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Hypoxia suppresses astrocyte glutamate transport independently of amyloid formation

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Abstract

Sustained hypoxia alters the expression of numerous proteins and predisposes individuals to Alzheimer's disease (AD). We have previously shown that hypoxia *in vitro* alters Ca^{2+} homeostasis in astrocytes and promotes increased production of amyloid β peptides (Aβ) of AD. Indeed, alteration of Ca^{2+} homeostasis requires amyloid formation. Here, we show that electrogenic glutamate uptake by astrocytes is suppressed by hypoxia (1% O_2, 24 h) in a manner that is independent of amyloid β peptide formation. Thus, hypoxic suppression of glutamate uptake and expression levels of glutamate transporter proteins EAAT1 and EAAT2 were not mimicked by exogenous application of amyloid β peptide, or by prevention of endogenous amyloid peptide formation (using inhibitors of either β or γ secretase). Thus, dysfunction in glutamate homeostasis in hypoxic conditions is independent of Aβ production, but will likely contribute to neuronal damage and death associated with AD following hypoxic events.

Keywords: Glia; Glutamate transport; Hypoxia; Alzheimer's; Amyloid

Sustained hypoxia is an important consequence of numerous cardiorespiratory diseases such as emphysema, congestive heart disease and chronic obstructive pulmonary disease, and is also a key factor in other conditions such as cancer [1,2]. In the brain, as in other tissues, hypoxia can lead to adaptive remodeling of numerous cellular processes through the activation of a variety of transcription factors [3]. Some aspects of these remodeling processes may be considered protective, whilst others may be pathophysiological. Indeed, clinical evidence suggests hypoxia can predispose individuals to dementias such as Alzheimer's disease (AD; [4–6]). Amyloid precursor protein (APP), from which amyloid peptide (Aβ) of Alzheimer's disease is derived, is one of the few gene products whose expression is increased following a period of cerebral ischemia [7,8]. Since a major cleavage product of APP, sAPPα, is neuroprotective (e.g. [9]), increased expression of APP can be considered a defence mechanism against ischemia. However, increased APP levels also provide increased substrate for formation of neurotoxic Aβs [9] and Aβ production is indeed increased following both mild and severe ischemia [10,11].

We have previously shown that chronic hypoxia *in vitro* promotes Aβ formation in various cell types, including PC12 cells [12], central neurons [13] and astrocytes [14]. Increased Aβ formation resulting from hypoxia is associated with dramatic alterations in the functional expression of ion channels and Ca^{2+} homeostasis, key factors in the activity of both neurons and astrocytes. Crucially, these effects of hypoxia can be prevented by the application of inhibitors of β or γ secretases, which cleave Aβ from APP [13,14]. Most recently, we have demonstrated that chronic hypoxia suppresses glutamate uptake in astrocytes, an effect attributable to inhibition of the glutamate transporters EAAT1 and EAAT2 at the transcriptional level [15]. To date, conflicting reports exist as to the effect of Aβ on astrocytic glutamate uptake [16,17]. However these

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discrepancies can be attributed to various Aβ treatment protocols and cell culture methodology. The aim of the present study was to determine whether hypoxic inhibition of this key function of astrocytes was attributable to Aβ formation at both the functional and molecular level.

