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PROTECTION BY TRIS(HYDROXYMETHYL)-AMINOMETHANE AGAINST BEHAVIORAL AND NEUROCHEMICAL EFFECTS OF HYPOXIA

GARY E. GIBSON,* MASAHISA SHIMADA and JOHN P. BLASS*

Departments of Psychiatry and Biological Chemistry, Mental Retardation Research Center, University of California at Los Angeles, Center for the Health Sciences, Los Angeles, CA 90024, U.S.A.

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Abstract—Pretreatment of mice with the buffer tris(hydroxymethyl)aminomethane (THAM) at alkaline but not acid pH protected against effects of anemic and histotoxic hypoxia. THAM delayed the loss of the righting reflex (from 20.3 ± 0.9 to 27.4 ± 2.0 min, $P < 0.01$) and death (from 24 ± 4 to 32 ± 2 min, $P < 0.01$) in animals with anemic hypoxia (induced with $NaNO_2$). Alkaline THAM reduced the number of animals who lost the righting reflex after the induction of histotoxic hypoxia by injection of 4 mg/kg of KCN (from 10 of 12 to 2 of 12, $P < 0.01$). Pretreatment with alkaline THAM also prevented the fall in acetylcholine levels and the rise in cyclic GMP induced by anemic hypoxia, and ameliorated the reduction in the synthesis of acetylcholine from labeled precursors in anemic ($NaNO_2$-induced) hypoxia. Although the detailed molecular mechanisms by which THAM acts remain to be clarified, these observations may have direct clinical relevance for the care of the large number of patients in whom brain dysfunction results from conditions in which the supply of oxygen to the brain is impaired but not abolished.

The sensitivity of the brain to even a very brief interruption in its supply of oxygen has been recognized for decades [1–3] and hypoxic brain damage is a major cause of disability in both children and adults. Improved understanding of the mechanisms by which hypoxia damages the brain and identification of agents which ameliorate this damage have important practical implications.

It has become clear recently that there is a marked difference between conditions in which the supply of oxygen is abolished and conditions in which it is only impaired [4–16]. In anoxia, where the supply of oxygen is abolished, the homeostatic mechanisms of the brain fail. Within seconds, levels of ATP and creatine phosphate fall, ionic gradients fail, and cell death and autolysis follow promptly [1–3]. By contrast, hypoxia, in which the supply of oxygen is impaired but not abolished, can severely impair neural function without any demonstrable change in the levels of ATP or in the adenylate energy charge potential [4–16]. This finding has been confirmed in at least six laboratories and documented in great detail by Siesjö *et al.* in Lund [5–8]. The proposal has been made that energy-utilizing (anabolic) processes are more sensitive to hypoxia than are energy-producing (catabolic) processes [13]. It has been found recently that the biosynthesis of the neurotransmitter acetylcholine is exquisitely sensitive to conditions which impair oxidative metabolism by the brain [14–23]. Hypoxia too mild to alter the levels of lactate or of ATP in the brain leads to decreased synthesis of acetylcholine and to a rise in the level of cyclic GMP but not of cyclic AMP [19, 20]. The close link between carbohydrate oxidation and acetylcholine synthesis appears to depend in part on compartmentation of glucose metabolism with respect to acetylcholine synthesis [21, 22]. It may be mediated by changes in transmitochondrial potentials, since the reduction in acetylcholine synthesis in hypoxia or hypoglycemia was proportional to changes in transmitochondrial $NAD^+/NADH$ potentials *in vivo* and *in vitro* [14, 15]. To investigate further the relationship between alterations in redox potentials and effects of hypoxia, we have studied the effects on hypoxic mice of agents which alter H^+ concentrations. We report here that the effects of hypoxia on the synthesis of acetylcholine and on cyclic GMP in the brains of hypoxic mice [20, 23] are ameliorated by pretreatment with amine buffer THAM [tris(hydroxymethyl)aminomethane, also known as Tris] at alkaline pH. Alkaline THAM also delays or prevents the loss of the righting reflex in such animals. The direct clinical implications of these results for the care of patients with a number of common conditions are discussed below.

EXPERIMENTAL PROCEDURES

Materials. Trizma base (Tris or THAM) was purchased from the Sigma Chemical Co., St. Louis, MO. All other materials were exactly as described previously [15, 16, 21, 23]. Adult male Swiss–Webster mice were rendered hypoxic [15, 20] either by the subcutaneous injection of 225 mg/kg body weight of $NaNO_2$ (to oxidize hemoglobin to methemoglobin and induce anemic hypoxia) or by the intraperitoneal injection of 4 mg/kg body weight of KCN (to inhibit cytochrome oxidase and induce histotoxic hypoxia).

