.70, respectively (*Handbook of Chemistry and Physics*, 40th ed., CRC, 1958). Unless otherwise specified, all toxicity data a compilation of three cultures per condition from each of three replicate experiments.

For toxicity experiments, cells exposed to ambient air/5% CO_2 were handled on the bench top for glutamate application followed by incubation at 37°C in 95% air, 5% CO_2 for 24 hr. All solutions were warmed and equilibrated with 95% air, 5% CO_2. In control experiments, toxicity

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was equivalent for solutions actively equilibrated by bubbling with gas or for solutions passively equilibrated in a 5% CO_2 incubator.

Cells exposed to hypoxia were manipulated in a sealed plastic glove bag inflated with 95% N_2, 5% CO_2 and were rinsed in solutions equilibrated with 95% N_2, 5% CO_2 (actively bubbled). Atmosphere inside the bag was flushed at least three times (vacuum extraction followed by 95% N_2, 5% CO_2 influx at 5–10 psi) until a miniature oxygen electrode (Microelectrodes, Inc.) placed inside the bag registered an oxygen content of ≤1.0% (average 0.0 ± 0.2 in 16 experiments). In several experiments, the calibration range on the oxygen electrode was extended to twice normal to insure that the oxygen readings in this low range were reliable. Inflating the bag with 95% argon, 5% CO_2 resulted in identical toxicity, ruling out nitrogen specific effects.

For long term incubations in the hypoxic environment, uncapped culture dishes and the O_2 electrode and meter were placed inside the incubator at 37°C in an airtight chamber (Billups-Rothenberg Inc., Del Mar CA) filled with 95% N_2, 5% CO_2. After 24 hr, oxygen levels in the chamber remained below 1.0%, averaging 0.3 ± 0.3% (N = 18 experiments). In two experiments, oxygen content of the solutions over the cells was measured as 0.8 ± 0.08% (N = 18) during glutamate, 0.4 ± 0.05% at the beginning of the incubation period, and 0.1 ± 0.06% at the end of the 24 hr of hypoxia. In one experiment, pH measurements were made in cultures 24 hr after toxic exposure. Cultures were removed from the incubator and immediately encased, along with the pH meter, in the glove bag which was then inflated with either 95% air, 5% CO_2 or 95% N_2, 5% CO_2.

Calcium measurements. The fluorescence microscope and low light level detector system (Hartley and Dubinsky, 1993) were encased in the plastic glove bag along with the oxygen electrode and meter. Cultures incubated with 4 μM fura-2-AM for 15 min were rinsed in a basic salt solution (containing in mM: 139 NaCl, 3 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 10 NaHEPES, 27.75 glucose, 15 sucrose, 0.01 glycine, pH 7.4, 330 mOsm), and placed on the microscope stage. Camera gains and a neuronal field were selected for monitoring. The bag was sealed and the atmosphere was changed to one containing 95% N_2, 5% CO_2, by successive application of vacuum and entry of the nitrogen gas mixture. Four to five cycles of gas exchange were required (30–60 min) to reach an oxygen level of ≤0.5%. This was maintained without decrement until the bag was opened. During the first entry of the nitrogen atmosphere, the solution bathing the cells was changed to one equilibrated with 95% N_2, 5% CO_2 (in mM: 114 NaCl, 3 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 26 $NaHCO_3$, 27.75 glucose, 15 sucrose, 0.01 glycine, pH 7.4, 333 mOsm). Control experiments were performed in an identical manner but the bag was gassed (four exchanges) with 95% air, 5% CO_2.

Calcium concentrations were assessed using ratios of the emitted fluorescence (530 ± 15 nm) upon stimulation at 340 ± 10 nm and 380 ± 10 nm (Grynkiewicz et al., 1985). Calibrations were performed *in situ* by exposing untreated neurons to 5 μM ionomycin in the bicarbonate buffered salt solution (above) in 95% air, 5% CO_2 followed by addition of 15 mM K_2EGTA. Resulting values of R_{max}, R_{min}, and β are indicated in the figure captions.

Basal $[Ca^{2+}]_i$ after a 24 hr hypoxic and/or toxic exposure was monitored by loading dishes in 95% air, 5% CO_2, 37°C, with fura-2-AM. These measurements were necessarily made in ambient air after removal from the hypoxic chamber or ambient air incubator and assessment of survival. Cells were bathed in (in mM); 139 NaCl, 3 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 1.0 $NaHCO_3$, 27.75 glucose, 15 sucrose, 10 NaHEPES, and 0.01 glycine. Intracellular calcium responses to H_2O_2 and iron sulfate in naive cultures were also determined in ambient air in this HEPES buffered solution.