Methods

Astrocyte culture. Astrocytes were prepared exactly as previously described [15,18]. Briefly, cerebral cortices of 6–8-day-old Wistar rats were minced with a mechanical tissue chopper (McIlwain, Missouri, USA) and dispersed into PBS buffer containing 0.25 μg/ml trypsin (37 °C, 15 min). Digestion was halted by addition of an equal volume of buffer supplemented with 16 μg/mL soy bean trypsin inhibitor (type I-S; Sigma, Poole, Dorset, UK), 0.5 μg/mL DNase I (125 kU/mL; Sigma) and 1.5 mm $MgSO_4$. The tissue was then pelleted, triturated and the cell suspension pipetted off into media (Eagle's minimal essential medium supplemented with 10% fetal calf serum (v/v) and 1% (v/v) penicillin–streptomycin (Gibco, UK, Paisley, Scotland, UK)). The cell suspension was then aliquoted into 75 cm^2 flasks and also onto glass coverslips in 6-well tissue culture plates. Cells were kept in a humidified incubator (37 °C, 95% air; 5% CO_2). Only primary cell cultures were used, after 7–14 days in culture. Four to six hours following plating, cells were washed with fresh media to remove non-adhered cells, leaving a culture of >98% cortical astrocytes (confirmed by glial fibrillary acidic protein immunohistochemistry; data not shown). Cells exposed to chronic hypoxia were prepared identically but 24 h prior to experimentation were transferred to a humidified incubator equilibrated with 1% O_2, 5% CO_2 and 2.5% N_2. Corresponding control cells were maintained in a 95% air, 5% CO_2 incubator for the same period. For the secretase studies βS- II (30 nM) or γS-IV (3 μM) inhibitors (both Calbiochem, UK) were added to the culture medium at the stated concentration and incubated for 24 h prior to experimentation.

Electrophysiology. Fragments of coverslip with attached cells were transferred to a continuously perfused (3–5 ml/min) recording chamber and whole cell patch clamp recordings were obtained from individual astrocytes. The pipette solution (pH 7.25) consisted of (in mM): KSCN (140); EGTA (5); $MgCl_2$ (1); $CaCl_2$ (0.5); Hepes (10); MgATP (3); NaGTP (0.3). The perfusate (pH 7.4) was composed of (in mM): NaCl (150); KCl (5); $MgCl_2$ (2); Hepes (10); $CaCl_2$ (2); D-glucose (10). To evoke transporter currents, D-glutamate (10 μM) was applied to cells voltage-clamped at −70 mV. Patch pipettes had resistances 5–6 MΩ. Signals were acquired using a Multiclamp 700A amplifer controlled by Clampex 9.0 software via a Digidata 1322A interface (all Molecular Devices, Foster City, CA). NBQX, AP-5 and MCPG (all from Tocris, UK) were prepared as stock solutions and frozen. They were diluted to the given concentration in the extracellular solution and applied to the bathing medium. Offline analysis was carried out using the data analysis package Clampfit 9 (Molecular Devices, Foster City, CA) and data are expressed as mean ± SEM. P values are from two-tailed Student's t-test, where $P < 0.05$ was considered significant.

Western blotting. For Western blotting, astrocytes were grown to confluence as detailed above in 75 cm^2 flasks, washed free of media in ice cold PBS and then lysed in 800 μl M-per TM mammalian protein extraction reagent (Perbio Science, Tattenhall, Cheshire, UK) containing Complete Mini protease inhibitors (Roche Diagnostics UK Ltd., Lewes, East Sussex, UK). Protein levels of the cell lysates were then determined using a BCA assay kit according to manufacturers instructions (Pierce, Illinois, USA). Twenty-five microliters of sample buffer (125 mM Tris/

