
Survival Under Hypoxia. Age Dependence and Effect of Cholinergic Drugs

ANTONIA M. E. SCREMIN, M.D., OSCAR U. SCREMIN, M.D., AND THERESA BRECHNER, M.D.

SUMMARY Survival under hypoxia (5% O2; 95% N2) was tested in mice 1 day to 50-weeks-old. Survival Rate (ratio of number of animals that survived 30 min under 5% O2 to total number of animals exposed) and the time from onset of exposure until the last gasp (Survival Time) were maximum in newborn animals and decreased as a function of age. Survival Rate and Survival Time were strongly influenced by drugs affecting cholinergic transmission. Atropine decreased the high survival under hypoxia of 1-week-old mice in a dose-related manner. Physostigmine increased survival under hypoxia in mice 3 to 50-weeks-old. This effect was not related to a peripheral action of the drug since it was not mimicked by neostigmine, a cholinesterase inhibitor without central actions. Moreover, peripheral cholinergic blockade with glycopyrrolate, a quaternary cholinergic blocker, did not prevent the protective effect of physostigmine.

Since atropine impairs the ability of very young mice to survive hypoxia and physostigmine improves survival at later ages, it is concluded that a central cholinergic mechanism, possibly related to blood flow regulation, plays a significant role in the acute adaptation to hypoxia.

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IT IS KNOWN that the brain is particularly vulnerable to reduction of its energy supply but that its ability to withstand a hypoxic or ischemic insult is considerably greater in newborn animals.1-4 Physostigmine, a cholinesterase inhibitor, is able to prolong survival of mice subjected to hypoxic hypoxia,4 a possible indication of a role for cholinergic mechanisms in the adaptation of the brain tissue to this situation. It was of interest to explore the possible involvement of cholinergic mechanisms in the greater ability of young mice to withstand hypoxia. Therefore, the effects on hypoxic survival of cholinergic blockade with atropine and of enhancement of cholinergic transmission by physostigmine were evaluated in mice at different ages. In addition, experiments were performed to determine whether the protective effect of physostigmine relates to its peripheral or central actions.
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Material and Methods

Swiss-Webster mice one day to 50 weeks of age were used. Nursing animals were maintained with their mothers and older animals were allowed free access to food pellets and tap water until the moment of testing. Survival under hypoxia was tested by putting the animals into a 2 liter jar through which a continuous flow of 5 liters per min of a mixture of 5% O₂—95% N₂ was maintained. Gas composition inside the jar was monitored with a Beckman OM-11 Oxygen Analyzer. Air temperature was adjusted at 24 ± 0.5°C for animals over 3 weeks of age and at 26.5 ± 0.5°C for younger mice. Exposure to the hypoxic environment was limited to 30 min in all groups in order to establish a uniform finite duration for the experiment and, also, because in our previous experience, the cerebrovascular effects of physostigmine given in a single dose decrease considerably after that time. The time from onset of exposure until the last gasp (Survival Time) was measured for each animal. Mice that survived the 30 min exposure were assigned a survival time of 30 min, and were not tested again. Thus, if any animals survived for 30 min, the calculated mean survival time was an underestimation of the response to hypoxia of that group.

When the effects of drugs were tested, one-half of each litter was given an intraperitoneal injection of a solution of the drug in 0.9% NaCl, and the other half was injected with 0.9% NaCl only. The volume injected was always 0.01 ml/gram body weight. Drugs used were: physostigmine sulfate (Merck); atropine sulfate (Merck); neostigmine bromide (Sigma) and glycopyrrolate (Robinul® Robins Co.). Animals up to 3-weeks-of-age that received drug treatment were put into the jar either 2 at a time, along with 2 untreated controls, or 3 at a time with one control, to cancel any possible variation in temperature or oxygen concentration. With older animals, a single physostigmine-treated mouse was placed in the jar along with a single untreated control of the same age.

Heart rate was measured from the ECG obtained by use of subcutaneous needle electrodes and recorded on a Grass Model 7D Polygraph. In this case animals were exposed to hypoxia one at a time because of instrumental limitations.