Results

Toxicity studies were performed upon cultured hippocampal neurons in ambient air/5% CO_2, or an hypoxic environment of 95% N_2, 5% CO_2. Neuronal exposure to the hypoxic environment for 5 min produced little cell death by itself but exposure to the hypoxic environment for 24 hr killed about 40% of the initial population (Fig. 1*A*). Although not studied in detail, shorter hypoxic exposures produced less cell death. No damage to the astrocyte feeder layer was currently observed, in agreement with several laboratories (Vibulsreth et al., 1987; Goldberg et al., 1988; but see Yu et al., 1989). Five minute exposure to 500 μM glutamate was equally toxic in either ambient air or hypoxic

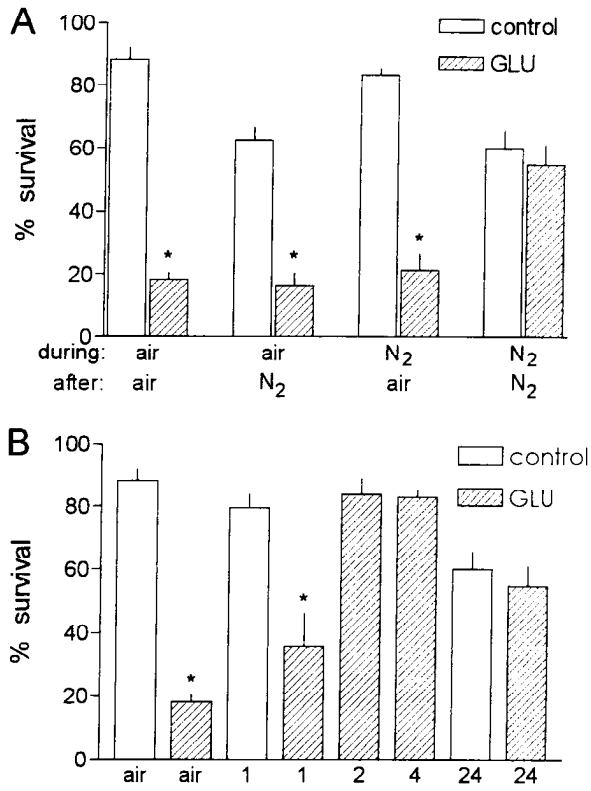

Figure 1. Oxygen depletion during and after glutamate overexposure was neuroprotective. *A*, Survival of neurons maintained in the indicated atmospheres for the 5 min glutamate exposure (*during*) and the following 24 hr period (*after*). *Hatched bars* represent cultures exposed to glutamate. *Open bars* represent cultures receiving only solution changes as controls. Mean ± SEM for 12–18 cultures per condition. Unless otherwise specified, statistical comparisons (one-way ANOVA followed by Bonferroni multiple comparisons test) were made between test conditions and the control bar to their immediate left. *Asterisk* indicates $p < 0.001$. *B*, Oxygen removal during glutamate exposure and for as little as 2 hr afterwards protected hippocampal neurons. *Numbered bars* represent the number of hours after glutamate treatment that cultures remained in the oxygen-depleted environment before reintroduction of ambient air. Bars labeled *air* represent cultures handled in ambient air/5% CO_2. All cultures were counted 24 hr following glutamate exposure.

environments if followed by recovery in ambient air. Likewise, glutamate exposure in air followed by the presence or absence of oxygen resulted in identical toxicity.

In contrast, removal of oxygen during both the glutamate challenge and the recovery period resulted in no additional cell loss beyond that induced by the hypoxic environment alone (Fig. 1*A*). Changes in the culture environment as a consequence of the extended hypoxia could not account for the observed protection. After 24 hr, lactate levels rose to 1.12 ± 0.04 mM in air and 1.21 ± 0.09 in the nitrogen environment (mean ± SEM, N = 9, not significantly different, two-tailed t test). These appeared to be well buffered as the color of the phenol red indicator did not change noticeably. pH of the culture media 24 hr after toxicity was 7.32 ± 0.003 in cultures exposed to glutamate in ambient atmosphere and 7.39 ± 0.002 in cultures exposed to glutamate and maintained in oxygen-depleted atmosphere (n = 12, $p < 0.0001$, two-tailed t test). In the absensce of lactate accumulation, it is difficult to ascribe the observed protection to a minor alkaline shift in external pH (see Discussion).

Further experiments showed oxygen removal during and for as little as 2 hr after glutamate exposure was sufficient to pro-

