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Hypoxic forebrain cholinergic neuron injury: role of glucose, excitatory amino acid receptors and nitric oxide

Michael P. Flavin*, Yuan Yang, Gerald Ho

Department of Pediatrics, Rm. 6-200, Doran 2, Kingston General Hospital, Queen's University, Kingston, Ont., Canada K7L 2V7

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Glucose depletion increased sensitivity to hypoxic insult in basal forebrain cultures in a dose-dependent manner as indicated by reduction of choline acetyltransferase (ChAT) activity, increased lactate dehydrogenase (LDH) release and disrupted morphology. The glutamate receptor antagonists 2-amino-5-phosphonovaleric acid (APV) and 6-cyano-2,3-nitroquinoxoline (CNQX) limited the degree of injury in combination and individually. The nitric oxide synthase (NOS) inhibitor *N*-nitro-L-arginine (NNLA) also either completely protected against mild injury or attenuated severe injury.

Cholinergic basal forebrain neurons can be protected from hypoxia by D,L-aminophosphonovaleric acid (APV) and 6-cyano-7-nitroquinoxalone (CNQX) [8], supporting data which suggest a key role for EAA receptor activation in hypoxic neuronal injury [12]. Nitric oxide (NO) appears to be important in glutamate neurotoxicity in cortical cultures [5]. However, nitric oxide synthase (NOS) inhibitors have caused both protection [3, 16] and exaggeration [20] of ischemic brain injury. Blood flow changes due to NOS inhibitors may be a confounding variable in these in vivo ischemia models but can be excluded in a cell culture system. *N*-Nitro-L-arginine (NNLA) competitively inhibits NOS [7], the synthetic enzyme for NO and this might be expected to reduce neuronal injury by direct effects.

We assessed the influence of glucose on hypoxic neuronal injury in basal forebrain cultures and examined the relative roles of EAA antagonists and a NOS inhibitor using reduction of specific choline acetyltransferase (ChAT) activity as the primary measure of injury and cell morphology and lactate dehydrogenase (LDH) release to support the data.

Basal forebrain tissue was harvested from the septal region of E14–15 Sprague–Dawley rat fetuses and dissociated and plated as previously described [8]. Cultures were exposed to hypoxia at 11 days in vitro. A salt solution (containing in mM: NaCl 120, KCl 5.4, CaCl 1.8 and tris-HCl 25) was used during exposure. Glucose was added in control unexposed cultures. Hypoxic exposure in 5% CO_2 at 37°C ranged from 3 to 8 h. Cultures were exposed to 0.5–1.5% O_2, glucose-free medium, APV 100 μM, CNQX 10 μM, NNLA 100 μM in various combinations. These doses of APV and CNQX caused minimal disruption of baseline ChAT activity. NNLA 100 μM was shown to prevent glutamate toxicity in cortical cultures [5]. The antagonists were present both during and after exposure unless otherwise stated. Serum-free MEM containing 2 mM glutamine and supplemented to 12 mM glucose was added after exposure. The cells were harvested 48 h after exposure (day 13 in vitro).

Prior to harvesting, cultures were inspected for signs of injury. Medium LDH concentration was measured by the method of Gay et al. [11]. ChAT activity was measured by modified method of Fonnum [9]. Protein assay was based on the method of Lowry [13].

Control, unexposed cultures showed clumps of phase bright cells with multiple processes on a monolayer of non-neuronal cells. Injured cells showed swelling, vacuolation, loss of processes or detachment. By inspection we could usually differentiate mild, moderate and severe injury which was primarily based on subsequent measurement of ChAT activity (mild = 70–80% control ChAT; moderate = 50–70%; and severe < 50%). Control cultures provided counts (dpm) which were always at least 10 times greater than background. Cultures tolerated up to 5 h hypoxia (H) or glucose removal (HG) with no significant change in morphology, ChAT activity or LDH release (data not shown).

*Corresponding author.