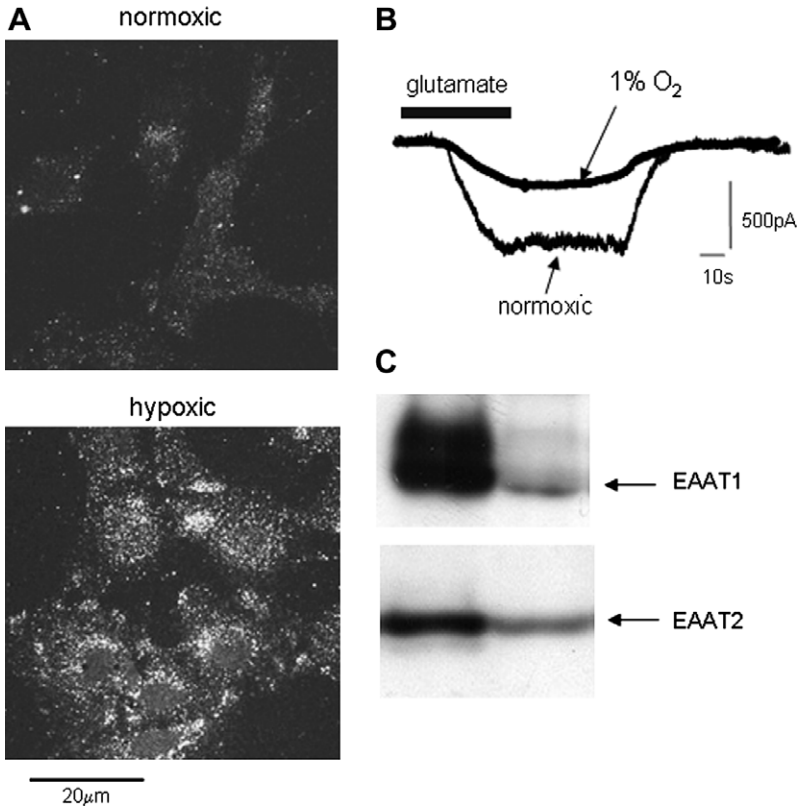

Fig. 1. (A) Representative images of astrocytes immunostained with the anti-Aβ antibody 3D6. Cells were cultured normoxically or in 1% O_2 for 24 h before immunostaining. Cells cultured in hypoxia (1% O_2) showed an increase in immunoreactivity. (B) Example inward current evoked by exposure of an astrocyte, voltage-clamped at −70 mV, to 10 μM L-glutamate (applied for the period indicated by the horizontal bar). Cells exposed to hypoxic conditions (1% O_2, 24 h) showed a reduction in the magnitude of the current amplitude. (C) Example Western blots showing EAAT1 and EAAT2 following 24 h normoxia (left lane) or hypoxia (right lane). Hypoxic conditions caused a reduction in protein expression for both EAAT1 and EAAT2.

HCl, pH 6.8, 2% (w/v) SDS, 20% (w/v) glycerol, 20 μg/μl bromophenol blue and 10% β-mercaptoethanol) was then added to 50 μl of each cell lysate. Samples (10–20 μg protein) were then loaded onto 12.5%, 0.75 mm thick polyacrylamide–sodium dodecyl sulphate gels and separated at 36 mA for 1 h before being transferred onto polyvinyl difluoride membranes (30 V overnight). Membranes were blocked with 5% non-fat milk protein in PBS–Tween (0.05%) for 1 h and immunostained with antibodies raised against the c-termini of rat EAAT 1 (AbCam, Cambridge UK; raised in rabbit against a 16 amino acid sequence), EAAT2 (AbCam, raised in rabbit against a 20 amino acid sequence). All antibodies were used at 1:1000 dilution for 3 h. Western blots were then washed and incubated with anti-rabbit Ig conjugated to horseradish peroxidase (1:2000, Amersham UK Ltd., Little Chalfont, Bucks, UK) and bands visualized using the enhanced chemiluminescence (ECL) detection system and hyperfilm ECL (Amersham, UK). Band intensities were measured using the Scion Image analysis software.

Immunocytochemistry. Astrocytes were rinsed three times with PBS and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 min. Cells were then blocked with PBS containing 10% goat serum. They were then washed 3 times in PBS before incubation overnight at 4 °C in primary antibody raised against Aβ either 3D6 (1:200, Elan Pharmaceuticals, USA) which recognises the first five residues of Aβ, or 4G8 (1:1000, Covance, UK) which recognises residues 18–22. The coverslips were then washed in PBS and incubated for 1 h at room temperature with anti-mouse secondary antibody conjugated to Alexa Fluor 555 (1:1000 in PBS). Coverslips were then washed in PBS and mounted onto glass slides using Vectashieid mounting medium containing 4′6′diamidino-2-phenylindole (DAPI) (Vector Labs) and the edges sealed with nail varnish. Slides were viewed under a Zeiss Laser Scanning Confocal Microscope (LSM 510). Fluorophores were excited by sequential scanning with Argon and UV lasers and composite images were processed using Zeiss AIM software. Images were taken for each condition using the same brightness, gamma and contrast settings.