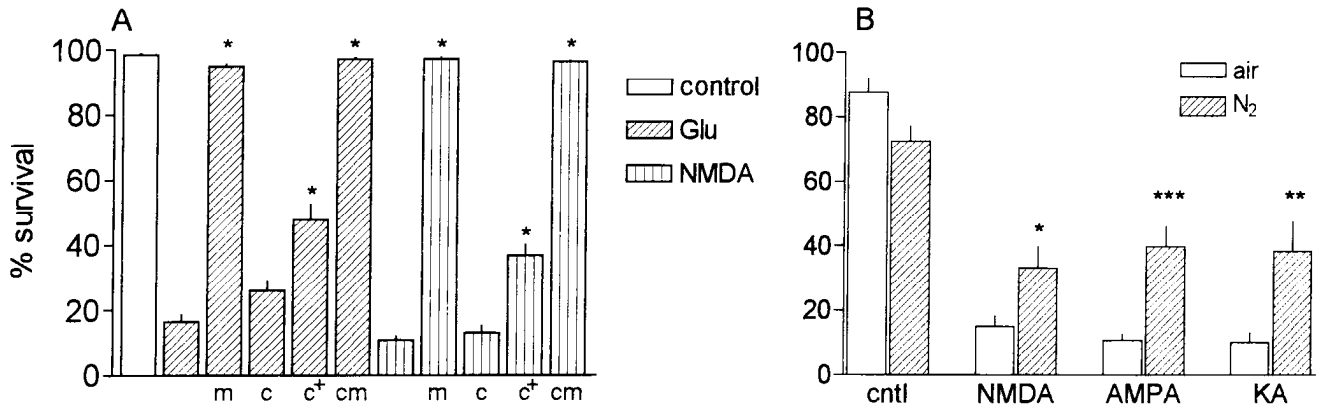

Figure 2. *A*, Both NMDA and non-NMDA receptors contribute to excitotoxic death. Hippocampal cultures received 5 min exposure to either 500 μM glutamate (*hatched bars*) or 500 μM NMDA (*striped bars*) accompanied by 20 μM MK-801 (*m*), 20 μM CNQX (*c*), or 20 μM MK-801 and 20 μM CNQX (*cm*). An additional set of cultures received 20 μM CNQX both during toxin exposure and for the subsequent 24 hr (*c*$^+$). *Asterisks* indicate $p < 0.001$ when compared to agonist alone. *B*, Oxygen removal partially protected against cell killing produced by different paradigms of glutamate receptor stimulation. Toxic exposures consisted of *NMDA*: 5 min of 500 μM NMDA followed by rinse in Earles' Balanced Salt Solution; *AMPA*: 24 hr of 30 μM AMPA plus 20 μM MK-801; *KA*: 24 hr of 30 μM kainate plus 20 μM MK-801. Bars represent 12–15 cultures per condition from five experiments. Statistical comparisons were made between toxic insults delivered in ambient air (*open bars*) and those in 95% N_2, 5% CO_2 (*hatched bars*) as described above. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

duce maximum protection (Fig. 1*B*). Reintroduction of oxygenated atmosphere after 1–4 hr of hypoxia by itself did not produce additional injury. Extending the hypoxia beyond 2 hr imposed additional cell killing attributable to oxygen depletion alone. Thus, oxygen availability during an initial critical period appears to play an obligate role in subsequent glutamate-induced injury.

Activation of different subtypes of ionotropic glutamate receptors each contribute to excitotoxic death, possibly by different mechanisms (Koh et al., 1990). MK-801, a noncompetetive NMDA antagonist, completely blocked toxicity induced by either glutamate or NMDA (Fig. 2*A*). CNQX, at concentrations that competetively inhibit non-NMDA receptors (Yamada et al., 1989), did not prevent toxicity when present during glutamate or NMDA exposure. However, when present both initially and during the 24 hr recovery period, CNQX was neuroprotective (Fig. 2*A*). Thus, while NMDA receptor activation was the primary event, non-NMDA receptor activation contributed to secondary toxic processes that occurred following both glutamate and NMDA overexposures.

Two different paradigms were employed to test the oxygen sensitivity of the toxicity produced by activation of each of these receptor subtypes. Transient NMDA receptor overstimulation mimicked the primary event in glutamate toxicity (Rothman and Olney, 1986) and long, low level activation of AMPA or KA receptors mimicked the slowly progressive neuronal death (Koh et al., 1990). Oxygen removal during and after NMDA exposure improved survival (Fig. 2*B*). Oxygen removal during 24 hr exposures to low doses of KA or AMPA provided greater, partial protection. Thus there appeared to be an oxygen dependent component to the excitotoxicity produced by activation of each type of ionotropic receptor.

One mechanism that must be considered to underlie any protective treatment is direct glutamate receptor antagonism. To assess glutamate receptor function in the absence of oxygen, glutamate-induced calcium increases were monitored in fura-2-AM loaded neurons during bath application of glutamate (Dubinsky, 1993). Glutamate receptor stimulation in the hypoxic atmosphere produced a rise in $[Ca^{2+}]_i$ that closely resembled that produced in ambient air (Fig. 3). $[Ca^{2+}]_i$ in both atmospheres rose rapidly to an initial peak and then decayed slowly towards basal levels. Half of all neurons monitored in either atmosphere displayed a plateau on the falling phase of the $[Ca^{2+}]_i$ changes, suggestive of the patterns attributed to mitochondrial sequestration and release in DRG neurons (Friel and Tsien, 1994; Werth and Thayer, 1994). Thus, glutamate receptor activation remained intact in the absence of oxygen.

Long term, permanent shifts in basal $[Ca^{2+}]_i$ were also not observed with the prolonged hypoxic treatments. No differences were observed in resting $[Ca^{2+}]_i$ between cultures protected from delayed, calcium-mediated glutamate toxicity by hypoxia and control cultures receiving similar solution changes but incubated in 95% air, 5% CO_2 for 24 hr (ratios of 0.296 ± 0.009, N = 110 from three dishes after hypoxia vs 0.294 ± 0.008, N = 128 from three dishes in controls, n.s. two-tailed t test). Neurons removed from hypoxic environments responded to glutamate with appropriate increases in $[Ca^{2+}]_i$.