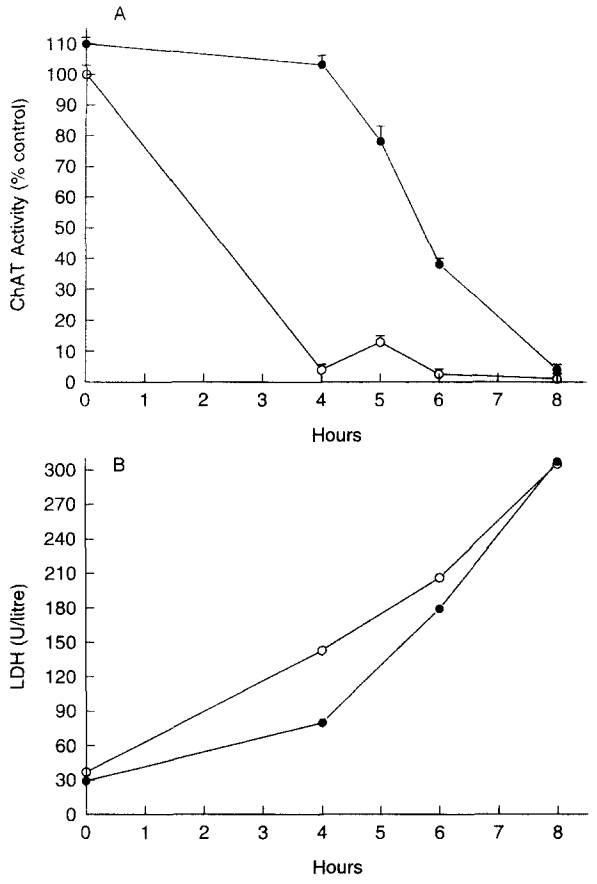

Fig. 1. A: the effect of increasing duration of H + HG insult from 4 to 8 h on ChAT activity expressed as percent control. Open circles connect H + HG data points while closed circles connect data points in which APV 100 μM and CNQX 10 μM have been added to the medium both during and after insult. At 4, 5 and 6 h, H + HG preservation of ChAT by APV + CNQX was significant ($P < 0.001$) ($n = 4$, mean ± S.E.). B: the effects of increasing H + HG on LDH concentration of a single aliquot of pooled culture medium from the same cultures as described in Fig. 3A. Open circles connect H + HG data points while closed circles connect data points in which APV 100 μM and CNQX 10 μM were added both during and after insult.

Combined insult (H + HG) significantly reduced ChAT activity. ChAT was significantly reduced after 4 h and was also very low in cultures exposed to 5, 6 and 8 h H + HG (Fig. 1A). Measurement of LDH on an aliquot of pooled medium in the same experiment showed that LDH concentration increased with increasing duration of H + HG (Fig. 1B). Thus an inverse relationship was noted whereby ChAT activity decreased in the cell layer as LDH concentration in the medium increased.

Combined APV 100 μM and CNQX 10 μM completely protected the cells from significant loss of ChAT activity at 4 h (Fig. 1A). Loss of ChAT activity after 5 and 6 h exposure was also severe but protection by APV and CNQX, although incomplete, was statistically significant ($P < 0.001$). Combined antagonists were ineffective in preventing the severe neuronal injury noted after 8 h H + HG when most neurons detached from the plate.

Coincidental with preservation of ChAT activity by APV and CNQX, we saw a reduction in LDH release with the combined EAA antagonists (Fig. 1B) although it was less impressive than the preservation of ChAT. Antagonists did not alter the high LDH concentration seen after 8 h H + HG.

The presence of glucose in the medium limited the degree of injury in a concentration-dependent manner (Fig. 2). ChAT activity was reduced to 13% when glucose was excluded from the medium and 47% in the presence of 1 mM glucose. Small reductions in ChAT activity after hypoxia in the presence of 2 mM and 6 mM glucose were not statistically significant. Combined APV 100 μM and CNQX 10 μM significantly attenuated the reduction in ChAT activity following hypoxia in the absence of glucose and also attenuated the moderate ChAT activity reduction in the presence of 1 mM glucose. APV 100 μM and CNQX 10 μM individually protected against moderate injury.

When applied both during and after insult, reduction of ChAT to 25% control was attenuated significantly by NNLA 100 μM (Table I). NNLA 250 μM or 500 μM caused no further protection. The presence of arginine 2.5 mM abolished any protective effect of NNLA. The addition of APV to NNLA in the culture medium provided a further small increment of protection.