Results

Age Dependence of Survival and Effect of Physostigmine. Survival under hypoxia was clearly age-dependent in animals that received no treatment. All mice tested on a Grass Model 7D Polygraph. In this case animals were exposed to hypoxia one at a time because of instrumental limitations.

described by 2 different slopes with a break between 5 and 7 weeks of age (fig. 2). A linear regression of Survival Time on age was found for the interval between 1 to 5 weeks of age: \( Y = 29.24 - 4.44X \), where \( Y = \text{Survival Time (min)} \) and \( X = \text{Age (weeks)} \). A linear regression of survival time on age was also found for the interval between 7 to 50 weeks of age (\( Y = 3.79 - 0.034X \)). Both regression coefficients were significantly different from zero at the level of \( p < 0.01 \).

Physostigmine significantly increased survival time at 3, 5, 7 and 50-weeks-of-age (fig. 2). Furthermore, at 5 and 7-weeks-of-age, when none of the untreated controls survived the complete test, 32% and 33%, respectively, of the animals treated with physostigmine did survive. At 50-weeks-of-age, however, although survival time was increased 4-fold by this drug, none of the mice survived 30 min.

Effects of Atropine on Survival of One-Week-Old Mice. A dose-dependent effect of atropine on hypoxic survival was observed, starting at 1 mg/kg (fig. 3). None of the mice treated with atropine at the dose of 10 mg/kg survived the test, and Survival Time decreased significantly in controls of the same age (one week) without treatment. A group of animals that received this same dose of atropine was introduced into the test...
Figure 2. Mean Survival Time of mice under 5% O₂ that received physostigmine (0.3 mg/kg) at different ages (hollow circles) is compared with survival time of untreated controls. Means ± Standard Errors and number of animals in the physostigmine groups were: 3 weeks = 24.48 ± 2.63, n = 14; 5 weeks = 18.76 ± 2.04, n = 19; 7 weeks = 17.63 ± 2.51, n = 18; 50 weeks = 7.49 ± 0.96, n = 10. Survival Rates were: 3 weeks = 0.64; 5 weeks = 0.32; 7 weeks = 0.33; 50 weeks = 0. Mean Survival Times and Survival Rates of untreated controls are listed in Fig. 1. Analysis of variance showed that physostigmine-treated animals differed from untreated controls of the same age at a level of p < 0.01 at all ages tested. Survival Time in the interval between 7 and 50 weeks of age needs further experimental exploration.

chamber and exposed to circulating air at the same rate and temperature as the ones subjected to hypoxia. These mice were still alive and showed normal behavior after several hours of exposure.

Heart Rate Changes. Untreated adult mice showed a pronounced bradycardia during hypoxia (fig. 4) and this effect was potentiated by physostigmine. Neostigmine, a cholinesterase inhibitor that does not cross the blood-brain barrier, also decreased heart rate to the level of physostigmine-treated animals during the hypoxic period, but did not increase Survival Time (mean Survival Time = 2.7 min., S.E. = 0.5; n = 6) over the value of untreated controls of the same age (mean Survival Time = 3.5 min., S.E. = 0.4; n = 21). Glycopyrrolate, a quaternary cholinergic blocker, was able to prevent the effect of physostigmine on the hypoxic bradycardia but did not affect its protective action against hypoxia. Survival Time of this group (mean = 15 min., S.E. = 4.2; n = 5) did not differ statistically from the value of animals of the same age treated with physostigmine only (mean Survival Time = 17.6 min, S.E. = 2.5; n = 18).

Discussion

The well documented ability of newborn and young animals to survive levels of hypoxia that are lethal for adults is confirmed here. Although the phenomenon was known more than a century ago, a more detailed account of the relation between age and anoxic survival was produced in the 'forties by Fazekas et al., Glass et al., and Britton and Kline. These authors proposed that an "adult pattern" of survival to anoxia or hypoxia was attained as early as 2 weeks after birth in dogs, cats, rabbits, rats and guinea pigs. Although survival time under hypoxia is also known to be longer in newborn as opposed to adult mice, no data are available, as far as we are aware, regarding

Figure 3. Bars represent Mean Survival Time under 5% O₂ of control and atropine-treated, 1-week-old mice. A Student-Newman-Keuls test for multiple comparisons among means showed that all group means of atropine-treated animals differed (p < 0.05) and that means of mice treated with 1 and 10 mg/kg atropine differed from the mean of control animals (p < 0.05). Numbers of animals in each group were: Control = 46; Atropine, 0.1 mg/kg = 18, 1 mg/kg = 17, 10 mg/kg = 20.

