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Effects of acute hepatic encephalopathy and in vitro treatment with ammonia on glutamate oxidation in bulk-isolated astrocytes and mitochondria of the rat brain

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Abstract. The metabolism of [1-^{14}C] glutamate to $^{14}CO_2$ and the glutamate dehydrogenase (GLDH) activity towards α-ketoglutarate (α-KG) formation were measured in bulk isolated astrocytes derived from control rats and rats with acute hepatic encephalopathy (HE) induced with thioacetamide. In addition, the effects of in vitro treatment of control and HE astrocytes and non-synaptic mitochondria with toxic (3mM) NH_4Cl concentration were followed. [1-^{14}C] glutamate oxidation measured as a whole was identical in control and HE astrocytes and was inhibited by ammonia to the same degree in either fraction. In the presence of a glutamate transamination inhibitor — 3mM aminooxyacetic acid (AOA), when only the GLDH-mediated part (25% of total) of the glutamate oxidation remained active, the inhibitory effect of ammonia treatment was much more pronounced in HE astrocytes than in control astrocytes. The ability of non-synaptic mitochondria to utilize glutamate to CO_2 was not changed in presence of 3mM NH_4Cl, whereas a substantial decrease of CO_2 production (about 80%) in both the control and HE preparations was observed in the presence of 3mM AOA. GLDH activity was not at all affected by either of the experimental conditions, both in astrocytes and purified non-synaptic mitochondria. Thus, the inhibition of glutamate oxidation in astrocytes by ammonia and the compounded inhibitory effect of HE, ammonia and AOA appeared to be located beyond the glutamate dehydrogenation step within the tricarboxylic acid cycle.

Key words: rat, hepatic encephalopathy, thioacetamide, astrocytes, glutamate oxidation, glutamate dehydrogenase

INTRODUCTION

Hepatic encephalopathy (HE) is a disease whose pathogenesis is related to the neurotoxic action of ammonia, and which ultimately leads to profound metabolic and morphological changes in astrocytes — the main compartment of ammonia incorporation in the central nervous system (Norenberg 1986). Glutamate is a key metabolite in astrocytes where it serves not only as a major ammonia trap (Cooper et al. 1988) but also as an important energy substrate (Yu et al. 1982). The observation that ammonia at pathological concentrations depresses glutamate oxidation in astrocytes in culture has been considered to reflect one of the causes of gliopathy accompanying HE and to substantiate the long disputed role of bioenergetic failure in this disease (Yu et al. 1984). However, HE related to toxic liver damage is not fully equivalent to hyperammonemia. Numerous other factors have been implicated in the pathogenesis of this disease (Zieve 1982). It thus appeared of importance to verify this in vitro finding in a HE model in vivo. In this study we have compared $^{14}CO_2$ production from ^{14}C-labeled glutamate in bulk isolated astrocytes derived from control rats and from rats with acute HE induced with thioacetamide (TAA). We have also measured the effect of in vitro addition of ammonia into control and HE astrocytes.

Glutamate flux via the tricarboxylic acid cycle may be initiated either by glutamate dehydrogenase (GLDH)-catalyzed oxidative deamination or by transamination mediated by aspartate aminotransferase, and the relative contribution of either of the two reactions in different CNS compartments is a matter of dispute (Hindfelt et al. 1977, Cooper and Plum 1987). Therefore, to distinguish between the two possibilities, the oxidation was carried out in the presence or absence of a transaminase inhibitor aminooxyacetic acid (AOA) (Meijer and Van Dam 1974). Moreover, we have measured in all the experimental conditions the GLDH activity of astrocytes and nonsynaptic whole brain mitochondria. An account of this work was reported previously (Albrecht et al. 1988).

METHODS

HE model

Female Albino Wistar rats weighing 160 to 200 g received 3 injections of 250 mg/kg TAA, i.p., at 24 h intervals and were killed 24 h after the last administration. As described previously, this treatment produced metabolic and pathophysiological changes typical of acute HE including an increase of blood ammonia by 124% and of brain ammonia by 27% (Albrecht and Hilgier 1984). The EEG changes in these animals were consistent with the precomatous stage (Pluta and Albrecht 1984).

Isolation of astrocytes and mitochondria

The fraction enriched in astrocytes (some 75% enrichment) was isolated from fresh cerebral hemisphers with the trypsin-aspiration-differential centrifugation method (Albrecht et al. 1982). The fraction was enriched in the astroglia–specific enzyme, glutamine synthetase, and showed a negligible activity of the neuronal marker, glutamate decarboxylase. Highly pure non-synaptic mitochondria were obtained from homogenates of cerebral hemispheres by Ficoll-sucrose gradient centrifugation (Lai and Clark 1979).