Extended periods of oxygen deprivation killed approximately 40% of the initial neuronal population. This might be attributable to loss of metabolic energy production (Siesjo, 1981; Kass and Lipton, 1982) or to indirect glutamate receptor activation as observed with combined hypoxic/hypoglycemic models of *in vitro* neuronal death (Goldberg et al., 1987, 1988; Tombaugh and Sapolsky, 1990). Glucose levels declined to 26.8 ± 0.1 mM (N = 6) by the end of 24 hr in air and 24.9 ± 0.2 mM (N = 9) in nitrogen ($p < 0.0001$, two-tailed t test). Further increasing the glucose concentration in the medium failed to protect against the hypoxic cell loss (77.1 ± 2.5% survival, N = 6 in 40 mM glucose compared to 75.0 ± 2.0% N = 6 in 27.75 mM glucose, n.s. Student-Newman-Keuls). Thus despite the increased consumption during hypoxia, glucose availability was not a limiting factor for survival during hypoxia in these experiments. Addition of other possible energy sources, 10 mM pyruvate or 20 mM fructose, were also not protective against the extended hypoxia (81.1 ± 3.6% survival N = 6, pyruvate; 75.0 ± 1.9% N = 6, fructose, n.s.).

In contrast, glutamate receptor antagonists, MK-801 and/or CNQX added at the time of oxygen depletion prevented the cell loss associated with severe hypoxia (Fig. 4*A*). Since oxygen depletion did not noticeably alter basal $[Ca^{2+}]_i$ during the initial

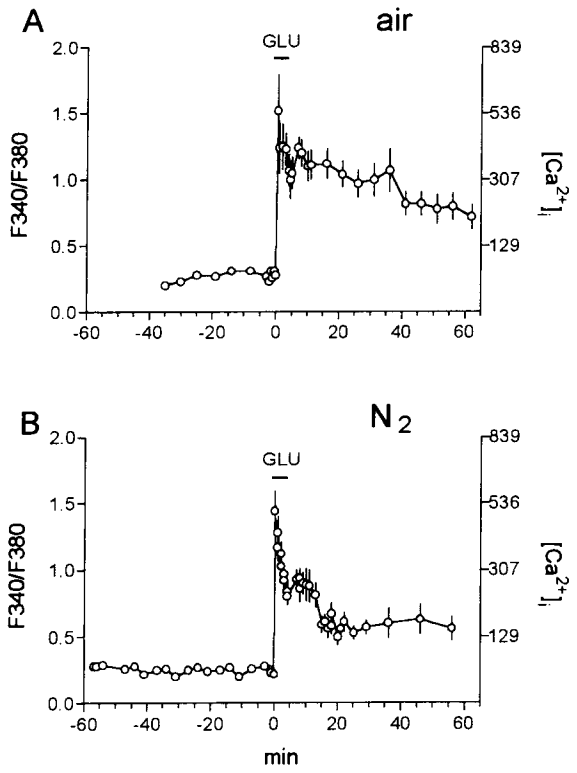

Figure 3. Similar intracellular calcium changes were induced by glutamate receptor activation in either the presence (95% air, 5% CO_2, *A*) or absence (95% N_2, 5% CO_2, *B*) of oxygen (11). $[Ca^{2+}]_i$ was monitored every 5 min during the changes in atmosphere prior to glutamate application (*horizontal line*). Statistical comparisons were made only between those data points that were acquired synchronously in both *A* and *B*. Data points at 0, 0.5, 1, 4, 5, 10, 11, 16, 21, 26, 31, 36, 46, and 56 min were compared, pairwise, by Student's *t* test. Only those points at 16, 21, and 26 min were significantly different at the $p < 0.05\%$ level. Data represent 35 neurons from three experiments in *A* and 24 neurons from three experiments in *B*. R_{max}, R_{min}, and β were 5.10, 0.06, and 5.99, respectively.

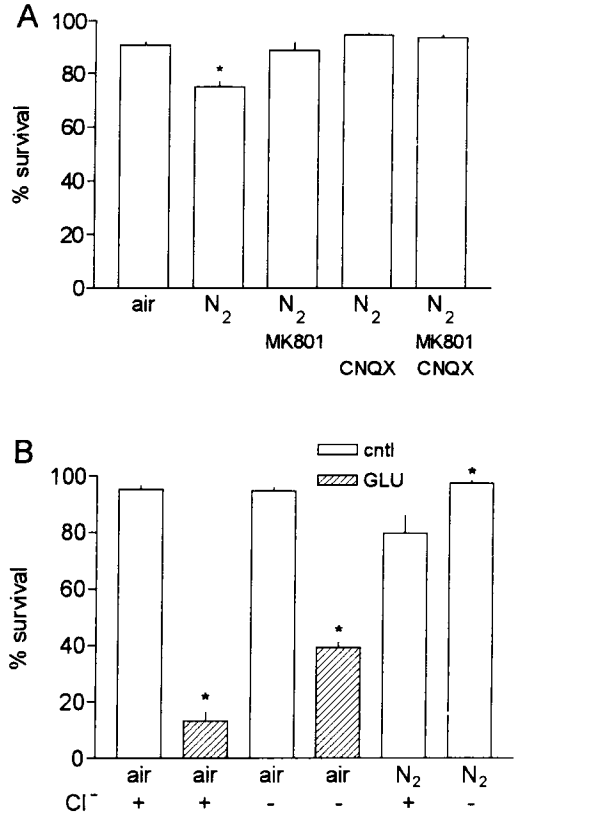

Figure 4. Neurotoxicity induced by 24 hr of severe hypoxia was attributable to low level glutamate receptor activation. *A*, Glutamate receptor antagonists, 20 μM MK801, 20 μM CNQX, or their combination prevented toxicity in cultures maintained for 24 hr in the hypoxic environment (N_2). Statistical comparisons were made between each oxygen-depleted condition and the air control using Dunnett's test. *Asterisk* indicates $p < 0.01$. *B*, Low chloride solutions ameliorated glutamate-induced toxicity in air and prevented toxicity associated with 24 hr of hypoxia. Statistics as in Figure 1*A*.