Figure 4. Mean Heart Rates and Standard Errors (beats per minute) of 7-week-old mice from 10 sec up to 2-4 min of exposure to 5% O₂, 95% N₂. Groups consisted of 5 animals each that received 0.3 mg/kg physostigmine (hollow circles), 0.3 mg/kg physostigmine and 0.1 mg/kg glycopyrrolate (hollow squares), 0.1 mg/kg neostigmine (filled squares) or no treatment (filled circles). Observation was limited to 2 min in untreated controls and animals that received neostigmine since they died shortly thereafter.
the resistance to hypoxia at different ages for this species. The present study reveals, at variance with the above cited evidence, that in mice hypoxic Survival Time continues to decrease even beyond the eleventh week of age. There is a rapid decrease in Survival Time as a function of age until the fifth week and a more gradual decrease thereafter.

The demonstration of a protective effect of physostigmine confirms and extends our previous observation made on 5 to 7-week-old mice under the same experimental conditions. Since this phenomenon is not reproduced by neostigmine, a quaternary compound which does not cross the blood brain barrier to a significant degree, and is not affected by a dose of glycopyrrolate that has peripheral but not central cholinergic blocking effects in mice, it is likely that the cholinergic receptors responsible for the protection against hypoxia are located within the central nervous system. The observation here reported that doses of atropine sufficient to produce peripheral cholinergic blockade do not affect the hypoxic survival of young mice, gives further support to that assumption. A decrease in hypoxic survival time is observed only at dose levels of atropine comparable to those required to induce other central cholinolytic effects of this drug.10

The heart rate change induced by physostigmine is not a necessary accompaniment of its protective effect, since the former can be blocked by glycopyrrolate without affecting the protection against hypoxia. Moreover, similar heart rate changes, i.e., potentiation of the hypoxic bradycardia, are observed with neostigmine, which does not protect against hypoxia (fig. 4).

It is known that physostigmine considerably increases cerebral blood flow11-12 and decreases cerebral oxygen consumption. Also, about the same high dose levels of atropine that are necessary to decrease hypoxic survival of young mice are required to block the cholinergic vasodilatation in rats and rabbits.13 It is tempting to speculate that the cerebrovascular effect of physostigmine might improve survival under hypoxia simply by increasing O₂ delivery to the brain. This argument is particularly relevant since the increased cerebral blood flow observed under hypoxia seems to be the only adaptive mechanism that the brain has for improving its oxygenation under hypoxia.14

Although the function of acetylcholine in the central nervous system is far from understood, it might play a role as a neurotransmitter in mechanisms essential for survival. Failure of transmission provoked by hypoxia would be compensated for by the increased availability of acetylcholine induced by physostigmine. Some support for this view is given by the finding that hypoxia decreases somewhat the synthesis of acetylcholine by brain tissue, although the total levels of acetylcholine are not affected even by a 20 min period of anemic hypoxia or hypoglycemia.15

The elucidation of the mechanism by which an enhanced central cholinergic transmission protects against hypoxia and, conversely, central cholinergic blockade decreases the chances of survival in this condition, must await further experimentation.

The phenomenon might have some practical implications, since the improvement of survival induced by physostigmine occurs in the absence of general anesthesia. The effect of atropine in survival of animals under hypoxia is also worth considering in view of the widespread use of drugs with potent central cholinergic blocking effects.

After this paper was submitted for publication, Artru and Michenfelder11 reported confirmation of our observation of a protective effect of physostigmine on hypoxic hypoxia and speculated on the reasons for an apparent quantitative disagreement between their work and ours. Their suggestion that small variations in the level of hypoxia might explain the disagreement seems unlikely, since in both cases basically the same measuring equipment has been used and control and treated animals were exposed in the same chamber. However, the body weight span of their mice (24-32 g) suggests that they were older than the ones we used in our first report (20-25 g., 5-7-weeks-old). Furthermore, the data they obtained compares well with the values reported in the oldest animals of the present series. Thus, age differences between animals in their series and those of animals in our first report might explain the apparent quantitative disagreement.