Oxidation of [1-^{14}C] glutamate

This was done essentially as described by Roeder et al. (1984). Astrocytes and mitochondria (2-3 mg protein/ml) were suspended in a buffer containing (mM): 0.21 EDTA, 1 KH_2PO_4, 3.5 $MgSO_4$, 70 Tris, 3.8 KCl, 103 NaCl, 80 sucrose. In samples where the effect of ammonium ions was studied, resuspension was done in the buffer supplemented with NH_4Cl to final concentration of 3 mM. The samples were then supplemented in a tight-fitted glass tube with 10 μl of 10 mM [1-^{14}C] glutamate (Amersham, UK, spec. radioactivity of the solution 5 μCi/ml), placed in the scintillation vials, closed and incubated for 30 min in the water bath at 37°C. Preliminary experiments revealed that the decarboxylation process was linear for up to 1 h. The reaction was terminated by injection of 20 μl of 20% trichloroacetic acid and the radioactivity of $^{14}CO_2$ trapped in the NCS solubilizer was measured by liquid scintillation spectrometry.

GLDH activity

GlDH activity was measured spectrphotometrically by following NADH synthesis in the reaction: L-Glutamate + NAD^+ + H_2O = α-ketoglutarate + NADH + NH_4^+ in the presence of hydrazine to remove the reaction product α-ketoglutarate (α-KG), with the spectrophotometric method of Williamson and Corkey (1986).

TABLE I

The effect of ammonia and AOA on (1–^{14}C) glutamate oxidation in astrocytes from control and HE rats

	Glutamate oxidation (nmoles $^{14}CO_2$x mg prot^{-1}x h^{-1})	
Additions	Control	HE
None	24.6 ± 9.4 (6)	23.2 ± 7.1 (6)
NH_4^+ (3mM)	14.2 ± 3.6 (6)	12.9 ± 5.9 (6)
AOA (1mM)	9.0 ± 1.5 (5)	9.4 ± 2.0 (5)
NH_4^+(3mM) + AOA (1mM)	4.4 ± 1.5 (5)	1.6 ± 0.5 (5)*

Each value represents mean ± SD, for the number of experiments in parentheses. *Different from respective control at $P<0.01$. (t–Student's test). For further details see the Methods section.

RESULTS

Oxidation of [1-^{14}C] glutamate by astrocytes (Table I)

Formation of $^{14}CO_2$ from [1-^{14}C] was inhibited by AOA down to 37% of the control level both in control and HE astrocytes. There was no effect of HE alone on glutamate oxidation either in the presence or absence of AOA. Ammonia alone inhibited the total oxidation by 52%. The combined effect of ammonia and AOA was stronger in HE astrocytes (92% inhibition) than in control astrocytes (82% inhibition).

TABLE II

The effect of ammonia and AOA on (1 – ^{14}C) glutamate oxidation in nonsynaptic mitochondria from control and HE rats

	Glutamate oxidation (nmoles $^{14}CO_2$ × mg prot^{-1} × h^{-1})	
Additions	Control	HE
None	35.2±14.6 (9)	39.6±11.0 (11)
NH_4^+ (3mM)	33.8±6.5 (8)	36.8±9.2 (10)
AOA (1mM)	4.4±2.7 (7)	8.3±5.6 (10)
NH_4^+ (3mM + AOA (1mM)	3.0±2.6 (7)	6.4±4.5 (9)

Each values represents mean ± SD, for the number of experiment in parentheses. All differences between the respective control and HE groups were statistically insignificant. For further details see the Methods section.

Oxidation of [1-^{14}C] glutamate by nonsynaptic mitochondria (Table II)

Utilization of labelled glutamate to CO_2 was inhibited by AOA to 86% and 78% respectively in control and HE mitochondria, but remained unchanged in the conditions of HE, ammonium chloride treatment, and combination of both.

TABLE III

The effect of ammonia on GLDH activity in astrocytes and mitochondria from control and HE rats

	GLDH activity (nmoles NAD^+ reduced × mg protein^{-1} × min^{-1})	
Treatment	Astrocytes	Mitochondria
Control	4.74±0.93 (6)	49.6±3.0 (5)
Control + NH_4^+ (3 mM)	4.70±0.65 (6)	49.2±2.0 (5)
HE	4.17±0.87 (6)	50.7±3 (5)
HE + NH_4^+ (3mM)	4.33±0.88 (6)	51.7±3.0 (5)

Each value represents mean ± SD, for the number of experiments in parentheses. All differences between treatments in the same preparation (astrocytes or mitochondria) were statistically insignificant. For further details see the Methods section.

GLDH activity in astrocytes and mitochondria (Table III)

The specific activity of GLDH in mitochondria was approximately 10 times higher than in astrocytes. There was no effect of ammonia, HE or combination thereof on the mitochondrial or astrocytic enzyme activity. Separate tests revealed no significant effect of 3 mM AOA on the enzyme activity in either control preparations or in any of the pathological variants (data not shown).