Methods. Animals were injected intravenously with a mixture of 3.3 mCi/kg body weight of [U-^{14}C] glucose (330 Ci/mole) and 20 μmoles/kg of [$^2H_4$] choline in 0.5 ml of neutral isotonic saline 1 min before killing by microwave irradiation [22, 20]. Acetylcholine

*Present adress; Dementia Research Service, Department of Neurology, Cornell University, Burke Rehabilitation Center, White Plains, NY 10605.

Table 1. Effect of THAM on behavioral sequelae of anemic hypoxia*

	Loss of righting reflex (min)	Death (min)
Control	20.7 ± 1.0 (24)	23.9 ± 3.6 (24)
Alkaline THAM	27.4 ± 2.0† (12)	31.8 ± 2.2† (12)
Acidic THAM	21.0 ± 1.1 (12)	24.0 ± 1.1 (12)

* Animals were injected with $NaNO_2$ to induce anemic hypoxia and with THAM, exactly as described in the text. Values represent the time after injection of $NaNO_2$ that the particular consequence of hypoxia appeared. Numbers in parentheses represent the number of animals.

† $P < 0.01$ by Student's t-test. The differences between controls and animals with acidic THAM were not significant.

metabolism was studied by the combination of radiochemical methods and gas–liquid chromatography–mass spectrometry [22], cyclic nucleotides by radioimmunoassays [20], and lactate and glucose [14, 15, 22, 24] by enzymatic fluorometric methods, exactly as described previously. THAM was given by intraperitoneal injection in a dose of 10 m-moles/kg body weight 30 min prior to the induction of hypoxia, as a solution of THAM at pH 10.3 or of THAM–hydrochloride at pH 4.5. Controls included both animals who received an equivalent volume of isotonic saline and animals receiving no intraperitoneal injections; no differences were noted between the two control groups.

RESULTS

Pretreatment with THAM at an alkaline but not an acid pH significantly delayed the loss of the righting reflex and death in animals with anemic hypoxia (Table 1). Pretreatment with alkaline THAM also markedly reduced the proportion of animals who lost their righting reflex from histotoxic hypoxia (Table 2).

The previously reported abnormalities in acetylcholine metabolism and cyclic GMP induced by hypoxia [19, 20] were ameliorated significantly by pretreatment with alkaline THAM (Fig. 1). THAM prevented the fall in cerebral acetylcholine concentration due to hypoxia. It reduced the inhibition of acetylcholine synthesis from labeled choline from 86 to 59 per cent. It caused a small but significant increase in the incorporation of [U-^{14}C] glucose into acetylcholine compared to the value for hypoxic animals. Pretreatment with alkaline THAM also prevented a statistically significant increase in the levels of cyclic GMP in the hypoxic brains. The alterations in acetylcholine metabolism induced by THAM were not due to changes in the specific activity of the precursors in the brain, since there was no significant change in the specific activity of glucose or of choline (Table 3). Treatment with alkaline THAM ameliorated the hypoxia-induced increase in lactate (Fig. 1) but actually increased the rate of glucose utilization. Neither the degree of hypoxia induced in these experiments nor the combination of hypoxia and THAM significantly altered cerebral glucose concentrations, the entrance of radioactive material into the brain (Table 3), or the levels of cyclic AMP (Fig. 1). Treatment with THAM in the absence of hypoxia did not alter significantly any of the parameters measured in these experiments except for glucose utilization (Table 3).

DISCUSSION

The experiments described above indicate that pretreatment with THAM ameliorates certain behavioral and neurochemical consequences in two models of hypoxia. They do not define, however, the mechanism by which THAM acts. Since the protective action of THAM depends on its being at an alkaline pH, its action appears to involve in some way a reduction in effective H^+ concentrations. Hydrogen ion concentrations and potentials influence the regulation of many facets of metabolism including transport across mitochondrial membranes [14, 25].

Nahas [26] found that THAM prevented the increase in arterial pCO_2, the drop in arterial pH, the increase in cerebral spinal fluid pressure and the progressive decrease in arterial oxygen saturation accompanying apneic oxygenation in dogs. Manfredi *et al.* [27] administered THAM to three patients suffering from pulmonary insufficiency. Immediate clinical improvement was observed in one patient, in one it was "believed" to be beneficial, and the third patient died. The most constant changes during infusion of THAM were an elevation of arterial blood and urinary pH, elevation of serum CO_2, and a significant decrease of arterial blood pCO_2. These investigators proposed that THAM produces its effects by promoting the loss of CO_2 from tissues to blood and by promoting CO_2 and bicarbonate excretion. Further experiments are needed to determine which among these actions of THAM are significant in modifying the effects of hypoxia.