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WISE YOUNG, PH.D., M.D.

SUMMARY

H2 clearance is a powerful method for monitoring blood flow. Simple and inexpensive to implement, the method allows multiple in situ determinations of blood flow from any tissue in which a small electrode can be implanted. There is, however, evidence to suggest that H2 clearance is neither as accurate nor as local a measure of blood flow as generally supposed. Both in theory and practice, it probably cannot accurately determine blood flow rates greater than 100 ml/100 gm/min or localize blood flow to tissue volumes of less than 5 ml. Moreover, its experimental application is complicated by many technical problems hitherto largely ignored by workers in the field. Some of these problems arise from the limitations of the steady state polarographic technique used to measure tissue H2 concentrations. Other problems stem from the failure to consider possible sources of error in H2 clearance monitoring; these include interference with the H2 signal by spurious electrode and tissue currents, and contributions from tissue ascorbate and O2. Nevertheless, with the appropriate safeguards and qualifications, H2 clearance is a valid and important approach to measuring blood flow.

The appropriate technology for monitoring tissue H2, however, had to be developed. In 1956, Misrahy and Clark recorded the potentials generated by spontaneous oxidation of inhaled H2 gas with platinum electrodes implanted in animal cerebral cortices. They found that under conditions generally known to increase blood flow, i.e., CO2 inhalation, their electrodes recorded larger positive potentials. Conversely, decreasing flow with barbiturate drugs resulted in smaller potentials. In 1959, Clark and Bargeron utilized similar electrodes, placed intravascularly, to detect left to right cardiac shunts in children. Although both of these attempts allowed a qualitative assessment of blood flow, they were limited because the potentiometric technique used was neither linear nor selective for H2 concentration changes. The appropriate technology for monitoring tissue H2, however, had to be developed. In 1956, Misrahy and Clark recorded the potentials generated by spontaneous oxidation of inhaled H2 gas with platinum electrodes implanted in animal cerebral cortices. They found that under conditions generally known to increase blood flow, i.e., CO2 inhalation, their electrodes recorded larger positive potentials. Conversely, decreasing flow with barbiturate drugs resulted in smaller potentials. In 1959, Clark and Bargeron utilized similar electrodes, placed intravascularly, to detect left to right cardiac shunts in children. Although both of these attempts allowed a qualitative assessment of blood flow, they were limited because the potentiometric technique used was neither linear nor selective for H2 concentration changes.

In 1961, Hyman introduced a quantitative polarographic technique of measuring tissue H2 concentration. Instead of recording the potentials generated by spontaneous H2 oxidation, he monitored the current generated by a platinum electrode, finding that it correlated with H2 concentration. Aukland et al. in 1964 modified this polarographic technique by polarizing the electrode to +250 mV, the voltage of a calomel reference electrode. With in vitro studies, they concluded that the technique did linearly estimate tissue H2 concentration and that interference by substances, such as O2, H+, and ascorbate, was acceptably small. Then they recorded the exponential

HYDROGEN (H2) CLEARANCE was introduced as a method of blood flow monitoring 17 years ago. Despite many advances both in the theory and practice of H2 clearance, no comprehensive review of the subject has appeared since the early pioneering work. In particular, little attention has been paid to the limitations of the polarographic technique used for measuring tissue H2 concentration.

Early Developments

The suitability of H2 gas as a blood flow tracer has been known for some time. In his classic 1951 treatise on blood flow tracers, Kety pointed out that H2 is metabolically inert and not normally present in body tissues. Furthermore, H2 dissolves readily in lipids and diffuses rapidly in tissues; thus, it should penetrate nervous tissues well. Because of its low water:gas partition coefficient of 0.018, the pulmonary circulation should rapidly remove it from arterial blood. Therefore, H2 fulfills the major criteria for tracer clearance studies of blood flow, developed by Kety and Schmidt.

From the Departments of Neurosurgery and Physiology and Biophysics, New York University Medical Center, 550 First Avenue, New York, NY 10016.
Reprints: Dr. Young, Department of Neurosurgery, NYU Medical Center, 550 First Ave., New York, NY 10016.
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A M Scremin, O U Scremin and T Brechner

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