Results

In the present series of studies, we firstly confirmed that hypoxia (1% O_2, 24 h) caused a striking increase in immunoreactivity for Aβ in astrocytes. This is illustrated in Fig. 1A, and similar data were obtained using either of two primary anti-Aβ antibodies, 3D6 and 4G8 which recognize different epitopes of Aβ. Identical exposures to hypoxia also markedly inhibited electrogenic glutamate uptake (e.g. Fig. 1B; see Fig. 3B for quantification), an effect attributable to selective suppression of the expression of glutamate transporters EAAT1 and EAAT2 (Fig. 1C; see also [15]).

Previous work from our laboratory has shown that many of the effects of hypoxia observed in cell lines, neurons and astrocytes can be mimicked by exposure of cells to exogenous Aβ (either the 1–40 or the 1–42 form; [13,14,19,20]). Exposure of astrocytes to Aβ (1–40) for 24 h was without effect on glutamate uptake currents,

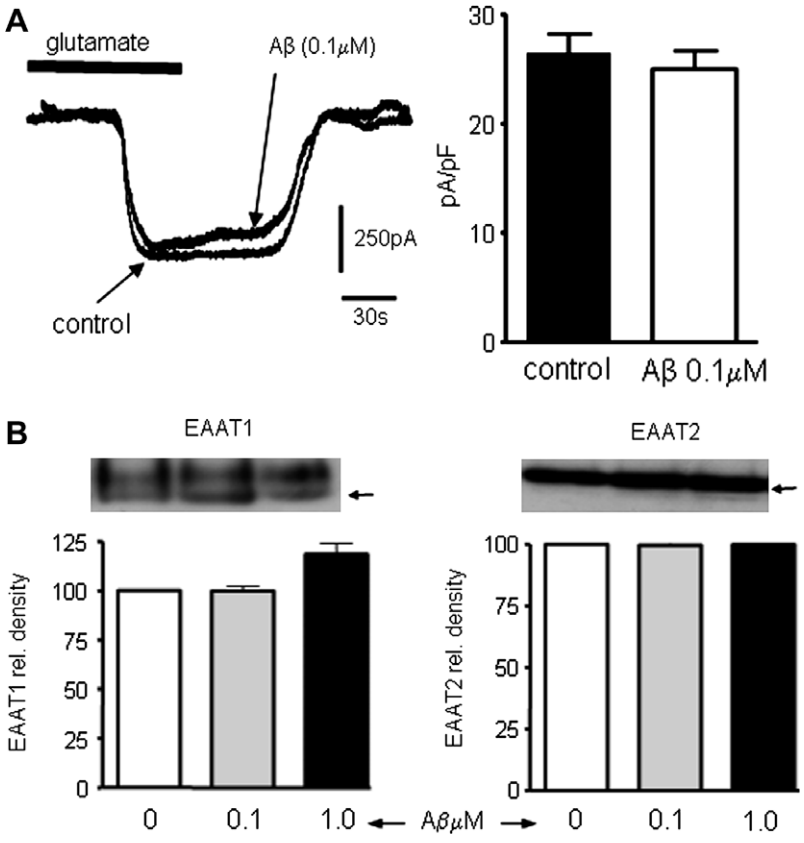

Fig. 2. (A) Example of glutamate evoked transporter current (recorded as in Fig. 1B) in the presence of exogenous Aβ. There was no difference in amplitude of the response in the presence of Aβ as compared to the control trace (left hand panel). Pooled data showing no difference in current density between Aβ treated and untreated cells (right hand panel). (B) Representative Western blots showing EAAT1 and EAAT2 expression in presence of Aβ (top panel). Bar graphs that illustrate relative protein expression normalised to control indicating that exogenous Aβ had no effect on expression of either EAAT1 or EAAT2 (bottom panel).

and this lack of effect was also observed at the protein level for both EAAT1 and EAAT2 (Fig. 2).