hour (Fig. 3*B*) or after 24 hr of hypoxia, calcium-independent mechanisms were considered. NMDA receptor overstimulation results in osmotic lysis following uninterrupted cation influx through open receptors, and the consequent passive Cl^- and H_2O entry (Rothman, 1985). Therefore, cultures were made hypoxic in solutions containing very low concentrations of Cl^- to prevent osmotic swelling (Rothman, 1985; Friedman and Haddad, 1994). Under these conditions, no cell death was observed (Fig. 4*B*). Thus, hypoxia alone appeared to produce a low level of extracellular glutamate accumulation that caused a slowly progressive neuronal death via osmotic lysis.

To eliminate the contribution of osmotically driven neuronal death, experiments were performed exposing cultures to different toxic doses of glutamate in the low chloride solutions (Fig. 5). Oxygen depletion during and for 24 hr after glutamate exposure fully protected these cultures against the remaining calcium-mediated degeneration. There was however, one exception. Prolonging the dose of glutamate to 30 min evidently produced neuronal damage that was too severe to be totally ameliorated by hypoxia. Thus, there appear to be extreme conditions in which the glutamate receptor mediated processes can be toxic without a contribution from oxygen-based reactions.

The relationship between calcium and oxygen dependence of neurotoxicity was explored by exposing cultures to a variety of

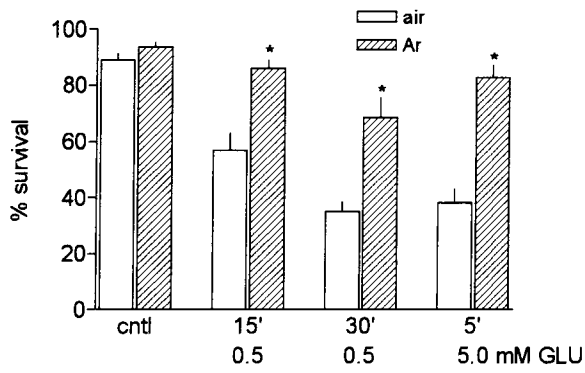

Figure 5. Oxygen depletion protected against more severe, calcium-dependent glutamate insults. Protection against prolonged glutamate exposures was substantial but less complete. Hippocampal cultures were exposed to 0.5 mM or 5 mM glutamate for the indicated times in ambient air (*open bars*) or in 95% argon, 5% CO_2 (*hatched bars*) and maintained in that atmosphere for 24 hr. These experiments were performed in low chloride solutions. Statistical comparisons were performed as described in Figure 1*A*.

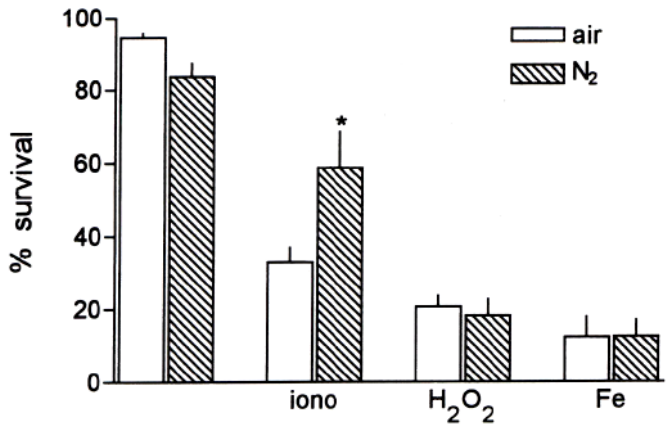

Figure 6. Oxygen depletion during and for 24 hr after 5 min exposure to other toxic agents provided partial protection against 1 μM ionomycin (*iono*), but not 1 mM H_2O_2 (*H_2O_2*) or a combination of 50 μM ferric sulfate, 50 μM ferrous sulfate and 1 mM ascorbate (*Fe*).

agents. To test if these additional forms of toxicity also involved oxygen-based processes, we exposed hippocampal cultures in the presence and absence of oxygen to (1) ionomycin, a calcium ionophore; (2) hydrogen peroxide, an exogenous, highly reactive oxygen species; or (3) a mixture of ferric and ferrous sulfate and ascorbic acid, a potent oxidant combination (Fig. 6). Application of H_2O_2 and iron sulfate were equally toxic in ambient air or hypoxic atmospheres (Fig. 6). Oxygen was apparently not required for the toxicity initiated by these highly reactive compounds. Oxygen depletion did protect against ionomycin-induced cell death (Fig. 6).