The effect of THAM in hypoxia, however, has direct clinical implication, whatever the detailed mechanism of action. There are a number of very common conditions in which the supply of oxygen to the brain is impaired but not abolished and brain dysfunction re-

Table 2. Effect of THAM behavioral sequelae of histotoxic hypoxia*

	Number of animals injected	Number of animals losing the righting reflex
KCN	12	10
KCN + alkaline THAM	12	2

* Animals were injected with KCN (4 mg/kg i.p.) to induce histotoxic hypoxia and with THAM, as described in the text. The differences between controls and animals with alkaline THAM were significant ($P < 0.01$).

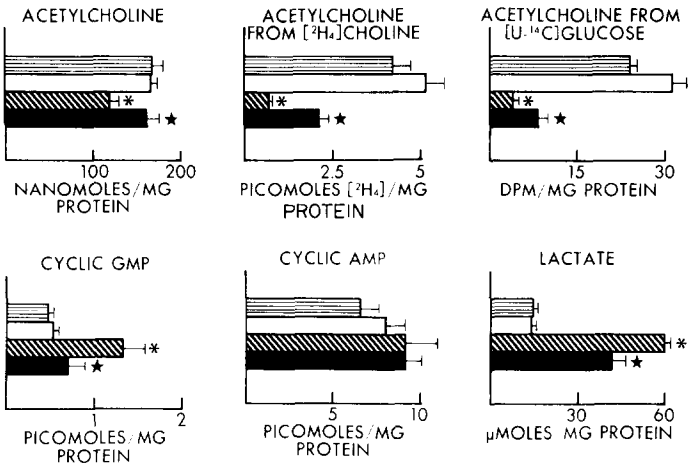

Fig. 1. Effect of alkaline THAM on brain metabolism in anemic hypoxia. Animals were killed by microwave irradiation 20 min after the injection of $NaNO_2$ and 1 min after the injection of a mixture of [U-^{14}] glucose and [$^2H_4$]choline, as described in detail in the text. THAM or an equivalent volume of isotonic saline was injected 30 min before the induction of hypoxia. Error bars represent S.E.M. key *, $P < 0.01$ vs controls; ★, $P < 0.01$ vs animals treated with $NaNO_2$ without prior treatment with THAM. Symbols represent: alkaline THAM ▤; saline-injected controls □; $NaNO_2$-induced hypoxia ▨; or $NaNO_2$-induced hypoxia and alkaline THAM ■.

sults. These include confusion and other neuropsychiatric abnormalities accompanying cardiac or pulmonary failure. It has been suggested recently that mental deterioration in aging may involve "brain failure secondary to cardiac failure" [28] with selective damage of cholinergic systems [28–33]. A relative increase in H^+ concentration in the brain may be a common mechanism in many kinds of coma due to hypoxia or metabolic brain disease [34]. The data presented above suggest that treatment with alkaline THAM might benefit patients with functional brain disease secondary to conditions which chronically impair but do not abolish the supply of oxygen to the brain. THAM has already been shown to penetrate cells [35] and to be safe in patients with metabolic acidosis, and in chronic pulmonary insufficiency, as mentioned above [27]. The protective effect of alkaline THAM in experimental hypoxia suggests the need for a clinical trial of the effects of THAM in patients in whom the supply of oxygen to the brain is impaired but not abolished.

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Table 3. Effect of alkaline THAM on brain metabolism in anemic hypoxia*

	Alkaline THAM	Saline	Hypoxia	Hypoxia and alkaline THAM
Glucose concentration (nmoles/mg protein)	19.1 ± 2.2	18.1 ± 1.2	15.5 ± 1.5	15.0 ± 2.7
Specific activity of glucose (dis./min/nmole)	2071 ± 116	2000 ± 212	1677 ± 200	1922 ± 300
Total dis./min in the brain (dis./min/mg protein × 10^{-3})	43.7 ± 3.4	47.4 ± 5.0	37.9 ± 8.4	48.5 ± 10.0
Glucose utilization (nmoles/min/mg protein)	2.1 ± 0.3†	5.6 ± 0.6	7.1 ± 0.9	9.3 ± 1.0†
Choline concentration (pmoles/mg protein	276 ± 42	230 ± 17	675 ± 85	355 ± 22
Choline specific activity [$^2H_4$] choline/total choline (per cent)	5.5 ± 0.8	7.6 ± 1.0	7.4 ± 0.9	8.5 ± 0.8
% Methemoglobin	3 ± 1	3 ± 1	70 ± 3	64 ± 5

* Animals were injected with $NaNO_2$ to induce anemic hypoxia and with THAM, exactly as described in the text. [U-^{14}C] glucose and [$^2H_4$] choline were injected 1 min before killing by microwave irradiation. Values represent means ± S.E.M. of twelve animals.

†$P < 0.05$ by Student's A-test.

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