DISCUSSION

The present results with bulk isolated astrocytes confirmed the earlier observation on astrocytes in culture that glutamate oxidation in these cells is sensitive to high, toxic concentrations of ammonia (Yu et al. 1984). The apparent absence of an effect of HE on this process is not surprising since neither blood nor brain ammonia reach toxic level in the present HE model (Albrecht and Hilgier 1984). An only moderate increase of brain ammonia was also observed in other HE models as well as in patients with acute or chronic HE (Denis et al. 1983, Zieve 1987). By contrast, acute hyperammonemia induced by MSO treatment resulted in a significant decrease of the activity of glutamate dehydrogenase as measured in the direction of α-ketoglutarate in astrocytes isolated with a similar procedure (Subbalakshmi and Murthy 1983). Hence, the present results reemphasize the fact that HE, which is a disease caused by a multitude of factors (Zieve 1982, Norenberg 1986), is not in all its aspects genuinely simulated by excess of ammonia.

AOA at 2 mM concentration has been reported to completely inhibit glutamate transamination (Tildon and Roeder 1984). As shown by the latter and confirmed in the present study (see the Results section), this treatment does not affect glutamate dehydrogenation.

A 63% inhibition of glutamate oxidation by AOA in astrocytes and about 80% inhibition in non-synaptic mitochondria are consistent with the observation made by Tildon and Roeder (1984) on brain homogenates and confirm the view that transamination is a major route of glutamate metabolism in cultured astrocytes (Yudkoff et al. 1987), neurones (Fitzpatrick et al. 1988), and purified mitochondria (Dennis et al. 1977). Similar inhibitory effects of AOA and NH_4Cl on glutamate oxidation including their additive character were observed in astrocytes in primary culture (Lai et al. 1989). It may be argued that, a fraction of CO_2 production from [1-^{14}C] glutamate in the astroglial preparations is due to glutamate decarboxylation mediated by glutamic acid decarboxylase (GAD) which is present in contaminating synaptosomes. However, this is unlikely to contribute to the observed effects of ammonia or HE, as the GAD activity in control astrocytes isolated with the present model was found not to exceed 3 nmoles $^{14}CO_2$/mg protein/h (Albrecht et al. 1982). Additionally, in order to verify the possibility of synaptosomal contamination we tested the influence of different concentrations of KCN, which is a very potent inhibitor of the respiratory chain, but is not expected to inhibit GAD, in astrocytes. At 3 mM KCN, an 80% decrease of CO_2 production was observed. At higher KCN concentrations the respiratory activity of this preparation was completely abolished (data not shown). This allows to qualify the observed CO_2 production to result from respiration but not from GAD-mediated decarboxylation. It is also worth recalling that HE in the present model did not significantly affect GAD activity (Wysmyk-Cybula et al. 1986).

A particularly interesting finding of the present study was that glutamic acid dehydrogenase-mediated (AOA-resistant) glutamate oxidation in HE astrocytes was more vulnerable to ammonia than in control astrocytes. This finds a clinical parallel in observations that both experimental animals (Gjedde et al. 1978) and patients with chronic HE (Lockwood et al. 1979) are more vulnerable to extra charge of ammonia than control subjects. It is tempting to speculate about a causal link between these observations.

The contribution of GLDH to the abnormalities of oxygen metabolism in astrocytes accompanying HE is not clear. Insofar, most studies have dealt with the enzyme activity in the presence of NADH, that is under conditions stimulating the reaction towards glutamate formation. Using these testing conditions, Murthy and colleagues observed a decrease of the astroglial enzyme activity in simple acute hyperammonemia (Subbalakshmi and Murthy 1983a), but a stimulation following inhibition of glutamine synthesis by MSO treatment (Subbalakshmi and Murthy 1983b). In the present study, in which GLDH was assayed under conditions favouring glutamate oxidation, the astroglial enzyme appeared refractory to HE, direct ammonia treatment, and combination of both. The lack of effect of all the factors on the enzyme in highly purified non-synaptic mitochondria, which are a better biological substrate than astrocytes themselves, further supported the notion that glutamate dehydrogenation is not the HE-sensitive step in astroglial glutamate oxidation. As such, the results are consistent with the recent observations that in astrocytes, ammonia affects the transport of reducing equivalents across the mitochondrial membrane by inhibiting the malate-aspartate shuttle (Subbalakshmi and Murthy 1983b, Palaiologos et al. 1988, Lai et al. 1989).

The absence of changes in the CO_2 production by nonsynaptic mitochondria in hyperammonemic conditions in vitro might be due to unrestricted delivery of glutamate to the inner transamination in mitochondria (Fitzpatrick et al. 1988, Lai et al. 1989), which is not the

case with astrocytes, where the delivery depends upon the malate-aspartate shuttle between mitochondria and cytoplasm (Ratnakumari and Murthy 1989). Increased glutamine formation from glutamate in astrocytes under excess of ammonia may interfere with normal level of glutamate and thus inhibit the shuttle transport of reducing equivalents across the mitochondrial membranes. It is noteworthy in this context that addition of glutamate or aspartate was observed to stabilize the malate-aspartate shuttle in cultured astrocytes in the presence of high ammonia (Murthy et al. 1988).

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