These other toxic agents produced very different intracellular calcium responses from that of glutamate. Ionomycin application is widely used to produce an uncontrolled calcium influx and maximal increases in $[Ca^{2+}]_i$. It is commonly applied to calibrate fura-2 fluorescence ratios (Grynkiewicz et al., 1985). In contrast, H_2O_2 and iron sulfate did not produce large initial, transient increases in $[Ca^{2+}]_i$ like glutamate or ionomycin (Fig. 7). Five minute application of H_2O_2 produced only minor perturbations

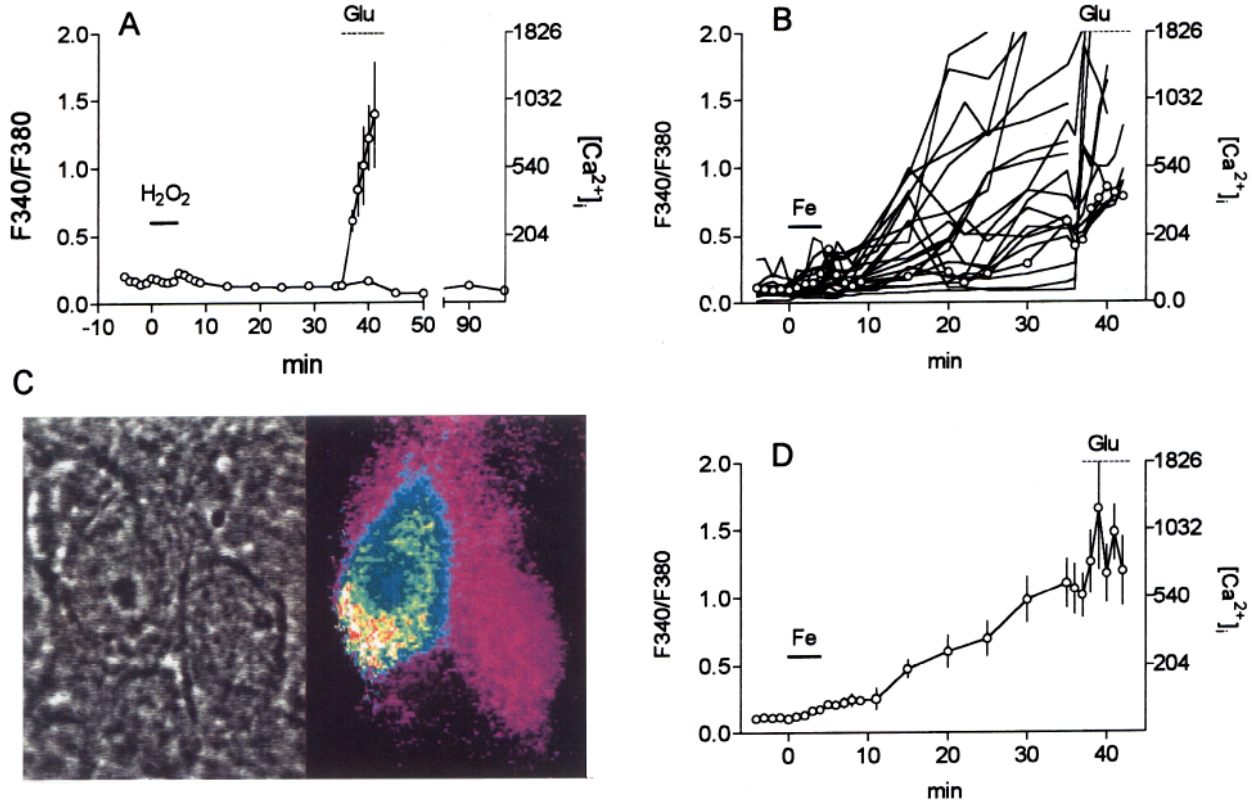

Figure 7. Fura-2 fluorescence responses to hydrogen peroxide (*A*) and a combination of 50 μM ferric sulfate, 50 μM ferrous sulfate and 1 mM ascorbate (*B, D*). *A*, Five minutes of 1 mM H_2O_2 produced no changes in $[Ca^{2+}]_i$ in 17 hippocampal neurons from three dishes. In one experiment (five neurons), $[Ca^{2+}]_i$ remained stable for over 90 min. In another experiment (eight neurons), 500 μM glutmate (*dotted line*) was added after 35 min. *B* and *D*, Five minutes of the iron sulfate mixture produced a slow rise in $[Ca^{2+}]_i$ in 20 neurons from three dishes. Thirty-seven minutes later, responses to 500 μM glutamate varied greatly (13 neurons). The highly variable responses of individual neurons are shown in *B* and the average response appears in *D*. *Circles* indicate the times ratios were acquired. In *B*, note that only those neurons with the lowest $[Ca^{2+}]_i$ demonstrated a clear response to glutamate. *C*, Ratio image of two neurons' response to iron revealed an initial increase in cytoplasmic $[Ca^{2+}]_i$ prior to nuclear calcium changes. The ratio image was taken at 20 min. By the time the bright-field image was taken, 25 min later, a prominent membrane bleb had developed in the plasma membrane of the leftmost neuron and dye had disappeared from the soma. Pseudocolor scale range is identical to the y-axis scale; black to red correspond to ratios of 0–2. In these experiments, R_{max}, R_{min}, and β were 3.6, 0.09, and 6.97, respectively.

in $[Ca^{2+}]_i$ for at least 90 min afterwards (Fig. 7*A*). Intracellular calcium in these neurons did rise sharply in response to subsequent glutamate addition. Higher concentrations of H_2O_2 produced slowly increasing cytoplasmic calciums accompanying somal swelling in a manner similar to iron.