It is conceivable that exogenously applied Aβ may not modulate cell function in the same manner as endogenously produced Aβ, so we also sought to determine the effects of inhibiting endogenous Aβ on glutamate transport and transporter expression. As exemplified in Fig. 3A and quantified in Fig. 3B, inhibition of either β or γ secretase using (β-secretase inhibitor II, 30 nM; γ-secretase inhibitor IV, 3 μM) had no significant effect on the ability of hypoxia to suppress glutamate uptake. Similarly, expression levels of EAAT1 (Fig. 4A) and EAAT2 (Fig. 4B) were unaffected by secretase inhibitors under either normoxic or hypoxic conditions.

Discussion

Insufficient O_2 supply to the brain is a consequence of numerous, often age-related diseases, and predisposes to the development of dementias, the most common of which

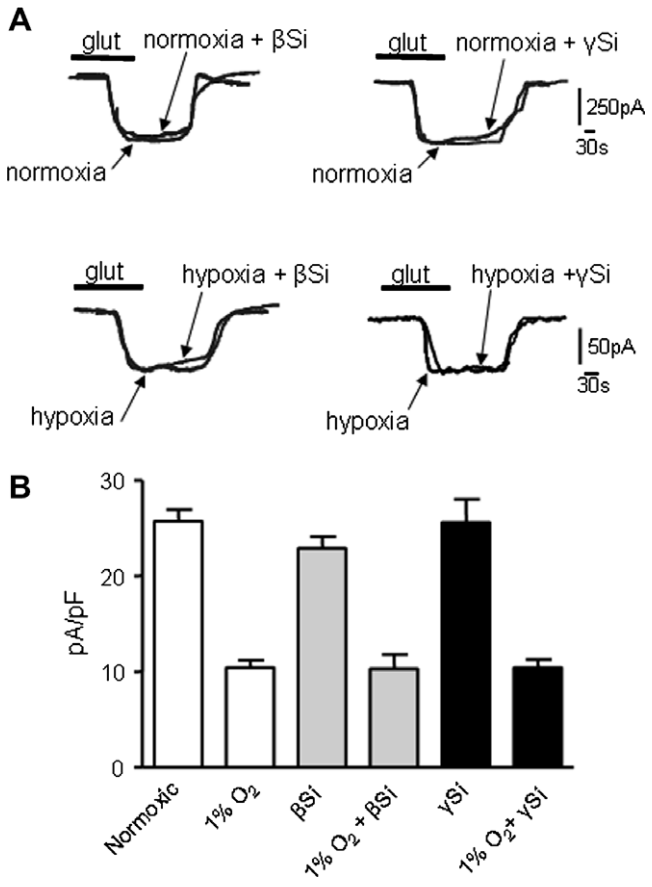

Fig. 3. (A) Superimposed example inward currents evoked by exposure of astrocytes to 10 μM L-glutamate (applied for the period indicated by the horizontal bar). Cells were either cultured under normoxic conditions, or 1% O_2 as indicated. In addition γ (3 μM) and β (30 nM) secretase inhibitors were included in the culture media as stated above. Upper and lower scale bars apply respectively to upper and lower traces. (B) Mean current densities (with SEM bars) evoked by glutamate in cells cultured under conditions indicated in (A).