Injury induced by combined H + HG in this system was accurately reproduced within experiments but significant variability was noted between experiments probably because of disproportionate time to achieve cell hy-

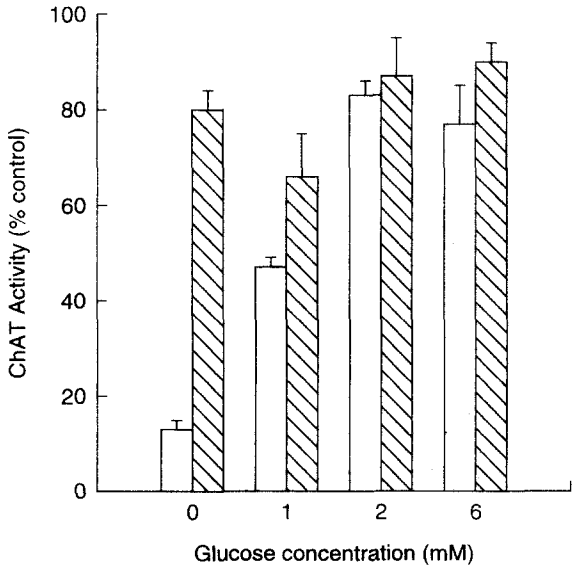

Fig. 2. The effects of 4.5 h hypoxia on septal cultures containing various concentrations of glucose (0–6 mM) in the medium without (open bars) and with addition of both APV 100 μM and CNQX 10 μM (hatched bars).

TABLE I

CHANGES IN ChAT ACTIVITY FOLLOWING H + hg WITH NNLA, NNLA IN THE PRESENCE OF ARGININE AND NNLA PLUS APV

Septal cultures exposed to 4.5 h H + HG at day 11 and harvested at day 13 in vitro. The effects of NNLA in varying doses of NNLA in the presence of arginine in excess and in the presence of APV on specific ChAT activity expressed as percent control. NNLA, APV or arginine were continued during and after insult. The preservation of ChAT at any dose of NNLA used was significant ($P < 0.05$). The addition of APV to NNLA during and after H + HG did not provide a statistically significant increment in ChAT activity ($n = 4$, mean ± S.E.).

Exposure	Additive	ChAT activity (% control)
Control	nil	100 ± 6
H + HG	nil	25 ± 3
H + HG	NNLA 100 μM	51 ± 2
H + HG	NNLA 250 μM	47 ± 1
H + HG	NNLA 500 μM	41 ± 2
H + HG	NNLA 100 μM + Arginine 2.5 mM	21 ± 1
H + HG	APV 100 μM	46 ± 1
H + HG	NNLA 100 μM + APV 100 μM	61 ± 5

poxia compared to time to be injured after onset of cell hypoxia.

Extremely low concentrations of glucose were able to minimize the degree of injury. These data reinforce other published data illustrating the critical importance of energy substrate availability in hypoxic ischemic brain insult [6]. Our cultures were fed with medium containing 25 mM glucose from day 1 through day 8 and 15 mM glucose medium in the 3 days leading up to injury (day 8–11). The protective effect of glucose may not be exerted solely by energy supply. The similar protection noted with APV and 2 mM glucose may be linked in that hypoglycemia induces EAA efflux [19] while glucose deprivation injury has been shown to be mediated by NMDA receptor activation [15].

Both NMDA and non-NMDA receptors have been implicated in hypoxic and ischemic neuronal injury [12, 18]. This appears to be true of hypoxic basal forebrain cholinergic neuronal injury as evidenced by the protective effects of APV and CNQX. The individual protective effect of APV suggests that at 11–13 days in vitro this region contains functional NMDA receptors contrary to findings in immature cultures [17].

The diagonal band which is incorporated in our culture system contains dense NOS immunoreactivity [2]. NADPH diaphorase positivity [14], which colocalizes with NOS, and acetylcholinesterase positivity are recognized features of neurons which are resistant to NMDA-mediated and hypoxic ischemic injury [10]. NNLA, as well as APV, individually protected cholinergic neurons in our system. The abolition of the protective effect of NNLA by arginine in excess suggests that the site of NNLA action is specific to NOS inhibition. The inability of increasing doses of NNLA to cause further protection suggests that NOS was saturated at 100 μM NNLA. NNLA applied during exposure may inhibit constitutive neuronal NOS and thus blocks deleterious effects of NO such as production of reactive nitrogen oxides. There was a modest additive effect when NNLA and APV were combined. To enable NOS to be activated downstream from NMDA receptor activation and Ca influx, one must assume that NMDA receptor blockade by APV was incomplete. Alternatively, inducible non-calcium-dependent NOS in microglia present in our culture may be the main site of action of NNLA [1]. Therefore one might expect additive effects with APV and NNLA particularly where NNLA was present for 48 h after insult (Table I).

A priority in further experiments will be to determine if NO is being produced in our cultures, to determine its source, and its contribution to neuronal death. Future experiments also need to control and define the contribution of glia. This system may allow further testing of possible separate and overlapping effects of glutamate and NO in hypoxic and glucose deprivation injury.

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