The iron sulfate mixture caused a slowly progressive increase in $[Ca^{2+}]_i$ that was quite variable in its onset and rate of increase (Fig. 7*B,C*). When $[Ca^{2+}]_i$ did rise, it never seemed to plateau or recover. The increase in $[Ca^{2+}]_i$ was accompanied by an overall decrease in intensity of fura-2 fluorescence when stimulated at both 340 and 380 nm. This often required continually increasing the camera gain to maintain realistic ratio values. Once $[Ca^{2+}]_i$ began to rise, neuronal somas also became noticably swollen or blebbed. In a few flat neurons, the increase in $[Ca^{2+}]_i$ appeared first in a crescent at the edge of the soma, then filled cytoplasmic areas (Fig. 7*D*), and only later spread throughout the nucleas as well. When glutamate was subsequently added to these cultures, responses were not always distinguishable from the already steadily increasing $[Ca^{2+}]_i$. These observations could be explained by a slowly progressive nonspecific increase in permeability of the plasma membrane which would let external calcium enter the cell while the fura-2 dye exitted. Thus iron sulfate appeared to act by directly damaging the neuronal plasma membrane. The hydrogen peroxide and iron sulfate toxicities were not associated with an early transient increase in $[Ca^{2+}]_i$ nor were they prevented by oxygen depletion. Ionomycin toxicity, as well as glutamate toxicity, required oxygen in addition to the influx of extracellular calcium.

Discussion

Our data demonstrates that delayed, calcium-mediated neuronal death requires the presence of oxygen. This was true when either glutamate or ionomycin were the toxins. In the case of glutamate, oxygen was required either during initial exposure or within a critical period of approximately 2 hr after glutamate removal. In the absence of oxygen, the changes initiated by disturbances in intracellular calcium homeostasis appeared insufficient to produce neuronal death.

The relative contributions of elevated $[Ca^{2+}]_i$ and oxygen-based reactions towards eventual toxicity can not be separated in the current set of experiments. The data demonstrate that the initial peak $[Ca^{2+}]_i$ values and the time course of recovery were largely similar during glutamate treatments in both aerobic and anaerobic atmospheres. This is consistent with the lack of correlation between peak $[Ca^{2+}]_i$ and eventual neuronal death (Michaels and Rothman, 1990; Randall and Thayer, 1992). At intermediate periods, some small differences were noted. No data are available concerning the relationship of $[Ca^{2+}]_i$ in this time period with eventual death, although a relationship between toxic outcome and the duration of calcium elevation has been suggested (Ogura et al., 1988). Further experiments are necessary to determine if the hypoxic protection results from an acceleration of calcium recovery.

Extended periods of glutamate receptor activation were able to overcome the protection provided by the anerobic environment. This observation is consistent with the notion that neuronal death can be produced by multiple glutamate-triggered pathways (Dubinsky et al., 1995), only some of which require oxygen. Multiple oxygen-based steps may contribute to the eventual toxicity since oxygen depletion provided full protection whereas blockade of calcium-stimulated enzyme reactions only provides partial protection (Rothman et al., 1993; Zhang et al., 1994).

Multiple processes certainly contribute to excitotoxicity since different types of glutamate receptors were activated at different times. Initial blockade of NMDA receptors provided full protection confirming that NMDA receptor activation was the primary toxic event. Continuous blockade of non-NMDA receptors also protected significantly indicating that they contribute to secondary excitotoxic processes. Oxygen-deprivation significantly improved survivals in paradigms mimicking this secondary process. Oxygen-deprivation was also effective in protecting against short term NMDA receptor overstimulation. Transient NMDA receptor overactivation is thought to result in death by a combination of rapid osmotic mechanisms and delayed calcium-mediated pathways (Rothman and Olney, 1986). In the NMDA experiments, the osmotic destabilization may have contributed to the overall neuronal death in addition to the calcium-mediated pathways. Thus the efficacy of oxygen-deprivation in preventing glutamate-induced toxicity probably represents a combined interruption of multiple pathways.

Hypoxia alone produced neuronal cell loss, attributable to glutamate-receptor stimulated osmotic lysis. Hypoxia did not produce short term or permanent increases in $[Ca^{2+}]_i$. This is in contrast to reports to the contrary after prolonged hypoxic exposures for $[Ca^{2+}]_i$ in freshly dissociated adult hippocampal neurons (Friedman and Haddad, 1993). Chronological age of the neurons and the acute nature of the latter preparation may account for this difference. Contrasts between the current result and the ^{45}Ca accumulation observed in hypoxic cortical cultures may reflect the differences between fura-2 and ^{45}Ca measurements throughout the excitotoxicity literature (Marcoux et al., 1990). Cellular energy requirements under severe hypoxia were presumably met by the high rate of glycolytic metabolism in cultured neurons (Pauwels et al., 1985; Lopes-Cardozo et al., 1986). Combined hypoxia/hypoglycemia-induced neuronal death and neuronal death induced by hypoxia alone have been attributed to activation of glutamate-based toxicity mechanisms (Goldberg et al., 1988; Kaku et al., 1991). In addition, hypoxic pretreatments predispose neuronal cultures to increased glutamate toxicity when the intervening period is relatively short (Kohmura et al., 1990; Dubinsky, 1992).