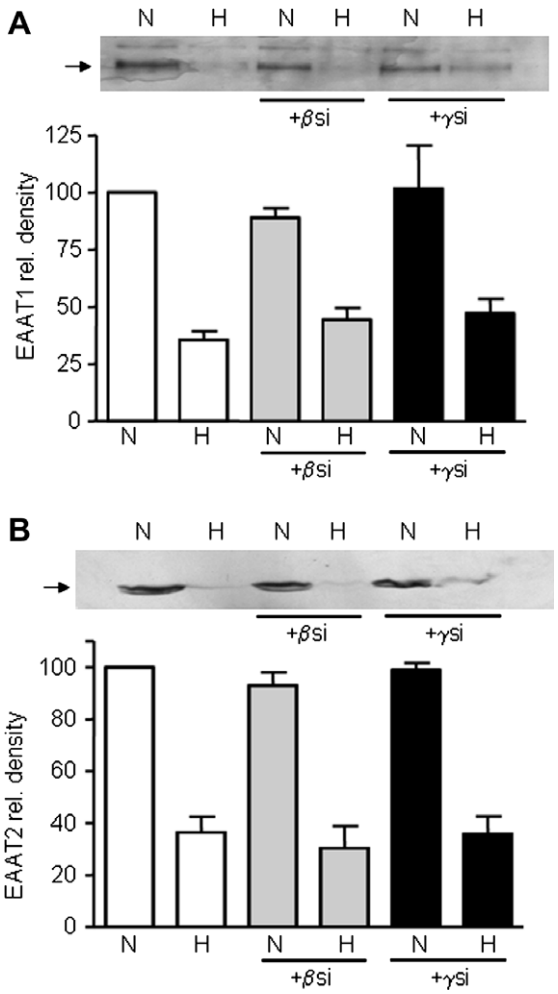

Fig. 4. (A) Western blot detection of EAAT1 (top panel) and bar graph illustrating relative levels of protein expression after normalisation of densitometric analysis relative to control (bottom panel). Cells were either cultured under normoxic conditions (N), or hypoxic (1% O_2) as indicated (H). In addition γ (γSi, 3 μM) and β (βSi, 30 nM) secretase inhibitors were included in the culture media as stated. (B) Western blot detection of EAAT2 (top panel) and bar graph illustrating relative levels of protein expression after normalisation of densitometric analysis relative to control (bottom panel). Cells were either cultured under the same conditions as in (A).

is Alzheimer's disease. Most strikingly, stroke survivors are up to 10-fold more likely to develop dementias than the rest of the population (e.g. [21]). The fundamental mechanisms underlying this important clinical association are not well understood. However, we have shown that, of the many physiological parameters disturbed are a consequence of stroke or other cardiorespiratory disorders, a reduction in available O_2 in itself can promote increased Aβ formation [12,20,22]. Our work has also indicated that hypoxic up-regulation of Aβ formation is associated with factors which disrupt intracellular Ca^{2+} homeostasis, a major proposed mechanism of cell death in Alzheimer's disease [23,24]. Thus, excessive levels of functional L-type Ca^{2+} channels in the plasma membrane arise under hypoxic conditions, a process which requires Aβ formation [13,25]. In addition, astrocyte Ca^{2+} homeostasis is disrupted by hypoxia, and is

partially reversed by inhibition of Aβ formation [14,26]. Here, we have identified the first functional alteration in astrocyte function arising from hypoxic conditions which appears to occur completely independently of amyloid formation. Previous cell functions (described above) altered by hypoxia could be partially or fully prevented by utilizing β or γ secretase inhibitors, and such effects of hypoxia were also mimicked by exogenous Aβ application. However, in these studies neither maneuver mimicked the effects of hypoxia on glutamate transport (Figs. 2–4).

Our results therefore indicate that of the multiple cell functions disrupted by hypoxia, not all are associated with formation of amyloid and therefore the onset of dementia. This suggests that dysfunction in glutamate homeostasis under hypoxia conditions is an independent event to the production of Aβ, at the level of the astrocytic glutamate transporters. However, this important effect of hypoxia to suppress the ability of astrocytes to clear glutamate is likely to contribute to glutamate-induced excitotoxicity, and so exacerbate neuronal damage in the hypoxic environment. This in turn is likely to contribute to the subsequent cellular pathology associated with hypoxia-associated dementias.

Acknowledgments

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