Many important cellular changes occur in the early hours following excitotoxic exposure. Appreciable neuronal death does not become evident before 2 hr after excitotoxin application (Dubinsky et al., 1995; Dawson et al., 1993). Full recovery of initial $[Ca^{2+}]_i$ requires up to 2 hr following glutamate exposure (Dubinsky, 1993). Applications of neuroprotective compounds become ineffective between 0.5 to 4 hr following excitotoxic insult, depending upon the toxin and culture system (Hartley and Choi, 1989; Levy and Lipton, 1990; Csernansky et al., 1994). In hippocampal cultures, MK-801 and CNQX offer significant protection only when added within 2 hr after glutamate (Dubinsky et al, 1995). Thus, the combined actions of elevated $[Ca^{2+}]_i$ and molecular oxygen appear to be acting during a critical period during which cell fate may still be undecided.

Considering the overshoot in oxygen tension upon reperfusion after ischemia, the contribution of oxygen-based damaging reactions may be an early step in the toxicity cascade (Silver and Erecinska, 1992). Although not all experiments have detected evidence of oxygen-based, free radical involvement in ischemic injury (Agardh et al., 1991; Lundgren et al., 1991; Folbergrova et al., 1993), some evidence exists (Cao et al., 1988; Oliver et

al., 1990). Gradual reoxygenation after ischemia improves recovery of intracellular metabolite levels, reduces inhibition of protein synthesis, and reduces lipid peroxidation in spinal cords (Danielisova et al., 1990; Burda et al., 1991; Fercakova et al., 1992). Similarly, neuronal survival in hypoxic murine cortical cultures improves following graded reoxygenation (Sher and Hu, 1992). Additionally, hyperoxia during reperfusion following cardiac arrest exacerbates neurological damage (Zwemer et al., 1994).

Oxygen depletion would be expected to inhibit all oxygen-based reactions; oxidative phosphorylation, oxygen-based free radical formation, and oxygen-based redox reactions. While we have no data to suggest which reactions might be contributing to excitotoxicity, oxygen-based free radicals have been implicated based upon the protection provided by free radical scavengers (Dykens et al., 1987; Monyer et al., 1990; Kinouchi et al., 1991; Clemens et al., 1993; Lafon-Cazal et al., 1993; Chow et al., 1994). Direct measurements revealed NMDA receptor-linked superoxide production 15–30 min after stimulation, associated with nitric oxide synthase activity and eventual cerebellar neuronal death (Culcasi et al., 1994). Chain reactions involving nitric oxide-stimulated superoxide, peroxynitrite, and hydroxyl radical formation may also be triggered by NMDA receptor stimulation (Dawson et al., 1993; Hammer et al., 1993; Lipton et al., 1993). Thus, the current studies are consistent with the previous experiments suggesting that oxygen-based reactions, perhaps involving free radicals, may contribute to ischemia and glutamate-induced toxic processes.

Our studies with other toxins suggest that oxygen may be essential for toxicities associated with rises in $[Ca^{2+}]_i$. Oxygen-deprivation also protected hippocampal cultures from toxicity associated with the calcium ionophore, ionomycin. Previously, *bcl-2*, superoxide dismutase and inhibitors of nitric oxide synthase have prevented neuronal death triggered by calcium ionophores (Dawson et al., 1993; Zhong et al., 1993), indicating that oxygen and free radical generation contribute to this type of calcium-triggered toxicity. Anoxia failed to protect hippocampal cultures from death produced by the potent external oxidants, H_2O_2 and iron sulfate. Neither of these later agents produced an immediate, transient increase in $[Ca^{2+}]_i$. Iron did cause a progressive increase in $[Ca^{2+}]_i$ concomitant with increased permeability of the plasma membrane. Since higher concentrations of H_2O_2 produced responses similar to iron, it is expected that the concentration used for toxicity would eventually produce a similar pattern of calcium increase at the time of plasma membrane breakdown. These results suggest that their lethality may be connected with structural damage to proteins or lipids of the plasma membrane and not a process dependent upon elevated internal calcium and oxygen-based reactions. Taken together, these experiments suggest that elevated internal calcium levels and production of intracellular oxygen-dependent events may be a potentially lethal combination.

It is unlikely that the small external alkalinization during hypoxia could possibly mediate the reported protection. External acidity decreases NMDA receptor operation in the physiological pH range. AMPA and kainate receptors do not show a pH-dependent decline in function until pH declines below pH 6.8 (Traynalis and Culcandy, 1991). Thus pH changes in the oxygen-depleted cultures were opposite to that expected for any protective pH effects upon NMDA receptor function. The observed pH difference may reflect the imprecise nature of CO_2 control in incubators compared to the more precisely mixed, bottled gas mixture used to fill the hypoxic chamber.

Whatever the mechanism, the oxygen-based step(s) in the toxicity cascade occur(s) very early after receptor stimulation during the time period when intracellular calcium remains elevated (Dubinsky, 1993). Calcium-mediated, delayed glutamate toxicity was prevented in the absence of oxygen. Thus, abnormally high elevations in $[Ca^{2+}]_i$ were not in themselves always sufficient to cause death. Molecular oxygen appeared to be an additional, important requirement for delayed glutamate neurotoxicity.

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